Chapter 3 Identification of the Sites of Incorporation of [^{3}H]3-Azioctanol in the nAChR

ABSTRACT

A photoactivatible derivative of a long chain alcohol, [^{3}H]3-azioctanol, has been used to localize the sites of interaction of alcohols with the *Torpedo* nicotinic acetylcholine receptor (nAChR). 3-Azioctanol inhibits the nAChR with an IC_{50} ~33 μM. The sites of [^{3}H]3-azioctanol incorporation in *Torpedo* nAChR-rich membranes were determined at 1 μM and 275 μM [^{3}H]3-azioctanol. At both concentrations, [^{3}H]3-azioctanol photoincorporated into all four nAChR subunits in the resting state. The incorporation of [^{3}H]3-azioctanol into the α-subunit in the absence of agonist was mapped to two large proteolytic fragments, one, ~20 kD, containing the first three transmembrane segments and one, ~10 kD, containing the fourth transmembrane segment. In the presence of agonist, there was enhanced incorporation in the α-subunit that was mapped to the ~20 kD fragment of α, while the incorporation in the ~10 kD fragment was similar to that seen in the absence of other ligands. Further digestion of the 20 kD fragment showed a primary site of incorporation at αGlu-262, at the extracellular end of the αM2 segment, that was labeled preferentially in the desensitized state. In the absence of other drugs, [^{3}H]3-azioctanol also photoincorporated into αTyr-190 and αTyr-198, residues within the binding site for agonists and competitive antagonists; this incorporation was blocked by the presence of carbamylcholine. No incorporation was attributable to the αM1 or αM3 segments. Digestion of the 10 kD fragment showed incorporation in the αM4 segment, at αHis-408 and αCys-412, residues known to be at the protein-lipid interface. These results indicate that the primary site of binding of 3-azioctanol is within the ion channel,

near the C-terminus of the αM2 segment, with additional lower affinity interactions within the agonist binding site and at the protein-lipid interface.

INTRODUCTION

In recent years, evidence for the direct interaction between general anesthetics and specific proteins has accumulated (Franks and Lieb, 1994). In particular, mutational analyses have identified amino acids in the inhibitory $GABA_A$ and glycine receptors as well as different positions within the nAChR which contribute to anesthetic action. Within the $GABA_A$ and glycine receptors, which, in general, are potentiated by general anesthetics, two residues, one in the M2 hydrophobic 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 (loreclezole and etomidate) (Krasowski *et al.*, 1998; Mihic *et al.*, 1997; Wingrove *et al.*, 1994; McGurk *et al.*, 1998; Moody *et al.*, 1997; Belelli *et al.*, 1997; Ye *et al.*, 1998; Wick *et al.*, 1998). The position in M2, 15' based on numbering from the conserved positive charge at the N-terminus of M2 (lysine in nAChR, arginine in $GABA_A$ and glycine receptors), 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 ~7 amino acids from the N-terminus of M3, but the orientation of this segment is not clearly established. However, the residue implicated in general anesthetic action on the $GABA_A$ and glycine receptors has been predicted to face the M2 helix, positioning it near the M2 15' residue (Wick *et al.*, 1998).

In contrast to the $GABA_A$ receptors, muscle and neuronal nAChR are inhibited by most general anesthetics. 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 (Dilger *et al.*, 1995). Site directed mutagenesis of the nAChR has shown that the nature of the residue at the M2 position 10', facing the lumen of the ion channel, can increase the potency of long chain alcohols and isoflurane as channel blockers (Forman *et al.*, 1995).

The residues implicated by the nAChR and $GABA_A$ receptor studies do not simply define a single, common binding site for long chain alcohols. Since alcohols potentiate most $GABA_A$ receptors but inhibit nAChRs, it is possible that long chain alcohols bind to different sites in the two receptors to exert their different actions. However, the sequence proximity of the positions in M2 that affect the binding of alcohols in the two receptors, only 5 amino acids apart, suggests that these positions both may affect a similar alcohol binding site, with one or both acting allosterically.

As an alternative to mutational analysis, the photoaffinity anesthetic 3-azioctanol was developed (Husain *et al.*, 1999) 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 EC_{50} of ~160 μM, an EC_{50} that is ~ 1/3 the potency of octanol. For the $GABA_A$ receptor, 3-azioctanol potentiates the response to submaximal concentrations of GABA, and inhibits agonist activation of muscle-type nAChR (IC_{50} ~30 μM).

[^{3}H]3-Azioctanol was used here as a photoaffinity probe to localize the sites of interaction of a long chain alcohol with *Torpedo* nAChR-rich membranes. Within the nAChR, incorporation was primarily within the α-subunit, with incorporation increased in the presence of agonist. The enhanced incorporation in the presence of agonist was within αGlu-262, at the C-terminus of αM2. In addition, [^{3}H]3-azioctanol incorporated at lower efficiency into the αM4 segment, at equal levels in the presence and absence of

agonist, and in the absence of agonist, [^{3}H]3-azioctanol also reacted with low efficiency with αTyr-190 and αTyr-198, residues implicated in the binding of agonist.

