Identification of Binding Sites in the Nicotinic Acetylcholine Receptor for
[3H]Azetomidate, a Photoactivatable General Anesthetic†

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†This research was supported by US Public Health Service Grant GM58448, by an award to
Harvard Medical School from the Howard Hughes Medical Biomedical Research Support
Program for Medical Schools, and by a Brooks Foundation Research Fellowship in
Neurobiology (to S.N.).

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Running Title: nAChR Amino Acids Photolabeled by [3H]azetomidate
ABSTRACT

To identify binding domains in a ligand-gated ion channel for etomidate, an intravenous general anesthetic, we photolabeled nicotinic acetylcholine-receptor (nAChR)-rich membranes from *Torpedo* electric organ with a photoactivatable analog, [³H]azietomidate. Based upon the inhibition of binding of the noncompetitive antagonist [³H]phencyclidine, azietomidate and etomidate bind with ten-fold higher affinity to nAChRs in the desensitized state (IC₅₀ = 70 µM) than in the closed channel state. In addition, both drugs between 0.1 and 1 mM produced a concentration dependent enhancement of [³H]ACh equilibrium binding affinity, but they inhibited binding at higher concentrations. UV irradiation resulted in preferential [³H]azietomidate photoincorporation into the nAChR α and δ subunits. Photolabeled amino acids in both subunits were identified in the ion channel domain and in the ACh binding sites by Edman degradation. Within the nAChR ion channel in the desensitized state, there was labeling of αGlu-262 and δGln-276, at the extracellular end, and δSer-258 and δSer-262, towards the cytoplasmic end. Within the ACh binding sites, [³H]azietomidate photolabeled αTyr-93, αTyr-190, and αTyr-198 in the site at the α-γ interface and δAsp-59 (but not the homologous position, γGlu-57). Increasing [³H]azietomidate concentration from 1.8 to 150 µM increased the efficiency of incorporation into amino acids within the ion channel by 10-fold and in the ACh sites by 100-fold, consistent with higher affinity binding within the ion channel. The state dependence and subunit selectivity of [³H]azietomidate photolabeling are discussed in terms of the structures of the nAChR transmembrane and extracellular domains.
INTRODUCTION

At clinically relevant concentrations, most general anesthetics modulate the responses of ligand-gated ion channels in the “Cystine-loop” superfamily that includes nicotinic acetylcholine receptors (nAChRs)\(^1\) and serotonin 5-HT\(_3\) receptors, with cation selective channels, and the GABA\(_A\) and glycine receptors with anion selective channels (1-3). General anesthetics enhance responses for submaximal concentrations of GABA and glycine, and at higher concentrations they can directly activate these receptors, while they noncompetitively inhibit nAChRs. Members of this superfamily contain five homologous subunits arranged about a central axis that is the ion channel (4, 5). Each subunit has a large N-terminal domain that contributes to the receptor extracellular domain and four transmembrane segments (M1-M4), organized as a four helix bundle, with M2 segments from each subunit contributing to the lumen of the ion channel (6). In the muscle-type nAChR, with a subunit stoichiometry \(\alpha_2\beta\gamma\delta\), the two agonist binding sites, which are in the extracellular domain at a distance 30 Å above the lipid bilayer, are at the interfaces between the \(\alpha\)-\(\delta\) and \(\alpha\)-\(\gamma\) subunits. The crystal structure of the molluscan ACh binding protein (AChBP), a soluble, homopentameric homolog of the nAChR extracellular domain, provides a general description of the extracellular domain and transmitter binding sites of nAChRs and other members of this protein superfamily (7).

R(+)etomidate, one of the most potent general anesthetics used clinically, acts at micromolar concentrations both as an anesthetic and as a potentiator of the responses to submaximal concentrations of GABA, while at concentrations above 10 \(\mu\)M, it inhibits GABA responses and directly activates GABA\(_A\) receptors (8), and it inhibits nAChRs (9). GABA\(_A\) receptors containing \(\beta2\) or \(\beta3\), but not \(\beta1\), subunits are most sensitive to etomidate, and site directed mutagenesis has identified a single amino acid within the M2 segment that determines
etomidate sensitivity in \( \text{GABA}_A \) receptors \textit{in vitro} (10) and for etomidate anesthesia \textit{in vivo} (11, 12). The position within \( \beta \text{M2} \) conferring etomidate sensitivity corresponds to that within M2 of GABA\(_A\) receptor \( \alpha \) subunits associated with sensitivity to volatile anesthetics (13).

In the absence of atomic resolution structures of these ligand-gated ion channels in the presence of anesthetics, it is difficult to distinguish whether the positions at which substitutions alter anesthetic potency contribute directly to anesthetic binding sites or are involved in the transduction mechanism and allosterically modulate anesthetic potency. Photoaffinity labeling provides a complementary approach to identifying amino acids contributing to drug binding sites (reviewed in (14, 15)). For the nACHR, available in high abundance from the electric organs of \textit{Torpedo}, photoreactive agonist and antagonists have provided extensive identification of amino acids contributing to the transmitter binding sites and to the ion channel (16, 17). Amino acids photolabeled by 3-[\(^3\)H]aziotocanol, a general anesthetic containing a photoreactive diazirine, have been identified at the extracellular end of the M2 ion channel domain, in the agonist binding site, and at the lipid interface (18).

Azietomidate, a diazirine derivative of etomidate, has been recently developed as a photoreactive analog to identify etomidate binding sites in GABA\(_A\) receptors and nACHRs (19). The two drugs are equipotent as anesthetics for tadpoles and as positive allosteric modulators of GABA responses, and azietomidate inhibits agonist activation of muscle type nACHRs with \( \text{IC}_{50} = 25 \mu \text{M} \). In addition, \(^3\)Hazietomidate at 1 \( \mu \text{M} \) was shown to photoincorporate preferentially into the \textit{Torpedo} nACHR \( \alpha \) and \( \delta \) subunits, with labeling of the \( \alpha \) subunit enhanced and the \( \delta \) subunit inhibited in the presence of agonist. Within the \( \alpha \) subunit, the agonist-enhanced labeling was localized to a 20 kDa fragment containing the M1, M2, and M3 transmembrane segments, while labeling inhibited by agonist was localized to an 18 kDa fragment containing ACh binding
site Segments A and B. We now characterize the effects of azietomidate and etomidate on the 
binding of radiolabeled drugs to the nAChR agonist sites and within the ion channel, and we use 
protein chemistry techniques to identify the amino acids contributing to [3H]azietomidate 
binding sites in the nAChR.
EXPERIMENTAL PROCEDURES

Materials. nAChR-enriched membranes were isolated from Torpedo californica electric organ (20). The final membrane suspensions were stored in 38% sucrose at -80 °C under argon. The membranes used here contained 1-2 nmol of [3H]acetylcholine (ACh) binding sites per milligram of protein. [3H]Azietomidate (11 Ci/mm) and nonradioactive R(+)-azietomidate were synthesized as described previously (19). [3H]Azietomidate was stored at a concentration of 1.5 μM at -80 °C in ethanol, which was removed via evaporation immediately prior to the addition of membranes or Torpedo physiological saline (TPS: 250 mM NaCl, 5 mM KCl, 3 mM CaCl₂, 2 mM MgCl₂, 5 mM sodium phosphate, pH 7.0). For studies of photoincorporation at 150 μM, [3H]azietomidate stock at 11 Ci/mm was isotopically diluted with an appropriate volume of a freshly made stock of nonradioactive azietomidate at 1 mg/mL in ethanol. This solution was then dried down prior to the addition of membranes as outlined above. R-(+)-etomidate was gift from Dr. David Gemell (Organon Labs, U.K.). [3H]Phencyclidine (PCP, 27 Ci/mm) was from New England Nuclear and [3H]ACh (3.6 Ci/mm) was synthesized from choline and [3H]acetic anhydride. Staphylococcus aureus glutamylendopeptidase (V8 protease) was from ICN Biomedical Inc; endoproteinase Lys C (EndoLys-C) from Roche Molecular Biochemicals. Trifluoroacetic acid was from Pierce, 10% Genapol C-100 was from Calbiochem.

Radioligand Binding Assays. The equilibrium of [3H]ACh and [3H]PCP in the presence or absence of 1 mM carbamylcholine (Carb) to Torpedo nAChR-rich membranes in TPS was measured by centrifugation in a TOMY MXT-150 microcentrifuge as described previously (21). Membrane suspensions were pretreated with diisopropylphosphofluoridate (~0.5 mM) for 15 min to inhibit acetylcholinesterase activity. For [3H]ACh (20 nM), binding was measured using dilute membrane suspensions (1 mL, 84 μg protein/mL, 40 nM ACh binding sites), whereas for
[\textsuperscript{3}H]PCP (6 nM), 200 \mu L aliquots at 0.7 mg protein/mL were used. Membrane suspensions were equilibrated with [\textsuperscript{3}H]ACh and [\textsuperscript{3}H]PCP for 30 min and 2 h, respectively, prior to centrifugation. Non-specific binding of [\textsuperscript{3}H]ACh and [\textsuperscript{3}H]PCP were determined in the presence of, respectively, 1 mM Carb and 1 mM proadifen (+ Carb) or 1 mM tetracaine (- Carb).