MATERIALS AND METHODS

Materials

nAChR-enriched membranes were isolated from *Torpedo californica* electric organ according to the method described by Sobel *et al.* (1977), modified as described by Pedersen *et al.* (1986). The final membrane suspensions were stored in 38% sucrose at -80 °C under argon. The membranes used here contained 0.5-2.0 nmol acetylcholine binding sites per milligram of protein. [^{3}H]3-Azioctanol and nonradioactive 3-azioctanol were a kind gift from Dr. Keith Miller, synthesized as described in Husain *et al* (1999). The specific activity of the [^{3}H]3-azioctanol as prepared was ~11 Ci/mmol. This stock was stored at –20 °C in CH_2Cl_2, 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}H]3-azioctanol, this stock was isotopically diluted with a stock of nonradioactive 3-azioctanol, 11 mM (concentration as reported in Husain, *et al* (1999), based on the absorbance of 3-azioctanol at 350 nm) in *Torpedo* physiological saline (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM sodium phosphate, pH 7.0; TPS), to a final specific activity of ~0.04 Ci/mmol. This dilution was prepared immediately before addition to membranes. *S. aureus* V8 protease was from ICN Biomedical Inc, EndoLysC from Boeringher Mannheim, phencyclidine (PCP) from Alltech Associates. Gallamine triethyl iodide was from Lederle, phenyltrimethylammonium (PTA) from Aldrich, and trifluoroacetic acid (TFA) was from Pierce. 1-Azidopyrene (1-AP) was purchased from Molecular Probes. 10% Genapol C-100 was from Calbiochem. Nicotine, d-tubocurare (dTC), 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}H]3-azioctanol

For analytical labeling experiments, freshly thawed *Torpedo* membranes (100 μg per condition) were diluted with TPS and pelleted (15000xg) for 30 minutes. The pellets were resuspended in TPS. Membrane aliquots were combined with [^{3}H]3-azioctanol in the absence or presence of other ligands, as noted in the figure legends. The final membrane concentration was 2 mg/ml (~1 μM nAChR). The final concentration of [^{3}H]3-azioctanol was as noted in the figure legends. When one of the conditions contained αBgTx, the samples were incubated for 2 hours in the dark at room temperature in glass vials; otherwise, the samples were irradiated within 3 minutes of the addition of drugs. The suspensions were irradiated at 365 nm (Spectroline lamp EN-16) for 10 minutes in a plastic 96-well plate on ice. The suspensions were diluted with sample loading buffer and directly submitted to SDS-PAGE.

For proteolytic mapping of [^{3}H]3-azioctanol labeled α-subunit with *S. aureus* V8 protease (Cleveland *et al.*, 1977; White and Cohen, 1992), labeling was carried out with 400 μg (analytical mapping) or 10 mg (preparative) nAChR-rich *Torpedo* membranes. For analytical mapping, samples were photolyzed in a 24-well plate while for preparative mapping, the samples were photolyzed in glass screw-top vials with a stir bar. Following photolysis, the membrane suspensions were pelleted as described above. For analytical mapping, samples were resuspended in sample buffer and submitted to SDS-PAGE. For preparative mapping, samples were resuspended in TPS (2 mg/ml). The samples were labeled further with 1-azidopyrene (1-AP) (Blanton and Cohen, 1994) to ease identification and isolation of subunits and fragments from gels. 1-AP (62.5 mM in DMSO) was added to a final concentration of 500 μM. After a 90 minute incubation, the

samples were photolyzed for 15 minutes on ice using a 365 nm lamp (Spectroline EN-16). Membranes were pelleted (15000xg) for 30 minutes, resuspended in sample buffer, and submitted to SDS-PAGE.

Gel Electrophoresis

SDS-PAGE was performed as described by Laemmli (1970), modified as described by Pedersen *et al.* (1986). For analytical gels, the polypeptides were resolved on a 1 mm thick, 8% acrylamide gel and visualized by staining with Coomassie Blue (0.25% w/v in 45% methanol and 10% acetic acid). For autoradiography, the gels were impregnated with fluor (Amplify, Amersham), dried, and exposed at -80 °C to Kodak X-OMAT film for various times (6-8 weeks). Additionally, incorporation of ^{3}H into individual polypeptides was quantified by scintillation counting of excised gel slices, as described in Middleton and Cohen (1991). For analytical V8 mapping gels, following electrophoresis, the gel was briefly stained with Coomassie Blue and destained to allow visualization of the subunits. The α-subunits were then excised and placed directly into individual wells 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 150V for two hours, and then the current was turned off for one hour. The gel was then run at constant current overnight until the dye front reached the end of the gel. The gel was stained, and the ^{3}H was quantified by liquid scintillation. For preparative labelings, the polypeptides were resolved on a 1.5 mm, thick 8% acrylamide gel. The α-subunit was identified in the 8% gels by 1-AP fluorescence, excised, and loaded directly onto the 1.5 mm mapping gels. The α-subunit proteolytic fragments of ~20 kD (αV8-20) and ~10 kD (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 M Na_2CO_4, 0.1% SDS (Blanton and Cohen, 1994; Hager and Burgess, 1980). The eluate was filtered (Whatman No. 1) and concentrated using Millipore 5K concentrators. To remove excess SDS, acetone was added to the concentrate, and, following incubation at –20°C overnight, the peptides were pelleted.

Proteolytic digestion:

For EndoLysC digestion, acetone-precipitated peptides were resuspended in 15 mM Tris, pH 8.1, 0.1% SDS. EndoLysC (1.5 mU 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, acetone 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, acetone-precipitated peptides were resuspended in a small volume (40 µl) of 100 mM NH_4CO_3, 0.1% SDS, pH 7.8. Genapol C-100 and trypsin were added, with a final concentration 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 [^{3}H]3-azioctanol labeled subunits were further purified by reverse-phase HPLC, as described by Blanton and Cohen (1992), using a Brownlee C4 Aquapore column (100 x 2.1 mm; 7 um particle size). Solvent A was

0.08% TFA in water, and solvent B was 0.05% TFA 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 minutes 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, 25 μL aliquots from the fractions were taken to determine the distribution of ^{3}H by liquid scintillation counting.

αV8-18 was purified by HPLC using solvents and gradients similar to those used by Cohen *et al.* (1991) to purify αV8-18 fragments, using a Brownlee C4 Aquapore column. Solvent A was 0.09% TFA in water, and solvent B was 0.1% TFA in acetonitrile. A linear gradient with several steps was used: 0 minutes, 10% solvent B; 10 minutes, 10% solvent B; 25 minutes, 25% solvent B; 45 minutes, 40% solvent B; 65 minutes 60% solvent B; 75 minutes, 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.