**Data Analysis.** The concentration-dependence of azietomidate or etomidate inhibition of radioligand binding and d-tubocurarine(dTC) inhibition of [\textsuperscript{3}H]azietomidate labeling of nAChR subunits was fit to:

\[
fx = f_0 / \left[1 + \left(x / IC_{50}\right)^n\right] + f_{ns}
\]

where \(f_x\) is the total [\textsuperscript{3}H]ACh or [\textsuperscript{3}H]PCP binding or [\textsuperscript{3}H]azietomidate subunit labeling in the presence of inhibitor concentration \(x\); \(f_0\) is the specific radioligand binding or [\textsuperscript{3}H]azietomidate incorporation in the absence of inhibitor; \(f_{ns}\) is a fixed parameter defined by the non-specific binding or labeling; \(IC_{50}\) is the total inhibitor concentration associated with 50 % inhibition of radioligand binding or subunit photolabeling; \(n\) is the Hill coefficient.

**Photolabeling of nAChR-enriched Membranes with [\textsuperscript{3}H]Azietomidate.** Freshly thawed nAChR-rich *Torpedo* membranes were diluted with TPS and pelleted, and the pellets were resuspended at 2 mg protein/ml (~1 \mu M nAChR) in TPS supplemented with 1 mM oxidized glutathione to serve as an aqueous scavenger. Membrane aliquots were combined with [\textsuperscript{3}H]azietomidate and agitated for 5 min prior to the addition of other drugs as noted in the figure legends. Samples were incubated for 1 h in the dark at 4 °C in polypropylene microfuge tubes after the addition of drugs. For photolabeling on an analytical scale, 200 \mu g aliquots were placed in a 96 well polypropylene microtiter plate (Falcon #3911), while preparative photolabeling (10 mg protein) was performed in a 2 cm glass petri dish with a stir bar. The suspensions, on ice, were irradiated for 25 min in a horizontal photochemical chamber reactor (Rayonet RPR-200,
Southern New England Ultraviolet Company, Branford, CT) using RPR-3500 bulbs that emit ~24 watts with an intensity maxima at 365 nm. In analytical experiments the suspensions were then diluted with 4X sample loading buffer and submitted to SDS-PAGE, while preparative samples were pelleted and solubilized in 1X sample buffer. In control analytical photolabeling experiments, we assessed by SDS-PAGE the dependence upon UV irradiation of the $^3$H incorporation into membrane polypeptides. No covalent incorporation occurred when samples were incubated for 90 min in the absence of irradiation, and no significant “dark reaction” was detected when samples were incubated in the dark for 30 min after irradiation for 15 min. When membrane suspensions containing 1 μCi were electrophoresed after incubation for 60 - 90 min in the absence of irradiation, a time-independent background of ~100 cpm was associated with each gel slice, compared to the 1000 to 10000 cpm incorporated after irradiation in gel slices containing nAChR subunits or other membrane polypeptides. When after irradiation samples were incubated in the dark for a further 30 min before dilution in sample loading buffer, the counts in the gel slices were within 5% of the nonincubated samples.

**Gel Electrophoresis.** SDS-PAGE was performed as described by Laemmli (22) with modifications (23). The polypeptides were resolved on a 1.5-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 Pharmacia Biotech) for 30 min, dried, and exposed at -80°C to Eastman Kodak X-OMAT film for various times (4-8 weeks). Incorporation of $^3$H into individual polypeptides was also quantified by scintillation counting of excised gel slices (20). For fragmentation of the nAChR $\alpha$ subunit by “in gel” digestion with *S. aureus* V8 protease, following electrophoresis, the gels were briefly stained with Coomassie Blue and destained to allow visualization of the subunits. The nAChR $\alpha$
subunit from a preparative scale labeling was then excised and placed directly into individual wells of a 1.5 mm mapping gel, composed of a 5-cm long 4.5% acrylamide stacking gel, and a 15 cm 15% acrylamide separating gel (23, 24). *S. aureus* V8 protease (200 μg) in overlay buffer (5% sucrose, 125 mM Tris-HCl, 0.1% SDS, pH 6.8) was added to each well. 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 with Coomassie Blue, and the proteolytic fragments of ~20 kDa (αV8-20), 18 kDa (α V8-18) and 10 kDa (α V8-10) were visualized and excised (23). The excised proteolytic fragments were isolated by passive elution into 15 mL 0.1 M NH₄HCO₃, 0.1% SDS, and 2.5 mM dithiotreitol. The eluate was concentrated using Vivaspin 15 Mr 5,000 concentrators (Vivascience Inc., Edgewood, NY). To remove excess SDS, acetone was added (70% final volume), and following incubation at -20 °C overnight, the polypeptides were pelleted.

**Proteolytic Digestions.** For digestion with EndoLysC or V8 protease, acetone-precipitated subunits or subunit fragments isolated from preparative scale labelings (10 mg protein) were resuspended in 200 μL of 15 mM Tris, 0.5 mM EDTA, pH 8.1, 0.1% SDS. For digestion of αV8-18, αV8-20 or δ subunit, EndoLysC (0.5 -1.5 units in 100 μL water) was added, and after 14 days at room temperature, the αV8-18 and αV8-20 digests were fractionated by HPLC, while the δ subunit digest was fractionated on 1.5 mm thick, Tricine SDS-PAGE gels (25, 26). δEKC-21 in resuspension buffer was digested with V8 protease (1 μg) at room temperature for 3-4 days before separation of fragments by HPLC.

**HPLC Purification.** Proteolytic fragments from the nAChR α and δ subunit digests were purified by reverse-phase HPLC on an Agilent 1100 binary HPLC system, using a Brownlee C4-Aquapore column (100 x 2.1 mm, 7 μM particle size) at 40 °C. Solvent A was
0.08% trifluoroacetic acid in water, and solvent B was 0.05% trifluoroacetic acid in 60% acetonitrile, 40% 2-propanol. A linear stepwise gradient at 0.2 mL/min was used (in % Solvent B): 0 min, 25%; 15 min, 28%; 30 min, 37%; 45 min, 52%; 60 min, 73%; 75 min 100%; 80 min, 100%; 85 min 25%; 90 min, 25%. Fractions were collected every 2 min (45 fractions/run) or 2.5 min (36 fractions/run). The elution of peptides was monitored by absorbance at 214 nm. Aliquots (2-4% by volume) from each fraction were taken to determine the \( ^3 \)H distribution by liquid scintillation counting.

**Sequence Analysis.** Automated N-terminal sequence analysis was performed on an Applied Biosystems Model 494 protein sequencer with an in-line 140C PTH analyzer. Samples were usually applied directly to Biobrene-pretreated, micro TFA glass fiber disks (Applied Biosystems #401111). For samples resuspended in 0.1% SDS, after drying, the filters were treated with gas trifluoroacetic acid (5 min) followed by an ethyl acetate wash (4 min) to remove detergent. HPLC samples (400 µL or 500 µl fractions) were loaded directly onto the glass fiber disks in 20 µl aliquots, allowing the solvent to evaporate at 40 °C between loads. Because sample loading under this condition results in cleavage on the N-terminal side of Trp residues (16), when fragments beginning at δThr-51, which contain a Trp in cycle 7, were sequenced (Figure 8B and Supplemental Figure 2B), the HPLC fractions were loaded directly onto the glass fiber disk, which was then dried and treated with Biobrene. Sequencing was performed using gas-phase trifluoroacetic acid to minimize possible hydrolysis. After conversion of the released amino acids to PTH-amino acids, the suspension was divided into two parts. One portion, usually one-sixth, went to the PTH analyzer, while the remaining five-sixths were collected for scintillation counting. For sequencing of fragments containing αM2 (Figure 7B), two-thirds went to the PTH analyzer and one-third was collected for scintillation counting. For these
samples, the plotted $^3$H release and PTH-amino acid release are those calculated based for the usual 1/6 mass analysis and 5/6 $^3$H determination. Yield of PTH-amino acids was calculated from peak height compared with standards using the Model 610A Data Analysis Program Version 2.1A. Initial and repetitive yields were calculated by a nonlinear least squares regression to the equation $M = I_0 \times R^n$, 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. Details concerning the values of $I_0$ and $R$ for each sequencing run as well as the cpm loaded on the filters and retained after sequencing are provided in the Figure legends for Supplemental Figs. 1 and 2.