Sequence Analysis

Automated amino terminal sequence analysis was performed on an Applied Biosystems Model 477A protein sequencer with an in-line 120A PTH analyzer. HPLC samples (450 μl per fraction) 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 TFA to minimize possible hydrolysis. After conversion of the released amino acids to PTH amino acids, the suspension was divided into two parts. One portion, approximately one-third, went to the PTH analyzer while the remaining two-thirds was collected for scintillation counting. Yield of PTH amino acids was calculated from peak height compared with standards using the program Model 610A

Data Analysis Program Version 1.2.1. Initial yield and repetitive yield were calculated by a nonlinear least squares regression to the equation $M=I_0*R^n$, where M is the observed release, I_0 is the initial yield, R is the repetitive yield, and n is the cycle number. PTH-derivatives known to have poor recovery (Ser, Arg, Cys, and His) were omitted from the fit.

Radioactivity incorporation in fragments and residues was quantified based on the results of sequence analysis. For the αV8-20 and αV8-10 fragments, approximately equal aliquots were subjected to either liquid scintillation counting or sequence analysis. These samples, since they contained SDS, were pre-treated on the sequencing filters for four minutes with gas-phase TFA, followed by a five minute wash with ethyl acetate. To estimate incorporation in large subunit fragments (αV8-20 and αV8-10) or in fragments isolated by HPLC, incorporation was calculated as the ^3H loaded divided by three times the observed initial yield of the sequence (three times because only one-third of the PTH-amino acids were measured for mass calculations). Because even under favorable conditions less than 50% of the material loaded is sequenceable, this calculation is an overestimate. For incorporation at specific residues, the mass of that residue was calculated from the initial yield and repetitive yield. The increased ^3H release in that cycle ($cpm_n–cpm_{n-1}$) was divided by twice the mass of that cycle (twice since two-fold more PTH-amino acids were assayed for ^3H than for mass). In this calculation, the radioactivity released and the mass levels reflect only the sequenced material.

RESULTS

Photoincorporation of [^{3}H]3-Azioctanol into nAChR-Rich Membranes.

Initial experiments were designed to characterize the general pattern of photoincorporation of [^3H]3-azioctanol and to test the sensitivity of photoincorporation to

various ligands. For these initial experiments, two concentrations of [^{3}H]3-azioctanol were used, 1 μM (11 Ci/mmol) and 275 μM (0.04 Ci/mmol). The IC_{50} of 3-azioctanol is ~30 μM. Therefore, 1 μM [^{3}H]3-azioctanol was well below the concentration necessary for inhibition of 50% of the nAChR, while 275 μM [^{3}H]3-azioctanol was a concentration sufficient to produce greater than 50% inhibition. Isotopic dilution of [^{3}H]3-azioctanol resulted in the presence of similar levels of ^{3}H in the samples containing 1 μM and 275 μM [^{3}H]3-azioctanol. Membranes (2 mg of protein/mL) were equilibrated with [^{3}H]3-azioctanol in the presence and absence of 2 mM carbamylcholine. After irradiation for 10 minutes 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 (Figure 3-1A), at both [^{3}H]3-azioctanol concentrations, in the absence of carbamylcholine the principal polypeptide labeled was a 34 kD polypeptide, identified as a mitochondrial chloride channel (VDAC) (Blanton *et al.*, 1998a). Of the nAChR subunits, α was labeled most strongly. The labeled band at 43 kD contained rapsyn, a peripheral polypeptide associated with the cytoplasmic aspect of the nAChR (Carr *et al.*, 1987). Incorporation of [^{3}H]3-azioctanol into the α-subunit was dependent on the conformational state of the nAChR, as the presence of agonist resulted in enhanced incorporation into the α-subunit, but not in non-nAChR polypeptides. Based on scintillation counting of excised gel slices, the increase in incorporation was, on average, ~5-fold at 1 μM [^{3}H]3-azioctanol (Figure 3-1B), and ~3-fold at 275 μM. The presence of agonist also increased the incorporation in the β-subunit, though only by ~1.4-fold at 1 μM. The radioactivity incorporation at two non-receptor polypeptides, rapsyn (43K), and the α-subunit of Na^+/K^+ ATPase (αNK), was not altered by the presence of carbamylcholine and appeared similar at 1 μM and 275 μM [^{3}H]3-azioctanol. Since the [^{3}H]3-azioctanol at 275 μM had an ~275-fold

lower specific activity, the observed similarity in the ^{3}H incorporation in the α-subunit at the two conditions indicated that the α-subunit labeled in the presence of 275 μM [^{3}H]3-azioctanol contained ~275-fold more moles of 3-azioctanol per mol subunit.

The dependence of the incorporation of [^{3}H]3-azioctanol on the time of photolysis was measured over a range of time in the presence of carbamylcholine (Figure 3-2). For short photolyses, the incorporation increased approximately linearly, while at 20 minutes the incorporation in the α-subunit in the presence of carbamylcholine appeared to saturate. The incorporation in the α-subunit in the presence of carbamylcholine without irradiation was 4% the levels seen following 10 minutes irradiation. For the remainder of the experiments, the photolysis was carried out for 10 minutes.

The concentration dependence for the enhancement of [^{3}H]3-azioctanol photolabeling by carbamylcholine (Figure 3-3) as well as the effects of other cholinergic agonists and competitive antagonists (Figure 3-4) was determined by quantification of ^{3}H incorporation in gel slices. The data for the concentration dependence of carbamylcholine was well fit by a single binding site, with a K=4 μM. While this value for the concentration of carbamylcholine producing 50% enhancement was higher than the directly measured Keq of 0.1 μM (Boyd and Cohen, 1980b), this was not unexpected, since the photolabeling experiment was carried out at a concentration of ACh sites of 2.2 μM. At concentrations sufficient to fully occupy the ACh site, the agonists phenyltrimethylammonium (PTA) and nicotine increased [^{3}H]3-azioctanol photoincorporation in the α-subunit to the same extent as carbamylcholine (Figure 3-4). The presence of the competitive antagonists dTC and gallamine, known to partially desensitize the receptor (Pedersen and Papineni, 1995; Medynski, 1983), resulted in incorporation in α-subunit ~60% of that seen in the presence of carbamylcholine, while for pancuronium, a competitive antagonist which is not known to desensitize the receptor,

[^{3}H]3-azioctanol incorporation in the α-subunit was similar to that seen in the absence of carbamylcholine. No effect of these cholinergic drugs was seen on the incorporation of [^{3}H]3-azioctanol into non-nAChR polypeptides including rapsyn (43 kD), calectrin (37 kD), or the Na^+/K^+ ATPase α-subunit (not shown).

The effects of several noncompetitive antagonists on the incorporation of [^{3}H]3-azioctanol at 1 μM were also tested (Figure 3-5A,B. For membranes equilibrated with carbamylcholine, the ^{3}H incorporation in α was insensitive to the presence of 1 mM octanol. At 100 μM, meproadifen, an aromatic amine noncompetitive antagonist, reduced the incorporation by ~50%. Two other aromatic amine noncompetitive antagonists, phencyclidine (PCP) and QX-222, failed to inhibit the incorporation of [^{3}H]3-azioctanol in the α-subunit (Figure 3-5B). The presence of noncompetitive antagonists did not affect the incorporation in the other nAChR subunits (not shown) nor the incorporation in non-nAChR polypeptides, including rapsyn (43 kD), VDAC (34 kD), and calectrin (37 kD) (not shown).

The effects of meproadifen were also studied in the presence of 275 μM [^{3}H]3-azioctanol (Figure 3-5C). At that concentration the presence of carbamylcholine resulted in a ~3-fold increase in the incorporation of [^{3}H]3-azioctanol in the α-subunit over that seen in the absence of carbamylcholine. In the presence of carbamylcholine, meproadifen reduced the incorporation by ~50%. In the absence of carbamylcholine, meproadifen actually enhanced the [^{3}H]3-azioctanol incorporation. In the presence of αBgTx, meproadifen did not alter the [^{3}H]3-azioctanol incorporation in the α-subunit. The incorporation in rapsyn (43 kD) was not affected by the presence of these cholinergic drugs.

The incorporation of [^{3}H]3-azioctanol in nAChR α-subunit was measured over a range of [^{3}H]3-azioctanol concentrations, using a constant specific activity of [^{3}H]3-

azioctanol (Figure 3-6). 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 nearly linearly up to ~ 1 mM and then appeared to saturate. At all concentrations, the incorporation in the presence of αBgTx was less than that seen in the absence of added drugs, though at ~2 mM the incorporation in the presence of αBgTx 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 higher incorporation in the absence of carbamylcholine showed high variability. The total ^3H incorporation at ~ 2 mM [^3H]3-azioctanol was ~0.25 mol [^3H]3-azioctanol/mol α, based on the reported counting efficiency (25%) of the toluene-based gel cocktail used (Middleton and Cohen, 1991).

Mapping of [^3H]3-azioctanol photoincorporation into α-subunit proteolytic fragments

The distribution of [^3H]3-azioctanol incorporation within the α-subunit was examined by digestion of the labeled subunit with *S. aureus* V8 protease under conditions which are known to generate four large, non-overlapping fragments resolvable by SDS-PAGE (Figure 3-7A). The largest fragment, a 20 kD peptide (αV8-20[1]), begins at αSer-

[1] Staining of the mapping gel with Coomassie Blue revealed the four expected proteolytic fragments in all conditions. Additionally, a band was present at ~22 kD in the conditioned labeled with 275 μM [^3H]3-azioctanol in the presence of carbamylcholine (not shown). This band was excised from this condition, and this region was also excised in the other conditions. This band was assumed to be αV8-20 that was highly labeled with [^3H]3-azioctanol, resulting in reduced mobility. Therefore, the ^3H present in this region was attributed to labeling in αV8-20 and was added to that of the αV8-20 band.

173 and contains the first three membrane spanning regions, αM1, αM2, and αM3 (Pedersen *et al.*, 1986). The 10 kD peptide (αV8-10) contains the fourth membrane spanning region, αM4, and begins at αAsn-339. The 18 kD (αV8-18) and 4 kD (αV8-4) peptides begin at αVal-46 and αSer-1, respectively. Membranes labeled with [^{3}H]3-azioctanol were subjected to SDS-PAGE, and the α-subunit was excised. This gel piece was loaded onto a mapping gel along with V8 protease. The α-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 μM and 275 μM [^{3}H]3-azioctanol. Similar levels of ^{3}H were used at the two concentrations, with a specific activity of 11 Ci/mmol at 1 μM and 0.04 Ci/mmol at 275 μM, resulting in a ~275-fold reduction in specific activity in the samples labeled in the presence of 275 μM [^{3}H]3-azioctanol compared to those labeled in the 1 μM condition. Based on liquid scintillation counting of these α-subunit proteolytic fragments, the main sites of photoincorporation in the absence of agonist were within the αV8-20 and αV8-10 fragments (Figure 3-7B). The ^{3}H incorporation in each fragment was similar at both concentrations of [^{3}H]3-azioctanol. In the absence of agonist, the incorporation in αV8-10 was ~60% that of αV8-20. The addition of agonist increased the labeling of the αV8-20 fragment, nine-fold at 1 μM and five-fold at 275 μM, while the ^{3}H incorporated in αV8-10 was unchanged by the presence of carbamylcholine. In the presence of carbamylcholine, the incorporation in αV8-20 accounted for ~90% of the incorporation at both [^{3}H]3-azioctanol concentrations, while αV8-10 contained ~6% of the total [^{3}H]3-azioctanol incorporation within the α-subunit fragments. The similar levels of ^{3}H incorporation in the fragments between the two concentrations, with [^{3}H]3-azioctanol at an ~275-fold lower specific activity at 275 μM, indicated that ~275-fold more molecules

of 3-azioctanol were incorporated at 275 μM [^{3}H]3-azioctanol. In all conditions, the incorporation in αV8-18 and αV8-4 was unchanged and lower than the incorporation in αV8-10.