$^3$H incorporation into nAChR residues was quantified based on the results of sequence analysis where the mass of that residue was calculated from the initial and repetitive yields. In most cases the increased $^3$H release in that cycle ($\text{cpm}_n - \text{cpm}_{(n-1)}$) was divided by five times the mass of that cycle, since five times more sample is measured for radioactivity than for mass. $^3$H release was divided by one-half the mass when two-thirds were analyzed for mass and one-third analyzed for $^3$H. In either of these calculations, the radioactivity released and the mass levels reflect only the sequenced material. In some cases, the sequencing run was interrupted and the material on the filter treated with $o$-phthalaldehyde as described previously (20). $o$-Phthalaldehyde reacts with primary amines preferentially over secondary amines (i.e. proline) and can be used at any sequencing cycle to block Edman degradation of peptides not containing an N-terminal proline at that cycle (27).
RESULTS

Etomidate and Azietomidate Inhibition of [3H]PCP and [3H]ACh Binding. We tested etomidate and azietomidate as inhibitors of the binding to *Torpedo* nAChR-rich membranes of [3H]phencyclidine ([3H]PCP), a positively charged, aromatic amine noncompetitive antagonist. [3H]PCP binds with high affinity (K\text{eq} = 1 \mu M) to a single site per nAChR in the desensitized state and more weakly (K\text{eq} = 7 \mu M) in the absence of agonist (28). In the absence or presence of agonist, azietomidate or etomidate at high concentrations inhibited the specific binding of [3H]PCP by >95% (Fig. 1). For nAChRs in the desensitized state, the concentration dependence of inhibition was fit to a single site model with IC\text{50} equal to 70 \mu M for azietomidate and 130 \mu M for etomidate. In the absence of agonist, both drugs inhibited [3H]PCP binding with IC\text{50} = 0.7 mM. We also characterized their effects on the equilibrium binding of [3H]ACh at a concentration sufficient to occupy ~20 % of sites, a condition that makes the assay sensitive to drugs that either increase or decrease ACh binding affinity (29). At concentrations between 100 and 600 \mu M, azietomidate increased [3H]ACh binding by 130%, with inhibition seen only at concentrations above millimolar. Etomidate also produced a similar increase and then decrease of [3H]ACh binding, but at three-fold higher concentrations (Fig. 1). In parallel assays, proadifen (100 \mu M), a well characterized desensitizing aromatic amine noncompetitive antagonist (29), also produced a maximal increase of [3H]ACh binding of 130 % (data not shown).

Photoincorporation of [3H]Azietomidate into nAChR-rich Membranes. The pattern of photoincorporation of [3H]azietomidate into nAChR-rich membranes was determined by incubating membranes with [3H]azietomidate at two concentrations: 1 \mu M (11 Ci/mmol) and 150 \mu M (0.07 Ci/mmol), with the isotope dilution for 150 \mu M chosen so that similar amounts of
$^3$H were added to the membranes at both concentrations. Membranes were photolabeled with $[^3H]$azietomidate in four conditions: (i) in the absence of other drugs; (ii) in the presence of proadifen, which binds to the ion channel and stabilizes nAChRs in the desensitized state without occupying the agonist sites; (iii) in the presence of the agonist Carb, which occupies the ACh sites and stabilizes the nAChR in the desensitized state; or (iv) in the presence of Carb and proadifen. After irradiation, the samples were fractionated on 8% SDS-PAGE. Samples were prepared in triplicate, with one gel prepared for fluorography (Fig. 2A) and two stained and cut into slices for determination of the $^3$H distribution by liquid scintillation counting (Fig. 2B).

At both concentrations $[^3H]$azietomidate was primarily photoincorporated within the nAChR in the $\alpha$ and $\delta$ subunits, and also in a 34 kDa polypeptide that is a mitochondrial voltage-dependent anion channel (VDAC) (30). Photolabeling of VDAC was inhibited by proadifen, but not by Carb.

Comparison of the photolabeling at 1 $\mu$M $[^3H]$azietomidate in the four conditions (Fig. 2A, lanes 2-5 and Fig. 2B, left) established that within the nAChR $\alpha$ and $\delta$ subunits, there were two components of labeling: agonist inhibitable and proadifen inhibitable. However, either proadifen or Carb alone increased the labeling within the $\alpha$ subunit by 1-1.5 fold compared to no drug addition, while labeling in the presence of both Carb and proadifen was at the same level as the no drug control. Within the $\delta$ subunit, proadifen increased incorporation by 80%, while Carb inhibited incorporation by 30% compared to no added drug. The proadifen inhibitable labeling in the $\delta$ subunit in the presence of agonist was ~20% the level of the agonist inhibitable labeling seen in the presence of proadifen. At 150 $\mu$M $[^3H]$azietomidate (Fig. 2A, lanes 6-9 and Fig. 2B, right), $^3$H incorporation in the $\alpha$ subunit was not altered by Carb or proadifen, but it was reduced by 30% in the presence of both drugs. In contrast, the $\delta$ subunit labeling was inhibited by ~60%
in the presence of Carb or Carb and proadifen. At either 1 μM or 150 μM [³H]azietomidate, the total ³H incorporation in the β and γ subunits was ~1/3 that in the δ subunit in the absence of drugs, with Carb or proadifen altering the labeling by <25%.

The efficiency of incorporation into the nAChR α and δ subunits was estimated by calculating the ratio between the pmol [³H]azietomidate incorporated in each subunit band and the pmol of nAChR subunit loaded on the gel. At 2 μM [³H]azietomidate, the ~4000 cpm of Carb or proadifen inhibitable labeling in the α subunit constituted specific labeling of ~0.3% of α subunits, while at 150 μM [³H]azietomidate, the ~1000 cpm of specific labeling in the α and δ subunits constituted labeling of 15% and 30% of subunits, respectively.

Photoincorporation into nAChR subunits was also measured from 10 to 150 μM [³H]azietomidate at a constant radiochemical specific activity. Membranes were photolabeled in the absence of any additional drugs, in the presence of Carb, or with Carb and proadifen (Fig. 3). In the absence of Carb, photoincorporation in the α subunit increased linearly, whereas in the presence of Carb incorporation increased up to ~75 μM [³H]azietomidate and then appeared to saturate. At 150 μM, ³H incorporation into the α subunit was the same in the absence and presence of Carb, and proadifen inhibited this incorporation by 50%. Incorporation into the δ subunit increased linearly in the absence of agonist, with labeling reduced by ~60 % in the presence of Carb. As seen at 1 μM [³H]azietomidate (Fig. 2), the proadifen inhibitable labeling in the δ subunit in the presence of Carb was <20 % the level of Carb inhibitable labeling. The ³H incorporation in the β and γ subunits was less than 20% that of the δ subunit labeling in the absence of Carb at all concentrations.
Inhibition of Photoincorporation by d-Tubocurarine. At 1 μM [3H]azietomidade, there was agonist inhibitable labeling in the α and δ subunits in the presence of proadifen, with any agonist inhibitable labeling in the γ subunit at less than 15% the labeling in the δ subunit. While the agonist-inhibitable labeling within the δ subunit was likely to reflect binding to the agonist site at the α-δ interface, we wanted to determine whether the labeling in the α subunit resulted from binding at that site or at the site at the α-γ interface. To address this, we took advantage of the fact that the competitive antagonist d-tubocurarine (dTC) binds with high affinity to the site at the α-γ interface (Keq = 50 nM) and with low affinity to the α-δ site (Keq = 4 μM) (31). We determined the concentration dependence of dTC inhibition of [3H]azietomidade photolabeling of nAChRs also equilibrated with proadifen (Fig. 4). After photolabeling and SDS-PAGE, bands containing the nAChR subunits were excised from the stained gels, and 3H photoincorporation was measured by scintillation counting. At high concentrations, dTC inhibited subunit labeling by the same extent as Carb, with the Carb-inhibitable labeling in the α, γ, and δ subunits of 1850, 280, and 3630 cpm, respectively. The concentration dependence of dTC inhibition of δ subunit labeling was fit by a single site model with IC50 = 9 μM, while for the α and γ subunits, IC50 = 1 μM (total concentration).

Inhibition of Photoincorporation by Etomidate. We also characterized by SDS-PAGE and gel slice analysis the effects of 300 μM (R+)-etomidate on the photoincorporation of 1μM [3H]azietomidade into nAChR-rich membranes (Fig. 5). As seen for proadifen (Fig. 2), in the absence of Carb, etomidate increased [3H]azietomidade incorporation into the nAChR α and δ subunits. In the presence of Carb, meprooacidifen, the quaternary ammonium analog of proadifen, inhibited α subunit photolabeling by 60% (as did proadifen, not shown), and etomidate reduced incorporation by half that amount. VDAC photolabeling was inhibited by 75% by
nonradioactive etomidate, but not by meproafen (in contrast to proadifen), and none of the drugs altered the photolabeling of the Na\(^+\)/K\(^+\)-ATPase \(\alpha\) subunit.

**Mapping \[^{3}\text{H}]\text{Azietomidate Photoincorporation into }\alpha\text{ and }\delta\text{ Subunit Proteolytic Fragments.}** For nAChRs labeled on a preparative scale (10 mg protein) with 1.8 \(\mu\text{M}\) \[^{3}\text{H}]\text{azietomidate} in the absence and presence of Carb, the \(^{3}\text{H}\) distribution within the \(\alpha\) and \(\delta\) subunits was first characterized by digestion of the isolated \(\alpha\) subunit with \(S.\text{ aureus}\ V8\) protease and the \(\delta\) subunit with EndoLysC, followed by fractionation of the digests by SDS-PAGE (Fig. 6). Digestion of nAChR \(\alpha\) subunit in gel by V8 protease generates four non-overlapping subunit fragments referred to as: \(\alpha\text{V8-4}\) (a 4 kDa fragment), containing the N-terminal 45 amino acids of \(\alpha\) subunit; \(\alpha\text{V8-10}\), (a 10kDa fragment), beginning at \(\alpha\text{Asn-338}\) and including the M4 transmembrane segment; \(\alpha\text{V8-18}\) (a 18 kDa fragment), beginning at \(\alpha\text{Val-46}\) and including segments A and B of agonist binding site; and \(\alpha\text{V8-20}\) (a 20 kDa fragment), beginning at \(\alpha\text{Ser-173}\) and containing segment C of the binding site as well as the M1-M3 transmembrane segments (23). For nAChRs photolabeled in the presence of Carb, \(^{3}\text{H}\) incorporation in \(\alpha\text{V8-20}\) was increased by 2.4 fold, while within \(\alpha\text{V8-18}\) it was inhibited by 85%. \(^{3}\text{H}\) incorporation into \(\alpha\text{V8-10}\) was the same in the absence or presence of Carb.