The carbamylcholine-dependent labeling of nAChR with [^{3}H]3-azioctanol was in the αV8-20 fragment containing αM1, αM2, and αM3. To further localize the site of labeling, 10 mg of membranes were labeled with 1 μM or 275 μM [^{3}H]3-azioctanol in the presence or absence of carbamylcholine, meproadifen, or αBgTx. 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 α-subunit with V8 protease, the αV8-20, αV8-18, and αV8-10 fragments were excised and eluted. To quantify the ^{3}H incorporation, the eluted αV8-20 and αV8-10 fragments were subjected to sequence analysis. Based on sequence analysis of the fragments, at 1 μM [^{3}H]3-azioctanol, in the absence of carbamylcholine ~0.008 moles of [^{3}H]3-azioctanol incorporated into a mole of αV8-20, and ~0.004 moles into αV8-10. In the presence of carbamylcholine, 0.06 moles incorporated into αV8-20 and 0.004 moles into αV8-10. At 275 μM [^{3}H]3-azioctanol, the incorporation increased, with ~0.55 moles incorporated per mole of αV8-20 and 0.24 moles per mole αV8-10 in the absence of carbamylcholine. In the presence of carbamylcholine at 275 μM [^{3}H]3-azioctanol, ~1.3 moles [^{3}H]3-azioctanol incorporated into αV8-20 and ~0.40 moles into αV8-10.

Localization of the sites of incorporation of [^{3}H]3-azioctanol in αV8-20 segment

M2

To determine if there was incorporation in the αM2 segment, the eluted αV8-20 fragment, labeled with [^{3}H]3-azioctanol, was digested with EndoLysC. Digestion with EndoLysC is known to create a ~10 kD fragment starting at αMet-243, the N-terminus of the αM2 segment, that can be purified by reverse-phase HPLC (Pedersen *et al.*, 1992), previous chapter). When the EndoLysC-digested αV8-20 which had been labeled with 275 μM [^{3}H]3-azioctanol in the presence of carbamylcholine was fractionated by reverse-phase HPLC, ~80% of the ^{3}H eluted in a peak centered at fraction 33 (~88% organic) (Figure 3-8A). For the samples labeled in the presence of αBgTx or the absence of other drugs, the ^{3}H 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 ^{3}H from the sample labeled in the presence of carbamylcholine, was subjected to Edman degradation (Figure 3-8B) which showed that the only sequence present was that beginning at αMet-243 (-carb: I_0=23 pmol; +carb: I_0=30 pmol). No other sequences were present at more than 10% the mass of the peptide beginning at αMet-243. For the sample labeled in the presence of carbamylcholine, there was a peak of ^{3}H 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 (not shown). Based upon the ^{3}H release in cycle 20, in the presence of carbamylcholine, there were ~0.33 mol [^{3}H]3-azioctanol incorporated per mol αGlu-262. In the absence of other drugs or the presence of αBgTx, there were ~0.14 mol [^{3}H]3-azioctanol incorporated per mol αGlu-262.

The HPLC profile of the EndoLysC-digest of αV8-20 labeled in the presence of 1 μM [^{3}H]3-azioctanol was similar to that at 275 μM (not shown). For the sample labeled in the presence of carbamylcholine, ~70% of the ^{3}H eluted as a single peak at ~90% organic. As with fraction 33 from the sample labeled in the presence of 275 μM [^{3}H]3-azioctanol, sequence analysis of the fraction containing the peak of ^{3}H from the samples labeled with 1 μM [^{3}H]3-azioctanol revealed the presence of a single sequence beginning at αMet-243 with release of ^{3}H in cycle 20 (not shown). In the presence of carbamylcholine, the release in cycle 20 was equivalent to 0.06 mol per mol αGlu-262, and that labeling was reduced by ~75% for the sample labeled in the presence of meproadifen and carbamylcholine (0.014 mol [^{3}H]3-azioctanol per mol αGlu-262). In the absence of carbamylcholine, the incorporation of [^{3}H]3-azioctanol at αGlu-262 (0.0012 mol [^{3}H]3-azioctanol incorporated per mol αGlu-262) was ~2% that seen in the presence of carbamylcholine.

Agonist site

For the αV8-20 labeled with either 1 μM or 275 μM [^{3}H]3-azioctanol in the absence of carbamylcholine, the HPLC chromatogram of the EndoLysC digest of αV8-20 (Figure 3-8A) contained a peak of ^{3}H at fraction 29 (69% 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: I_0=35 pmol; +αBgTx: I_0=55 pmol; +carb: I_0=36 pmol) (Figure 3-8C). This fragment contains residues contributing to the ACh site (α190-200) as well as the αM1 segment, since there is no lysine between αHis-186 and αLys-242, prior to αM2. At 275 μM [^{3}H]3-azioctanol, ^{3}H 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 ^{3}H in these cycles correspond to

αTyr-190 and αTyr-198, residues known to contribute to the agonist binding site (Chiara and Cohen, 1997; Middleton and Cohen, 1991; Dennis *et al.*, 1988; Sine *et al.*, 1994; Tomaselli *et al.*, 1991). The amount of incorporation in these residues was ~10% that in αGlu-262 in the absence of carbamylcholine, with [^{3}H]3-azioctanol only incorporating at ~0.013 mol per mol αTyr-190 and ~0.017 mol per mol αTyr-198. A similar pattern of release, though with lower levels of ^{3}H incorporation, was seen in the sample labeled with 1 μM [^{3}H]3-azioctanol in the absence of carbamylcholine (see Table 3-1 in Discussion). In the presence of carbamylcholine, while 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 αVal-189.

M1 and M3

As seen in Figure 3-8, EndoLysC digestion of αV8-20 generated two fragments, one beginning at αMet-243 and one beginning at αHis-186, which were separated by HPLC (Figure 3-8A) and sequenced (Figure 3-8B). The fragment beginning at αHis-186, which eluted in fraction 29, contained residues contributing to the agonist site as well as the αM1 segment. Based on the radioactivity in the fragment and the mass levels sequenced, this fragment from the sample labeled at 275 μM [^{3}H]3-azioctanol in the presence of carbamylcholine contained ~0.05 mol incorporated per mol fragment, ~4% that in the major radiolabeled fragment, recovered in fraction 33, that contained the αM2 and αM3 segments. Therefore, if there is any incorporation within the αM1 segment, it was less than 4% of the level of the incorporation in the αM2 segment.

Solution digestion of αV8-20 with V8 protease generates a ~9 kD fragment beginning at αLeu-263, the N-terminus of the αM2-M3 linker and containing the αM3 segment (Blanton and Cohen, 1994). To determine if [^{3}H]3-azioctanol incorporated into

the αM3 segment, αV8-20 labeled with 275 μM [^{3}H]3-azioctanol was digested with V8 protease, and the fragments were separated by HPLC (Figure 3-9). V8 protease cleaves at the C-terminal side of glutamates, and in order to generate the fragment beginning at αLeu-263, cleavage must occur at αGlu-262, which is labeled by [^{3}H]3-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 ^{3}H eluted at fraction 33 (Figure 3-9, 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 ^{3}H in the fraction, this fragment should have contained the αM2 segment. The fragment beginning at αLeu-263 was expected to elute at ~55% organic (Blanton and Cohen, 1994). A small peak of ^{3}H was present in fraction 23 (~50% organic), and one half of this fraction from each condition was subjected to Edman degradation (not shown). Two sequences were present, one the fragment beginning at αLeu-263 (–carb: I_0=4.8 pmol; +αBgTx: I_0=3.4 pmol; +carb: I_0=1.5 pmol) as well as a fragment beginning at αThr-52 (–carb: I_0=72 pmol; +αBgTx: I_0=24 pmol; +carb: I_0=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 ^{3}H in this fraction were attributable only to the sequence beginning at αLeu-263, then, in the presence of carbamylcholine, 0.08 mol [^{3}H]3-azioctanol incorporated per mol 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.

Incorporation of [^{3}H]3-azioctanol into αV8-18

To characterize the levels of incorporation in the αV8-18 fragment compared to the incorporation in αV8-20, αV8-18 was purified by reverse-phase HPLC (Figure

3-10A). A peak of ^{3}H eluted at fraction 23, as well as in two hydrophobic peaks. The hydrophobic peaks corresponded to contamination by αV8-20. Sequence analysis of fraction 23 showed two sequences present at similar levels, one beginning at αVal-46 (–carb: I_0=41 pmol; +αBgTx: I_0=19 pmol; +carb: I_0=32 pmol) and the other beginning at αThr-52 (–carb: I_0=38 pmol; +αBgTx: I_0=24 pmol; +carb: I_0=31 pmol) (Figure 3-10B). These two peptides are the known N-termini of αV8-18. Radioactivity release was evident in the 6th cycle. Since these two sequences were present at similar levels, however, it was unclear to which sequence the release was attributable. Similar levels of release were seen in the presence or absence of carbamylcholine or αBgTx. The residue, either αGlu-51 or αArg-57, was labeled by ~0.003 mol [^{3}H]3-azioctanol per mol residue.

Additional incorporation is also present within αV8-18, though at an undetermined site(s). EndoLysC digestion of αV8-18 followed by HPLC separation showed a peak of ^{3}H that contained a single sequence, that beginning at Lys-77 (-carb I_o=22 pmol, +αBgTx I_o=15 pmol, +carb I_o=13 pmol) (not shown). The fragment showed ~ 10% incorporation in each of the three conditions. Based on the radioactivity released in the cycle containing αTyr-93, a residue contributing to the agonist binding site, this position is labeled by less than 0.00003 mol [^{3}H]3-azioctanol per mol residue (not shown).

Localization of the sites of incorporation of [^{3}H]3-azioctanol in αV8-10

At 275 μM [^{3}H]3-azioctanol, the αV8-10 fragment labeled in the presence or absence of other cholinergic drugs showed similar levels of ^{3}H incorporation. Additionally, the levels of incorporation in αV8-10 labeled with 1 μM [^{3}H]3-azioctanol were similar in the presence and absence of other drugs. HPLC purification of αV8-10

labeled with 275 μM [^{3}H]3-azioctanol revealed that ~60% of the incorporated ^{3}H eluted in the flow-through (Figure 3-11A, inset), while only ~20% eluted in a broad peak between fractions 32-35, where intact αV8-10 was known to elute (Blanton and Cohen, 1992). Sequence analysis confirmed the presence of αV8-10 in these fractions. The presence of ^{3}H in the flow-through indicated that most of the [^{3}H]3-azioctanol incorporated into αV8-10 was not stably incorporated under the conditions of HPLC.