A solution digest of \(\delta\) subunit by endoproteinase Lys C (EndoLys-C) generates several large subunit fragments, including a 10kDa fragment (\(\delta\text{EKC-10}\)) (32), beginning at \(\delta\text{Met-257}\) at the N-terminus of \(\delta\text{M2}\) and including \(\delta\text{M3}\), and a 21 kDa fragment (\(\delta\text{EKC-21}\)), beginning at \(\delta\text{Glu-48}\) and containing ACh binding site segments D, E and F (33). \(^{3}\text{H}\) incorporation into \(\delta\text{EKC-10}\) was increased 2.6 fold in the presence of Carb, while incorporation into \(\delta\text{EKC-21}\) was reduced by \(\sim\)80%.
[^3]H]Azietomidate Photolabels αGlu-262 in αM2. To identify amino acids labeled by 1.8 μM[^3]H]azietomidate, αV8-20 was digested in solution with EndoLysC, which is known to generate fragments starting at αMet-243 (the N-terminus of αM2) and at αHis-186 (that contains ACh binding site Segment C) that can be purified by reverse phase HPLC (18, 34). When the EndoLysC digests of[^3]H]azietomidate-labeled αV8-20 were fractionated by HPLC, for nAChRs labeled in the presence of Carb there was a broad hydrophobic [H] peak (~98% organic), while for the sample labeled in the absence of Carb, the [H] was reduced by 90% in those fractions (Supplemental Fig. 1A). When these fractions were pooled and sequenced (Fig. 7A), the primary sequence began at αMet-243 and a secondary sequence at αSer-173, the N-terminus of αV8-20. For the sample labeled in the presence of Carb, there was a single peak of [H] release of 2220 cpm in cycle 20, while for the sample labeled in the absence of Carb, there was only 70 cpm released. Since no [H] release was detected above background when intact αV8-20 (14,000 cpm), beginning at αSer-173, was sequenced for 23 cycles, the release in cycle 20 corresponds to labeling of αGlu-262 at the extracellular end of αM2.

[^3]H]Azietomidate Photolabels αTyr-190 and αTyr-93 in the ACh Binding Site. The concentration dependence of dTC inhibition of[^3]H]azietomidate photolabeling in the nAChR α-subunit (Fig. 4) indicated that, despite its low affinity interaction with the ACh binding site,[^3]H]azietomidate might be photolabeling amino acids within the agonist site at the interface of the α and γ subunits. When the EndoLysC digests of αV8-20 were fractionated by HPLC (Supplemental Fig. 1A), the sample labeled in the absence of Carb contained a peak of [H] eluting at ~73% organic that was not present in the sample labeled in the presence of Carb. When these fractions were pooled and sequenced (Fig. 7B), the primary sequence began at αHis-186, which contains αTyr-190 in the fifth cycle of Edman degradation and extends through αM1, and the
secondary sequence began at \( \alpha \text{Ser-173} \). There was \(^3\text{H}\) release in cycles 5 (340 cpm) and 13 (50 cpm) that was reduced by \( >90\% \) for the sample labeled in the presence of Carb. The \(^3\text{H}\) release in these cycles corresponded to labeling of \( \alpha \text{Tyr-190} \) and \( \alpha \text{Tyr-198} \) in ACh binding site Segment C.

\( \alpha \text{Tyr-93} \) of ACh binding site Segment A was identified as the amino acid labeled in \( \alpha \text{V8-18} \) by sequence analysis of fragments isolated by reversed phase HPLC from an EndoLysC digest. The \(^3\text{H}\) eluted in a broad peak at \( \sim 64\% \) organic for the sample labeled in the absence of Carb (Supplemental Fig. 1B). When the fractions were pooled and an aliquot sequenced (Fig. 7C), there was a single sequence beginning at \( \alpha \text{Lys-77} \). The \(^3\text{H}\) release in cycle 17 (730 cpm) for the sample labeled in the absence of Carb, which was not seen for +Carb sample, corresponded to labeling of \( \alpha \text{Tyr-93} \). The labeling of \( \alpha \text{Tyr-93} \) was confirmed by the \(^3\text{H}\) release also seen in cycle 17 when another aliquot was sequenced, with this sample treated in the fifth cycle of Edman degradation with \( o \)-phthalaldehyde, which reacts selectively with the free N-termini of amino acids other than proline, a secondary amine, blocking Edman degradation (not shown).

**[^3\text{H}]Azietomidate Photoincorporation within the \( \delta \text{M2 Segment} \).** The initial mapping with EndoLysC of the distribution of \(^3\text{H}\)azietomidate incorporation in the \( \delta \) subunit (Fig. 6) established that there was Carb-enhanced labeling in a \( \sim 10 \) kDa fragment and Carb-inhibitable labeling in a 21 kDa fragment. When the 10 kDa gel bands (\( \delta \text{EKC-10} \)) isolated from nAChRs labeled with 1.8 \( \mu \text{M} \) \(^3\text{H}\)azietomidate in the absence or presence of Carb were further purified by reverse phase HPLC, for the +Carb sample, the \(^3\text{H}\) eluted in a single, hydrophobic peak (\( \sim 79 \% \) organic) which was reduced by \( >90\% \) in the -Carb sample (Supplemental Fig. 2A). When these fractions were pooled and sequenced (Fig. 8A), there was a single fragment beginning at
δMet-257, the N-terminus of δM2. For the +Carb sample, there was a major peak of $^3$H release at cycle 20 (2100 cpm), and additional release in cycles 2 (740 cpm) and 6 (160 cpm), all of which were reduced by more than 90% in the -Carb sample. The $^3$H release in cycles 2, 6, and 20 corresponded to labeling of δSer-258, δSer-262, and δGln-276.

$[^3]$H]Azietomidate Photolabels δAsp-59 in the Agonist Binding Site. Sequence analysis of an aliquot of gel-purified δEKC-21 (-Carb, 1500 cpm) isolated from nAChRs labeled with 1.8 μM $[^3]$Hazietomidate revealed the presence of δ subunit fragments beginning at δHis-20 (3pmol), δHis-26 (3 pmol) and δGlu-47 (1 pmol), as reported previously (33). During 17 cycles of Edman degradation, there was $^3$H release above background only in cycle 13 (20 cpm), which would be consistent with labeling of δAsp-59 in ACh binding site segment D in the fragment beginning at δGlu-47. Further evidence for labeling of δAsp-59 was seen when an aliquot of δEKC-21 (-Carb, 1500 cpm) was sequenced after digestion with V8 protease, which cleaves δ subunit after δGlu-50 (35), positioning δAsp-59 in cycle 9. After digestion, there was 95 cpm released in cycle 9, with no release above background remaining at cycle 13.

To confirm $[^3]$Hazietomidate labeling of δAsp-59 and to determine whether there was additional agonist inhibitable incorporation in the δ subunit, δEKC-21 was isolated from nAChRs labeled with 150 μM $[^3]$Hazietomidate (0.073 Ci/mm mol). When the material eluted from this band was digested in solution with V8 protease and the digest fractionated by reverse phase HPLC, the major peak of $^3$H eluted at ~54% organic (Supplemental Fig. 2B). When these fractions were pooled and sequenced (Fig. 8B), the primary sequence began at δThr-51 and a secondary sequence began at δVal-30. For the sample labeled in the absence of Carb, there was
a major peak of $^3$H release at cycle 9 (490 cpm), corresponding to $\delta$Asp-59, and that $^3$H release was reduced by more than 90% in the sample labeled in the presence of Carb.