To localize the ^{3}H incorporation within αV8-10 that is stably incorporated, [^{3}H]3-azioctanol labeled αV8-10 that had been eluted from gel was digested with trypsin, under conditions known to cleave the fragment at αArg-400 (Blanton and Cohen, 1992). HPLC purification of the digest showed the major peak of ^{3}H in the flow-through, as well as a peak of ^{3}H at fractions 30-33 (Figure 3-11A). Based upon the ^{3}H elution profile seen when intact αV8-10 was purified by HPLC, the ^{3}H in the flow-through, ~60% of the eluted ^{3}H, was assumed to result from [^{3}H]3-azioctanol incorporation which was unstable to HPLC conditions. The ^{3}H present between fractions 30-33 accounted for ~15% of the total eluted ^{3}H. Sequence analysis of the pooled fractions 30-33 showed the presence of a primary sequence beginning at αTyr-401 (-carb: I_0=502 pmol; +αBgTx: I_0=457 pmol; +carb: I_0=423 pmol), near the beginning of αM4, along with a secondary sequence beginning at αSer-388 (-carb: I_0=68 pmol; +αBgTx: I_0=70 pmol; +carb: I_0=72 pmol) (Figure 3-11B). In all conditions tested, ^{3}H release was observed in cycles 8 and 12, indicating incorporation in αHis-408 and αCys-412. Additional low level release was seen reproducibly in cycle 3, corresponding to αAla-403. [^{3}H]3-Azioctanol incorporated into αHis-408 and αCys-412 at ~0.0025 mol per mol residue at 275 μM, at ~1% that level at 1 μM. However, at both concentrations most of the ^{3}H eluted with the flow-through of the HPLC, and this ^{3}H 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}H]3-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 the αM4 segment, at αHis-408 and αCys-412, and in the agonist binding site, at αTyr-190 and αTyr-198, as well as minor incorporation elsewhere. While the incorporation in αM4 was independent of the presence of other drugs, the incorporation at αGlu-262 increased for nAChR in the desensitized state, while incorporation at αTyr-190/αTyr-198 was seen only in the absence of carbamylcholine 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 carbamylcholine. This increased incorporation was due to the desensitization of the nAChR since other agonists also increased the incorporation, while the incorporation was lowest in the presence of pancuronium or αBgTx. The competitive antagonists dTC and gallamine caused only a partial increase in the incorporation in the α-subunit. Sequence analysis showed that the increased photoincorporation in the presence of carbamylcholine was due to increased incorporation at αGlu-262, at the C-terminus of αM2. The incorporation of 1 μM [^{3}H]3-azioctanol at αGlu-262 in the absence of carbamylcholine or αBgTx was ~2% the incorporation in the presence of carbamylcholine, likely due to the fact that even in the absence of agonist ~10% of the nAChR are in the desensitized state in isolated *Torpedo* membranes (Boyd, 1994).

While the primary pharmacology observed at the level of the intact subunit was the increased incorporation in the presence of carbamylcholine, attributable to increased incorporation at αGlu-262, analysis of subunit fragments revealed that there was also photolabeling of αTyr-190 and αTyr-198 inhibitable by the presence of carbamylcholine or αBgTx. These residues have both been labeled previously by competitive antagonists, such as [^{3}H]d-tubocurare (Chiara and Cohen, 1997) and DDF (Dennis *et al.*, 1988), and agonists, such as [^{3}H]nicotine (Middleton and Cohen, 1991). The efficiency of incorporation in these residues, 0.012% of Tyr-190 labeled at 1 μM and 1.3% labeled at 275 μM [^{3}H]3-azioctanol, was lower than the incorporation in αGlu-262 even in the absence of carbamylcholine, 0.12% at 1 μM and 14% at 275 μM [^{3}H]3-azioctanol. The inhibition of this incorporation by the presence of carbamylcholine or αBgTx established that the occupancy of the agonist binding site prevented the accessibility of [^{3}H]3-azioctanol to these side chains.

In the presence of carbamylcholine, the aromatic amine noncompetitive antagonist meproadifen partially inhibited the incorporation in the α-subunit, although two other aromatic amine noncompetitive antagonists, PCP and QX-222, did not. The presence of meproadifen resulted in an ~60% inhibition in the incorporation at the subunit level, as well as an ~70% inhibition at αGlu-262. This residue was also labeled by [^{3}H]meproadifen mustard (Pedersen and Cohen, 1990). While charged noncompetitive antagonists fully inhibit the incorporation of [^{3}H]meproadifen mustard, indicating that these drugs bind in a mutually exclusive manner, it is not known whether a charged and an uncharged noncompetitive antagonist can bind the channel simultaneously. Therefore, further studies of the binding of these noncompetitive antagonists are necessary before the partial inhibition of the incorporation can be clearly understood.

Although the photoincorporation of most drugs studied which photoincorporate into residues in M2 in the ion channel domain, including [^{3}H]chlorpromazine, [^{3}H]triphethylphosphonium, [^{125}I]TID, and [^{3}H]tetracaine, are inhibited by the presence of high concentrations of the nonradioactive analog, the presence of 1 mM octanol did not inhibit the incorporation of 1 μM[^{3}H]3-azioctanol in the α-subunit in the presence of carbamylcholine. However, if octanol behaves similarly to 3-azioctanol, this result is not unexpected. As shown in Figure 3-6, the incorporation of [^{3}H]3-azioctanol in the α-subunit in the presence of carbamylcholine increased approximately linearly up to ~1.5 mM [^{3}H]3-azioctanol. This linearity indicates a lack of saturation of [^{3}H]3-azioctanol incorporation in the entire subunit (although the incorporation at certain sites may have been saturated). Therefore, if the concentration of [^{3}H]3-azioctanol were held constant while the concentration of non-radioactive 3-azioctanol was increased, then the incorporation of [^{3}H]3-azioctanol would appear constant in this range (for example, see Figure 3-1). While 1 mM octanol would be expected to inhibit the incorporation at sites that were fully bound below 1 mM octanol, it would not inhibit the incorporation at the several sites that were not fully bound at 1 mM octanol.

The IC_{50} of 3-azioctanol is ~30 μM (Husain, *et al*, 1999). The incorporation of [^{3}H]3-azioctanol in α-subunit in the presence of carbamylcholine, however, was half-maximum at ~1 mM, well above the concentration at which the binding at the inhibitory site should have been fully occupied. As was demonstrated, multiple sites of labeling contributed to the total incorporation in the α-subunit at 1 μM [^{3}H]3-azioctanol. A comparison of the incorporation of [^{3}H]3-azioctanol at 1 μM and 275 μM (Table 3-1) showed that the only site of incorporation which did not increase approximately linearly with concentration was αGlu-262 which was labeled at ~6% at 1 μM and ~33% at 275 μM, an increase of only ~6-fold. The other sites, those in αM4 and the agonist site,

which were labeled similarly in the absence and presence of carbamylcholine, showed an ~100-fold increase between 1 μM and 275 μM, indicating a lack of saturation at these sites at these concentrations. More detailed studies are required to determine the concentration of [^{3}H]3-azioctanol necessary to produce half-maximum incorporation in αGlu-262 as well as the maximum labeling at this residue.

Between 1 μM and 275 μM [^{3}H]3-azioctanol there was a linear increase in the level of incorporation at αTyr-190/αTyr-198 in the agonist site. Although no studies have been carried out with [^{3}H]3-azioctanol, octanol at concentrations up to 4 mM did not inhibit [^{3}H]ACh binding, while butanol, which could be studied at high concentrations, inhibited the binding with a K_I of ~80 mM (Firestone *et al.*, 1994). Since [^{3}H]3-azioctanol labeled the agonist site, but only in the absence of carbamylcholine or αBgTx, it is possible that the inhibition of agonist binding by alcohols is due to direct, low affinity competition of the alcohol with the agonist for the agonist binding site. Further studies are required to determine whether the labeling at the agonist site saturates at higher concentrations of [^{3}H]3-azioctanol or increases linearly over the range of accessible concentrations.

Although the non-M2 sites did not show saturating incorporation between 1 and 275 μM [^{3}H]3-azioctanol, it is possible that the levels of incorporation in a given residue were underestimated. While the incorporation in αV8-20 and αV8-18 appeared stable under the HPLC conditions used, ~60% of the ^{3}H in the αV8-10 fragment was eluted in the flow-though of the HPLC. Therefore, at all concentrations the incorporation at αHis-408 or αCys-412, or possibly another site, was most likely underestimated, due to instability of the photoadduct.

The high efficiency of the incorporation of [^{3}H]3-azioctanol into αGlu-262 could be due to preferential reactivity of [^{3}H]3-azioctanol with glutamates. However, while the

only other reported amino acid labeled by an aliphatic diazirine was a glutamate of a hexosaminidase (Liessem *et al.*, 1995), in our study [^{3}H]3-azioctanol photoincorporated into a variety of side chains, including histidine, cysteine, alanine, valine, and tyrosine. Acidic side chains at some binding sites showed no [^{3}H]3-azioctanol incorporation. For example, in αM4 there was reactivity with alanine, histidine, and cysteine, but no reaction with αAsp-407 at the N-terminus of αM4. Therefore, the high reactivity with αGlu-262 is most likely due primarily to a higher affinity of [^{3}H]3-azioctanol for that region of the ion channel.

The preferential labeling of the M2 segment of the α-subunit by [^{3}H]3-azioctanol contrasts with the labeling seen in the desensitized state for other NCAs (including chlorpromazine, triphenylphosphonium, and 3-trifluoromethyl-3-phenyl diazirine (Karlin and Akabas, 1995)) which labeled amino acids in the M2 segment of each subunit in the desensitized state. Meproadifen mustard also reacted selectively with αGlu-262, however. Although the observed preference may be partially attributable to higher reactivity of the reactive intermediate with glutamates, in the β-subunit the equivalent residue is an aspartate, which should not react very differently from a glutamate. This position in β, however, when mutated to a cysteine, is not modified by water-soluble modification reagents, while a cysteine at α262 is (Akabas *et al.*, 1994; Zhang and Karlin, 1998). Therefore, the three-dimensional structure of the nAChR β-subunit, and perhaps the γ- and δ-subunits, is not similar to that of α in this region of the ion channel domain, and the preferential incorporation into the α-subunit may reflect a unique conformation of the α subunit in this region.

Previous studies aimed at elucidating the site of long chain alcohol binding on the nAChR have implicated residues at the 10' position (Forman, 1997; Forman *et al.*, 1995), while studies with the $GABA_A$ receptor have indicated the contribution of a residue in

M2 at the 15' position, which faces away from the channel lumen on a turn of the helix between the 13' and 17' residues (Wick *et al.*, 1998). Modeling the M2 segment as an α-helix with the azi group of 3-azioctanol near αGlu-262 (Figure 3-12) shows that the carbon chain of 3-azioctanol only reaches to the 13' residues, and is incapable of reaching to this level on the opposite face of the helix. The photolabeling and electrophysiology results, then, are in apparent disagreement. However, the electrophysiology studies measure octanol inhibition of the open state of the receptor, while the photoaffinity labeling studies are done with a desensitized receptor. It is possible that octanol binds differently in the two states, perhaps 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 nature of the region near αGlu-262.

The studies presented here provide strong evidence that, in the desensitized state of the nAChR, [^{3}H]3-azioctanol binds within the ion channel domain near αGlu-262. Further studies, such as photoincorporation of [^{3}H]3-azioctanol in the open channel or the effects of site directed mutagenesis of the N-terminal end of the M2 segment on the inhibition of the nAChR by octanol, will be necessary to further refine the site of action of long chain alcohols on the nAChR.