**Efficiency of $[^3\text{H}]$Azietomidate Labeling at 1 and 150 $\mu$M.** The percentage incorporation per residue was compared for the labeled amino acids in the ion channel domain and the agonist sites in nAChRs photolabeled at 1.8 and 150 $\mu$M $[^3\text{H}]$azietomidate (Table 1). For the amino acids in the ion channel domain, at each concentration $\alpha$Glu-262 was labeled at ~10-fold higher efficiency than $\delta$Gln-276 and at 100-fold higher efficiency than $\delta$Ser-258 or $\delta$Ser-262. However, between 1.8 and 150 $\mu$M, for each amino acid the labeling increased by only 10-20 fold. At each concentration the amino acids in the agonist binding sites ($\alpha$Tyr-93, $\alpha$Tyr-190, and $\delta$Asp-59) were labeled at similar efficiencies, and the efficiency of labeling increased linearly (100-fold) with concentration from 1.8 to 150 $\mu$M. At 150 $\mu$M $[^3\text{H}]$azietomidate, $\alpha$Tyr-93 and $\alpha$ Tyr-190 were labeled at 21% and 10% efficiency, $\alpha$Glu-262 at 29%, and $\delta$Asp-59 at 32%. The observed dependence of amino acid photolabeling on $[^3\text{H}]$azietomidate concentration is generally consistent with the results of the radioligand binding assays, with azietomidate binding with ~15-fold higher affinity to the ion channel in the desensitized state ($[^3\text{H}]$PCP binding, IC$_{50}$ = 70 $\mu$M) than to the ACh sites ($[^3\text{H}]$ACh binding, IC$_{50}$ ~1 mM).
DISCUSSION

Radioligand binding assays provided evidence for at least two azietomidate binding sites within the nAChR: (i) a site in or near the nAChR ion channel, where azietomidate binds with ten-fold higher affinity to nAChRs in the desensitized state ([3H]PCP inhibition, IC50 = 70 μM) than in the closed channel state; and (ii) the agonist sites, where azietomidate binds with low affinity, inhibiting [3H]ACh binding with IC50 ~1 mM. Analysis of the pharmacologic specificity of [3H]azietomidate photolabeling into the nAChR subunits also provided evidence for two components of labeling. First, there was labeling in the transmembrane domain as evidenced by the inhibition of labeling in the α and the δ subunits by proadifen, a desensitizing noncompetitive antagonist. For nAChRs in the desensitized state and equilibrated with agonist, at 1 μM [3H]azietomidate, ~60% of the α and 20% of the δ subunit labeling were inhibitable by proadifen. Second, agonist binding site labeling was revealed in the presence of proadifen when ~50% of the labeling in the α subunit and 75% of the labeling in the δ subunit were inhibitable by agonist (Carb) or competitive antagonist (dTC). Analysis of the distribution of [3H]azietomidate within large α and δ subunit fragments revealed that the Carb and proadifen inhibitable components of labeling were contained within different nAChR structural domains. The Carb inhibitable labeling was within fragments that contain most of the extracellular domains of those subunits (αV8-18 and δEKC-21), while the proadifen inhibitable labeling was within fragments that contain much of the subunit transmembrane domain (αV8-20 and δEKC-10).

Effects of etomidate on [3H]azietomidate incorporation in the α and δ subunits.

Etomidate at 300 μM increased the incorporation of 1 μM [3H]azietomidate in the nAChR α and δ subunits (Fig. 5). This increased labeling can be explained by the fact that etomidate and
Azietomidate are desensitizing noncompetitive antagonists. They bind to the nAChR ion channel domain with higher affinity in the desensitized state than in the closed state, and they stabilize the desensitized state at lower concentrations than are necessary to occupy the ACh sites (Fig. 1). Etomidate at 300 μM occupies less than 20% of the ACh binding sites, and the effect of nAChR desensitization predominates over competition at the agonist sites. The increased [3H]azietomidate labeling in the presence of etomidate or proadifen (Fig. 2) indicates that azietomidate binds with higher affinity to one or both ACh binding sites in the nAChR desensitized state than in the closed channel state. In contrast, in the presence of Carb, 300 μM etomidate inhibited the [3H]azietomidate labeling of the α subunit by ~50% of the maximal inhibition produced by proadifen or meproadifen. The extent of this inhibition is consistent with the observed concentration dependence of [3H]PCP inhibition, and as noted previously (19), this etomidate inhibitable labeling is contained within the α subunit transmembrane domain (αV8-20). Taken together, these results indicate that etomidate competes with azietomidate in the nAChR ion channel domain and that both drugs bind to the agonist binding sites.

**Azietomidate interactions in the nAChR transmembrane domain.** In the desensitized state, [3H]azietomidate photolabeled amino acids at two different levels of the M2 ion channel domain. Incorporation was most efficient at the extracellular end of the ion channel, with αGlu-262 (αM2-20) labeled at 10 to 20-fold higher efficiency than δGln-276 (δM2-20) at both 1.8 and 150 μM [3H]azietomidate (Table 1). At the cytoplasmic end of the ion channel [3H]azietomidate also clearly reacted with δSer-258 (δM2-2) and δSer-262 (δM2-6), albeit at 1% the efficiency of αGlu-262. Although not visible as plotted in Fig. 7A, the observed ³H release during sequence analysis of αM2 indicates that αSer-248 (αM2-6) is also labeled at ~1% the efficiency of
labeling of αGlu-262, so [3H]azietomidate labeling at the cytoplasmic end of the ion channel domain is not restricted to the δ subunit.

Each of the labeled amino acids in αM2 and δM2 have been previously identified by mutational analyses and affinity labeling as contributing to the lumen of the ion channel (reviewed in (4)). The only structure of the nAChR transmembrane domain that is of sufficiently high resolution to interpret our results is that of the closed state structure (6). With this limitation in mind, in Fig. 9 the amino acids labeled by [3H]azietomidate in the desensitized state are highlighted in green in the closed state structure. Also highlighted are δM2-15 (in magenta), the position in the GABA$_A$ receptor β3 subunit identified as an affinity determinant for etomidate modulation of GABA responses (10), and δTyr-228 (in yellow) in δM1, the amino acid in the nAChR transmembrane domain photolabeled by the volatile anesthetic halothane (36). With a distance of 20 Å between M2-6 and M2-20, the labeling of those positions can not be accounted for by azietomidate binding in the ion channel in a single orientation. Before discussing the likely origin of the labeling at the extracellular and cytoplasmic ends of the ion channel domain, we discuss features of the labeling at the extracellular end.

We do not know whether αGlu-262 is labeled in one or both α subunits, but the labeling of δGln-276 makes it likely that the adjacent αGlu-262 is labeled. Within the closed state structure (Fig. 9), δGlu-280 (blue), which is unlabeled, is positioned one helical turn above the labeled δGln-276, with the intervening δPro-279 producing a kink in the helix. The fact that δGlu-280 is not labeled in the desensitized state suggests that between the closed and desensitized states there is movement around δPro-279 so that δGlu-280 and δGln-276 are no longer in register in the desensitized state. It is also striking that in the closed state structure, the side chains of αGlu-262 and δGln-276 (as well as βAsp-268 at βM2-20) are oriented towards the
lumen of the channel in an environment accessible to azietomidate. The lack of labeling of these amino acids in the closed state indicates either that: (i) the local structure in this region differs significantly between the closed and desensitized states; or (ii) a part of the nAChR extracellular domain blocks access or occludes this apparent pocket.

Although the labeling at δM2-2 and δM2-6 is only at 10 and 1% the efficiency of the labeling at δM2-20 and αM2-20, respectively, the labeling efficiency at all four positions increased by the same extent (10 to 20 fold) as the azietomidate concentration was increased from 1.8 to 150 μM. Thus, there is no evidence that the different efficiencies of labeling result from a different concentration dependence of occupancy of sites at the extracellular and cytoplasmic ends of the ion channel. It is possible that the binding site for azietomidate in the ion channel in the desensitized state is only at the cytoplasmic end near M2-2 and M2-6, but that these residues, δSer-258 and δSer-262, have low intrinsic reactivity and reaction is favored with αGlu-262 and to a lesser extent with δGln-276 as photoactivated azietomidate diffuses out of the ion channel. Such an interpretation appears consistent with previous reports that in the nAChR desensitized state the binding site for [3H]chlorpromazine is near the cytoplasmic end of the ion channel, based upon its photolabeling of amino acids at M2-6 in each subunit (37-39). However, we recently reexamined [3H]chlorpromazine photolabeling in the nAChR desensitized state and found that photolabeling at M2-17 and M2-20 (δLeu-273 and δGln-276) occurred at 10-fold higher efficiency than the labeling at M2-6 (δSer-262) (M. Ziebell and J. B. Cohen, unpublished), a fact that was not seen in the earlier studies where samples were sequenced for fewer than 17 cycles. Thus, based upon photolabeling, for nAChRs equilibrated with agonist, although azietomidate and chlorpromazine have access to a site near the cytoplasmic end of the ion channel, the highly efficient labeling of amino acids at the extracellular end indicates that this
is the binding site with highest occupancy. Further studies, using rapid-mixing and freeze-clamp techniques to trap nAChRs in transient, agonist-stabilized states, will be necessary to determine whether the photolabeling near the cytoplasmic end is favored when most nAChRs are not in the equilibrium desensitized state.

**Azietomidate interactions with the ACh binding sites.** Based upon the inhibition of $[^3]$H]ACh binding, azietomidate binds with only low affinity (IC$_{50} \sim$1 mM) to the ACh binding sites, and interactions with the agonist site are therefore not expected to contribute significantly to the pharmacological inhibition of nAChRs seen at azietomidate concentrations below 100 μM. However, it is the low affinity interactions of $[^3]$H]azietomidate with the agonist binding sites that account for the observed photoincorporation in the nAChR extracellular domain. Thus, at 1 μM $[^3]$H]azietomidate, photolabeling in the α and δ subunits is ~doubled when nAChRs were stabilized in the desensitized state by proadifen, and we established that this labeling, which is agonist-inhibitable, results from labeling of the core aromatics within the ACh binding site (αTyr-93, αTyr-190, and αTyr-198 (Figs. 7B and 7C)) and from reaction with δAsp-59 in ACh binding site Segment D (Fig. 8B).

Based upon the concentration dependence of dTC inhibition of $[^3]$H]azietomidate labeling at the subunit level (Fig. 4), for the agonist binding site at the α-γ interface there was preferential labeling of amino acids in the α subunit, while for the agonist binding site at the α-δ interface, there was preferential labeling of amino acids in the δ subunit. This preferential labeling of amino acids in the α subunit for one agonist binding site and of amino acids in the δ subunit for the other was unexpected. In terms of the structures of the ACh binding sites in a nAChR homology model based upon the structure of the AChBP (Fig. 10), the same α subunit core aromatics contribute to each ACh binding site, and with γGlu-57 the amino acid equivalent to
\(\delta\text{Asp-59}\), there is no reason to expect a difference in the intrinsic reactivities of those side chains. Any labeling of \(\gamma\text{Glu-57}\) must be \(\leq 10\%\) the labeling of \(\delta\text{Asp-59}\), based upon the amount of Carb (or dTC) inhibitable labeling in the \(\gamma\) and \(\delta\) subunits. Interestingly, azietomidate can be docked in either agonist binding site in a fixed atom model with the 3-azibutyl group within the aromatic pocket (which predicts labeling of the core aromatics) or with the benzene ring in the aromatic pocket and with the diazirine oriented towards \(\delta\text{Asp-59}\) (or \(\gamma\text{Glu-57}\)). However, it would be surprising for a ligand with such low affinity to have dramatically different modes of binding in the two ACh binding sites.

Although etomidate does not activate nAChRs, etomidate and azietomidate can directly activate GABA\(_A\) receptors in the absence of agonist (19). Our results with the nAChR indicate that it will be important to determine whether etomidate or azietomidate binding to the GABA binding sites contributes to the direct gating of that receptor.

**Selectivity of Photolabeling with Aliphatic Diazirines.** At 1 \(\mu\text{M}\), \([\text{H}]\)azietomidate photolabeled \(\alpha\text{Glu-262}\) at 10-fold higher efficiency than either \(\delta\text{Gln-276}\) or \(\delta\text{Asp-59}\), but the preferential reaction with \(\alpha\text{Glu-262}\) results at least in part from the higher affinity interaction of azietomidate with the ion channel domain than with the ACh binding sites. Thus, at 150 \(\mu\text{M}\), \([\text{H}]\)azietomidate photolabeled \(\alpha\text{Tyr-93}\), \(\alpha\text{Tyr-190}\) and \(\delta\text{Asp-59}\) in the ACh sites at the same efficiency as \(\alpha\text{Glu-262}\) (Table 1). In contrast, at both 1 and 150 \(\mu\text{M}\) azietomidate, \(\delta\text{Gln-276}\) was labeled at 10\% and \(\delta\text{Ser-258}\) and \(\delta\text{Ser-262}\) at 1\% the efficiency of \(\alpha\text{Glu-262}\). Thus, the reduced labeling at these positions does reflect the lower intrinsic reactivities of these side chains with the photoreactive intermediate.

Although there have been no studies to our knowledge of the relative rates of reaction of photoactivated *aliphatic* diazirines with individual amino acids or with amino acids in test
peptides, our results with azietomidate in conjunction with the previously characterized nAChR amino acids photolabeled by \[^3\text{H} \text{azioctanol}\] (18) begin to define the range of side chains photolabeled in membrane proteins by aliphatic diazirines. \[^3\text{H} \text{Azioctanol}\] and \[^3\text{H} \text{azietomidate}\] both labeled amino acids in the nAChR ion channel domain and in the ACh sites, with differences that establish that the observed patterns of labeling reflect the orientations of the bound ligands and not simply the intrinsic side chain reactivities. Both drugs at low concentrations labeled \(\alpha\text{Glu-262}\) most effectively, but within the ACh binding site azioctanol reacted with \(\alpha\text{Tyr-190}\) and not with \(\alpha\text{Tyr-93}\) or \(\delta\text{Asp-59}\). While we did not characterize \[^3\text{H} \text{azietomidate}\] photolabeling at the lipid interface, \[^3\text{H} \text{azioctanol}\] reacted with \(\alpha\text{His-408}\) and \(\alpha\text{Cys-412}\) in \(\alpha\text{M4}\). Thus the aliphatic diazirines react most efficiently with Asp, Glu and Tyr, but also with Gln, Ser, Cys, and His. For \[^3\text{H} \text{azioctanol}\], there was possible labeling of several aliphatic side chains (18), but this was not seen with \[^3\text{H} \text{azietomidate}\].

UV irradiation of aliphatic diazirines can form reactive intermediates either by the elimination of molecular nitrogen to form a carbene or by conversion of the diazirine to a diazo intermediate which in the presence of a proton donor can form potentially long-lived electrophilic intermediates (15, 40, 41). The side chains that are labeled by azietomidate or azioctanol are consistent with the formation of a photoactivated electrophile, which could be either a singlet carbene or a carbonium ion. However, the selective labeling of a subset of Tyr, Glu or Asp within the ACh binding sites or at the entry to the ion channel indicate that the labeling results from occupancy of binding sites and not diffusional encounters with generally reactive side chains. Further evidence consistent with this conclusion is provided by studies with adenylate kinase, where 3-azioctanol reacted with a His and 7-azioctanol reacted with an Asp that in the crystal structure are in a pocket within 5 Å of each other (42).
The broad range of side chain reactivities seen for azietomidate indicates that this photoreactive anesthetic is well-suited for the identification of etomidate binding sites in GABA_A receptors, the pharmacological target responsible for general anesthesia (11, 12).
REFERENCES


   *J. Biol. Chem.* **252**, 1102-1106


FOOTNOTES

1 Abbreviations: azetomidate, 2-(3-methyl-3H-diaziren-3-yl)ethyl 1-(phenylethyl)-1H-imidazole-5-carboxylate; Carb, carbamycholine; dTC, d-tubocurarine; EDTA, ethylenediamine tetraacetate; EndoLys-C, endoproteinase Lys-C; etomidate, 2-ethyl 1-(phenylethyl)-1H-imidazole-5-carboxylate; GABA_\text{A} receptor, \(\gamma\)-aminobutyric acid type A receptor; HPLC, high-pressure liquid chromatography; nAChR, nicotinic acetylcholine receptor; OPA, \text{o-}phthalaldehyde; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate; TPS, Torpedo physiological saline; Tricine, N-tris-(hydroxymethyl)methylglycine; Tris, Tris-[hydroxymethyl]aminomethane; V8 protease, Staphylococcus aureus endopeptidase Glu-C; VDAC, voltage dependent anion channel.
FIGURE LEGENDS

Figure 1. Effects of azietomidate (open symbols) and etomidate (closed symbols) on the equilibrium binding to *Torpedo* nAChR-rich membranes of $[^3]$H$\text{ACh}$ (▲, △) and $[^3]$H$\text{PCP}$ in the presence (■, □) or absence (●, ○) of carbbamylcholine. For $[^3]$H$\text{PCP}$ (6 nM), specific binding was determined as the difference between total binding and nonspecific binding in the presence of tetracaine (-Carb) or proadifen (+Carb). For $[^3]$H$\text{PCP}$ the inhibition curves were fit to Eq. 1 (Methods). In the presence of Carb, IC$_{50}$ = 73±5 and 130 ±10 μM for azietomidate and etomidate, respectively. In the absence of Carb, IC$_{50}$ = 650 ±150 μM for both drugs. In the absence of azietomidate or etomidate, total binding was 4825 ±330 cpm (+Carb) and 1330 ± 90 cpm (-Carb); nonspecific binding was 430 ±10 cpm (+Carb) and 590 ± 40 (-Carb). Specific binding was 1 and 0.3 nM. For $[^3]$H$\text{ACh}$ (10 nM) total binding was 7500 ± 200 cpm and non-specific was 180 cpm. In parallel experiments, 100 μM proadifen produced a maximal potentiation of $[^3]$H$\text{ACh}$ binding, which was 230% of control.

Figure 2. Pattern of $[^3]$H$\text{azietomidate}$ photoincorporation into nAChR-rich membranes in the presence and absence of Carb and/or proadifen. nAChR rich membranes (200 μg at 2 mg/mL) were equilibrated with 1 μM (11 Ci/mmol) (A: lanes 2-5 and B: left) or 150 μM (A: lanes 6-9 and B: right) (0.073 Ci/mmol) $[^3]$H$\text{azietomidate}$ in TPS in the absence of any drug (lanes 2 and 6), in the presence of 100 μM proadifen (lanes 3 and 7), in the presence of 2 mM Carb (lanes 4 and 8) and in the presence of Carb and proadifen (lanes 5 and 10). After equilibration, samples were irradiated at 365 nm for 25 min and membrane polypeptides separated by SDS-PAGE and visualized by Coomassie Blue (A, lane 1). The gel was processed for fluorography, and exposed to film for 4 weeks (A, lanes 2-9). Indicated on the left are the...
mobilities of nAChR subunits (α, β, γ, δ), rapsyn (Rsn), the α subunit of the (Na⁺/K⁺)-ATPase (αNK), and the mitochondrial voltage dependent anion channel (VDAC). B), ³H incorporation in the absence or presence of Carb and/or proadifen at 1 and 150 μM [³H]azietomidate was quantified by scintillation counting as described under "Experimental Procedures". Bars shown are the mean ± S.D. of duplicate samples.

Figure 3. Concentration dependence of [³H]azietomidate photoincorporation into nAChR-rich membranes. nAChR-rich membranes (200 μg at 2 mg/mL) were equilibrated with varying concentrations of [³H]azietomidate (0.11 Ci/mmol) in the absence of other drugs (○, ●), in the presence of 2 mM Carb (▲, ▼), and in the presence of Carb and 100 μM proadifen (□, △). After irradiation at 365 nm for 25 min, polypeptides were separated by SDS-PAGE and visualized by Coomassie Blue stain. A), Bands corresponding to the nAChR α (●, ▲, ▼) and β (○, □, ▼) subunits, and B), the γ (●, ▲, ▼) and δ (○, □, ▼) subunits were excised, and ³H incorporation quantitated by scintillation counting. Error bars are from the average of two replicates.

Figure 4. Concentration dependence of d-tubocurarine inhibition of [³H]azietomidate photoincorporation into nAChR subunits. nAChR-rich membranes (240 μg aliquots, 245 pmol ACh binding sites) were equilibrated in 240 μl TPS with 0.6 μM [³H]azietomidate, 100 μM proadifen and the indicated concentrations of dTC (0.5 to 30 μM, closed symbols) or 0. 3 mM Carb (open symbols). Each sample was then divided into two aliquots and irradiated at 365 nm for 25 min. The samples were then fractionated by SDS-PAGE on two gels (duplicate experiments), and the protein bands visualized by Coomassie stain. Bands containing the α (●,
○), δ (■, □) and γ subunits (▲, △) were excised, and the \(^3\)H incorporation determined by scintillation counting. The averages with standard deviations of two experiments for each dTC concentration are shown. When fit by Equation 1 (Methods), the inhibition of \(^3\)Hazietomidate labeling of α, δ, and γ subunits by dTC was characterized by IC\(_{50}\) values of 1.1 ± 0.2, 9.3 ± 1.5 and 1.2 ± 0.5 \(\mu\)M, respectively. Nonspecific photolabeling in the presence of proadifen and Carb was 1385 ± 20, 660 ± 20 and 424 ± 10 cpm for α, δ and γ subunits, respectively. Photolabeling of the α subunit of the Na\(^+\)/K\(^+\)-ATPase (90 kDa band) by \(^3\)H azietomidate was 550 ± 30 cpm in the absence or presence of Carb or dTC.

**Figure 5. Effect of etomidate on the photoincorporation of \(^3\)Hazietomidate into nAChR-rich membranes.** nAChR-rich membranes (200 \(\mu\)g at 2 mg/mL in TPS) were equilibrated with 1.5 \(\mu\)M \(^3\)Hazietomidate in the absence of other drugs (-Carb) or with 300 \(\mu\)M (R+) etomidate (-Carb+etom), and in the presence of 300 \(\mu\)M Carb with no other drugs (+Carb), with etomidate (+Carb+etom), or with 100 \(\mu\)M meproadifen (+Carb +mep). Samples were irradiated for 20 min at 365 nm and then fractionated by SDS-PAGE. Polypeptides were visualized by Coomassie Blue, and the bands corresponding to the nAChR α and δ subunits, the α subunit of the Na\(^+\)/K\(^+\)-ATPase and VDAC were excised and \(^3\)H incorporation quantified by scintillation counting.

**Figure 6. Mapping the sites of \(^3\)Hazietomidate incorporation into the nAChR α and δ subunits.** nAChR-rich membranes (10 mg at 2 mg/mL) were labeled with 1.8 \(\mu\)M \(^3\)Hazietomidate in the absence and presence of 2 mM Carb. After irradiation at 365 nm for 25 min, membranes were subjected to SDS-PAGE and the gel stained with Coomassie Blue. A), the stained strips corresponding to the nAChR α subunit were excised and transferred to the well of...
a 15% mapping gel and digested in-gel by *S. aureus* V8 protease, and *B*, the δ subunit was excised, eluted and concentrated, and digested in solution (200 μL; -Carb, 220,000 cpm; +Carb, 36,000 cpm) with EndoLysC (1.5 units) for 2 weeks, and the digest was then fractionated by Tricine gel SDS-PAGE. After electrophoresis, the gels were cut into twenty five 0.5 cm strips, and the $^3$H and polypeptides were eluted and concentrated. For the nAChR α subunit, the peaks of $^3$H corresponded to αV8-10, αV8-18, and αV8-20. For the δ subunit labeled in the absence of Carb, the peak of $^3$H was at ~20 kDa (strips 17-19, δEKC-21), while in the presence of Carb the $^3$H peak was at ~10 kDa (strips 9 and 10, δEKC-10). Eluates from strips containing peaks of $^3$H were pooled and concentrated to 200 μL and $^3$H recovery was determined by counting 1 μL aliquots. Indicated in the Diagrams above are the nAChR α-subunit (upper) and δ-subunit (lower) proteolytic fragments produced by digestion of V8 protease (23) and EndoLysC (33), respectively.

**Figure 7. Sequence analysis of [3H]azietomidate-labeled α subunit fragments isolated by HPLC from EndoLysC digests of αV8-20 (A, B) and αV8-18 (C).** For αV8-20 and αV8-18 isolated from nAChRs labeled with 1.8 μM [3H]azietomidate (Fig. 6), EndoLysC digests were fractionated by reverse phase HPLC (Supplemental Fig. 1). Samples from the HPLC were sequenced for αV8-20 that contained the peak of $^3$H for the +Carb sample (*A*, fractions 39-43) and for the -Carb sample (*B*, fractions 29-31), and from αV8-18 the peak of $^3$H for the -Carb sample (*C*, fractions 21-24). Plotted are the $^3$H (○, ●) and mass released (□, ■) for samples labeled in the absence (○,□) or presence (●,■) of Carb. *A*, The primary sequence began at αMet-243, the N-terminus of αM2, and a secondary sequence at αSer-173, the N-terminus of αV8-20 (+Carb: αMet-243 $I_0 = 12$ pmol, αSer-173 $I_0 = 6$ pmol; -Carb: αMet-243 $I_0 = 8$ pmol,
αSer-173 $I_0 = 7$ pmol). The $^3$H release in cycle 20 (2220 cpm) was consistent with agonist-dependent $[^3]$Hazietomidate incorporation into αGlu-262 (180 cpm/pmol).  

B) For fractions 29-31 the primary sequence began at αHis-186 and a secondary sequence began at αSer-173 (-Carb: αHis-186 $I_0 = 11$ pmol, αSer-173 $I_0 = 6$ pmol; +Carb: αHis-186, $I_0 = 13$ pmol, αSer-173 $I_0 = 7$ pmol) The $^3$H release in cycles 5 (340 cpm) and 13 (50 cpm) was consistent with agonist-inhibitable $[^3]$Hazietomidate incorporation into αTyr-190 (11 cpm/pmol) and αTyr-198 (4 cpm/pmol).  

C) For the labeled fragment from the EndoLysC digest of αV8-18, a single sequence was detected beginning at αLys-77 (-Carb: $I_0 = 33$ pmol; +Carb: $I_0 = 13$ pmol). Any secondary sequences were at <5% that level. The $^3$H release in cycle 17 (730 cpm) was consistent with agonist-inhibitable $[^3]$Hazietomidate incorporation into αTyr-93 (17 cpm/pmol).  

For A through C, the primary sequence for each sample is shown on the top axis.

**Figure 8. Sequence analysis of $[^3]$Hazietomidate-labeled δ subunit protease fragments identifies the labeling of δSer-258, δSer-262, and δGln-276 in δM2 (A) and of δAsp-59 in the ACh binding site (B).** $^3$H (○, ●) and mass released (□, ■) when fractions containing the peak of $^3$H from the HPLC purifications of δEKC-10 (fractions 25-26, Supplemental Fig. 2A, labeling at 1.8 μM $[^3]$Hazietomidate) and of a *S. aureus* V8 protease digest of δEKC-21 (fractions 18-19, Supplement Fig. 2B, labeling at 150 μM $[^3]$Hazietomidate) were sequenced for samples labeled in the absence (○,□) or presence (●,■) of Carb.  

A) For δEKC-10, a single sequence was detected beginning at δMet-257, the N-terminus of δM2, with any other sequence at <5% that level (+Carb: $I_0 = 80$ pmol; -Carb: $I_0 = 50$ pmol). The $^3$H release in cycles 2 (740 cpm), 6 (160 cpm) and 20 (2100 cpm) was consistent with agonist-dependent $[^3]$Hazietomidate
incorporation into δSer-258 (2.2 cpm/pmol), δSer-262 (0.6 cpm/pmol) and δGln-276 (17 cpm/pmol). **B**, The primary sequence for the labeled fragment from δEKC-21 began at δThr-51 (-Carb: I₀ = 20 pmol; +Carb: I₀ = 28 pmol), and there was a secondary sequence beginning at δVal-30 (-Carb, I₀ = 8 pmol; +Carb, I₀ = 7 pmol). For these samples, the HPLC fractions were applied directly to the glass fiber sequencing filter before treatment with Biobrene. The ³H release in cycle 9 (490 cpm) was consistent with agonist-inhibitable [³H]azietomidate incorporation into δAsp-59 (22 cpm/pmol). For **A** and **B** the primary sequences are shown on the top axes.

**Figure 9. The location of amino acids in the nAChR ion channel photolabeled by [³H]azietomidate.** A tube and ribbon representation of the δ subunit transmembrane domain and the M2 α helices that form the lumen of the ion channel is shown based on the structure of the nAChR transmembrane domain in the closed state(6), viewed from extracellular domain (upper) and in cross section (lower, extracellular surface up, with βM2 removed and the approximate level of the lipid headgroups indicated). Shown in green are αGlu-262(αM2-20) and δSer-258 (δM2-2), δSer-262 (δM2-6), and δGln-276 (δM2-20) that are labeled by azietomidate in the desensitized state and in blue δGlu-280, that is not labeled. Shown in magenta is δM2-15, a position in the GABA_A receptor β subunit identified as an etomidate affinity determinant and in α subunits as an affinity determinant for alcohols and volatile anesthetics. Shown in yellow is δTyr-228, in δM1, that is labeled by [¹⁴C]halothane (36). Azietomidate, which measures 13 Å in an extended conformation, is shown at the same in a space filling representation.
Figure 10. The location of amino acids in the ACh binding sites photolabeled by \[^{3}H\]azietomidate. A ribbon representation of the two agonist binding sites at subunit interfaces from a *Torpedo* nAChR homology model (16) based on the crystal structure of the AChBP (7), showing the site at the α-γ interface (upper) with α subunit in red and γ subunit in blue, and the site at the α-δ interface (lower) with α subunit in red and δ subunit in grey. β strands are numbered in white using the nomenclature from the AChBP crystal structure. β strand-9 and ACh binding site segment C are in the foreground with a portion made transparent in order to help visualize the core aromatic residues αTyr-93 from segment A, αTrp-149 from segment B, αTyr-190 and αTyr-198 from segment C, and γTrp-55/δTrp-57 from segment D. Also indicated are γGlu-57 and δAsp-59. The side chain of δAsp-59 (or γGlu-57) is 10 Å from αTyr-190 and 14 Å from αTyr-93. The concentration dependence of dTC inhibition of \[^{3}H\]azietomidate photoincorporation indicate that αTyr-93 and αTyr-190 at the α-γ interface were labeled, while at the α-δ interface δAsp-59 was labeled. The primary labeled binding site residues are colored green and the unlabeled residues are colored yellow.
Table 1. Concentration dependence of $[^3]$Hazietomidate incorporation into residues of nAChR α and δ subunits (pmol $[^3]$H/pmol PTH-amino acid (%)).


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<tr>
<th>Ion Channel Domain (Incorporation +Carb)</th>
<th>$[^3]$H-Azietomidate</th>
<th>Amino Acid</th>
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Identification of Binding Sites in the Nicotinic Acetylcholine Receptor for
\[^{3}\mathrm{H}]\text{Azietomidate, a Photoactivatable General Anesthetic}

Michael R. Ziebell, Selvanayagam Nirthanan, S. Shaukat Husain, Keith W. Miller, and Jonathan B. Cohen

SUPPLEMENTAL FIGURES 1 and 2

Supplemental Figure 1. HPLC purification of \[^{3}\mathrm{H}]\text{azietomidate-labeled} \alpha \text{ subunit fragments from the EndoLysC digests of } \alpha \mathrm{V8-20} \text{ and } \alpha \mathrm{V8-18}

EndoLysC digests of gel-purified of \(\alpha \mathrm{V8-20}\) (A) and \(\alpha \mathrm{V8-18}\) (B), from nAChRs labeled with 1.8 \(\mu\mathrm{M}\) \[^{3}\mathrm{H}]\text{azietomidate} (Figure 6) in the absence (\(\bigcirc\)) or presence of Carb (\(\bullet\)), were fractionated by reverse phase HPLC. \(\text{Upper}\), \[^{3}\mathrm{H}\text{] elution profiles (2\% of each fraction counted) and elution gradient (\% solvent B);}\ \text{Lower, absorbance (—) profile. For A), 77,000 cpm (Carb) and 187,000 cpm (Carb) from the EndoLysC digest of } \alpha \text{V8-20 were loaded. For the +Carb sample } \sim 15\% \text{ of } \[^{3}\mathrm{H}\text{ applied to the column eluted in a single broad hydrophobic peak (fractions 39-42, 98 \% organic). These fractions from both +Carb and –Carb samples were pooled separately and sequenced (Fig. 7A) (+Carb: } \alpha \text{Met-243 } I_0 = 12 \text{ pmol, R = 92\%, } \alpha \text{Ser-173 } I_0 = 6 \text{ pmol, R = 84\%, 25,000 cpm loaded and 6740 cpm remaining on the filter after 24 cycles; –Carb } \alpha \text{Met-243 } I_0 = 8 \text{ pmol, R = 2\%, } \alpha \text{Ser-173 } I_0 = 7 \text{ pmol, R = 89\%, 3000 cpm loaded and 760 cpm remaining)). For the -Carb sample, } \sim 12\% \text{ of the } \[^{3}\mathrm{H}\text{ applied to the column eluted in fractions 21-24 (—64 \% organic) and}
~7% eluted in fractions 29-31 (~73% organic). Fractions 29-31 were pooled from each condition separately and sequenced (Fig. 7B -Carb: αHis-186 I₀ = 11 pmol, R = 90%, αSer-173 I₀ = 6 pmol, R = 94%, 4800 cpm loaded and 970 cpm remaining after 22 cycles; +Carb αHis-186, I₀ = 13 pmol, R = 89%; αSer-173 I₀ = 7 pmol, R = 89%, 1540 cpm loaded, 330 cpm remaining on the filter). Additional sequence analysis revealed that the shoulder of ³H in fractions 35-36 also contained low levels of the fragments beginning at αMet-243 (+Carb, I₀=0.8 pmol) and αSer-173 (+Carb, I₀=0.5 pmol). Sequence analysis of the hydrophilic peak of ³H (fractions 21-24) from the HPLC fractionation of the EndoLysC digests of αV8-20 revealed a single sequence starting at αLys-77, a fragment that was present because αV8-20 isolated by SDS-PAGE also contains some contaminating αV8-18 (23). Since the ³H in fractions 2 and 3 (flow-through) was not retained on sequencing filters, this ³H was not stably incorporated in α subunit fragments. For B) 9,000 cpm of the +Carb sample and 55,000 cpm of the -Carb sample from the EndoLysC digest of αV8-18 were loaded. For the -Carb sample 30% of ³H loaded on the column eluted in a single peak (fractions 21-24, ~64 % organic). These fractions from both +Carb and –Carb samples were pooled separately and sequenced (Fig. 7C (αLys-77: -Carb: I₀ = 33 pmol, R = 92%, 4500 cpm loaded and 1025 cpm remaining after 25 cycles; +Carb: I₀ = 13 pmol, R = 88%, 1000 cpm loaded, and cpm remaining)).
Supplemental Figure 2. HPLC purification of $[^3H]$azietomidate-labeled nAChR $\delta$ subunit fragments. 

A), $\delta$EKC-10 isolated from nAChRs labeled with 1.8 $\mu$M $[^3H]$azietomidate (Figure 6) and B), an S. aureus V8 protease digest of $\delta$EKC-21 isolated from nAChRs labeled with 150 $\mu$M $[^3H]$azietomidate in the absence (○) or presence of Carb (●) were purified by reverse phase HPLC. Upper, $^3$H elution profiles (2 or 4% of each fraction counted) and elution gradient; Lower, absorbance (—) profile. For A), 63,000 cpm (+Carb) and 24,000 cpm (-Carb) from $\delta$EKC-10 were loaded onto the HPLC, and 21,000 cpm (+Carb) and 2200 cpm (-Carb) were recovered in fractions 25-26 (~79 % organic). These fractions from both conditions were pooled separately and sequenced (Fig. 8A (δMet-257: +Carb: $I_0 = 78$ pmol, R = 94%, 21,000 cpm loaded and 2090 cpm remaining on the filter after 30 cycles; -Carb: $I_0 = 51$ pmol, R = 94%; 2700 cpm loaded and 200 cpm remaining)). For B), the $\delta$ subunit (-Carb, 188,000 cpm; +Carb, 90,000 cpm) was isolated from nAChRs labeled with 150 $\mu$M $[^3H]$azietomidate (0.073 Ci/mmol) in the absence and presence of Carb and digested by EndoLysC. A 21 kDa band was isolated from the digest by Tricine SDS-PAGE, and the material eluted from this band (-Carb, 19,500 cpm; +Carb, 2,800 cpm) was digested in solution (200 $\mu$L) with V8 protease (1 $\mu$g) for 4 days. 90% of these samples were fractionated by reverse phase HPLC, with 600 cpm (+Carb) and 5000 cpm (-Carb) recovered in fractions 18 and 19 (47 % organic). These fractions from each condition were pooled separately and sequenced (Fig. 8B (δThr-51: -Carb: $I_0 = 20$ pmol, R = 84%, δVal-30: $I_0 = 8$ pmol; 5100 cpm loaded, 1100 cpm remaining on filter after 20 cycles; +Carb: δThr-51 $I_0 = 28$ pmol, R = 76%, δVal-30 $I_0 = 7$ pmol, 630 cpm loaded, 110 cpm remaining)).
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J. Biol. Chem. published online February 3, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M313886200

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