Time-Resolved Photolabeling of the Nicotinic Acetylcholine Receptor by \([^3]H\)Azietomidate, an Open-State Inhibitor

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ABSTRACT

Azietomidate is a photoreactive analog of the general anesthetic etomidate that acts as a nicotinic acetylcholine receptor (nAChR) noncompetitive antagonist. We used rapid perfusion electrophysiological techniques to characterize the state dependence and kinetics of azietomidate inhibition of Torpedo californica nAChRs and time-resolved photolabeling to identify the nAChR binding sites occupied after exposure to \([^3]H\)azietomidate and agonist for 50 ms (open state) or at equilibrium (desensitized state). Azietomidate acted primarily as an open channel inhibitor characterized by a bimolecular association rate constant of \(k_+ = 5 \times 10^7 \text{ M}^{-1} \text{s}^{-1}\) and a dissociation rate constant of \(<3 \text{ s}^{-1}\). Azietomidate at 10 \(\mu\text{M}\), when perfused with acetylcholine (ACh), inhibited the ACh response by \(~50\%\) after 50 ms; when preincubated for 10 s, it decreased the peak initial response by \(~15\%\). Comparison of the kinetics of recovery of ACh responses after exposure to ACh and azietomidate or to ACh alone indicated that at subsecond times, azietomidate inhibited nAChRs without enhancing the kinetics of agonist-induced desensitization. In nAChRs frozen after 50-ms exposure to agonist and \([^3]H\)azietomidate, amino acids were photolabeled in the ion channel [position M2–20 (\(\alpha\text{Glu-262, } \beta\text{Asp-268, } \delta\text{Gln-276}\)), in \(\delta\text{M1 (}\delta\text{Cys-236), and in } \alpha\text{MA/} \alpha\text{M4 (}\alpha\text{Glu-390, } \alpha\text{Cys-412) that were also photolabeled in nAChRs in the equilibrium desensitized state at approximately half the efficiency. These results identify azietomidate binding sites at the extracellular end of the ion channel, in the \(\delta\) subunit helix bundle, and in the nAChR cytoplasmic domain that seem similar in structure and accessibility in the open and desensitized states of the nAChR.

General anesthetics of diverse structures, including volatile and intravenous anesthetics and alcohols, modulate the function of members of the “Cys-loop” superfamily of neurotransmitter-gated ion channels either by potentiating agonist responses at inhibitory GABA\(_\Lambda\) receptors and glycine receptors or by inhibiting responses of the excitatory serotonin 5-HT\(_3\) and nicotinic acetylcholine receptors (nAChRs) (Yamakura et al., 2001; Arias et al., 2003; Hemmings et al., 2005). Each Cys-loop receptor is a pentamer of homologous or identical subunits that associate at a central axis that is the ion channel. Structural models can be derived from the structure of the muscle-type nAChR based upon cryoelectron microscopy images of the Torpedo marmorata nAChR (Unwin, 2005), which provides a definition of the tertiary structure of the nAChR but lacks the resolution necessary to reliably define the positions of individual amino acids. The N-terminal half of each subunit contributes to the extracellular domain, containing the neurotransmitter binding sites that are located at subunit interfaces (\(\alpha-\gamma\) and \(\alpha-\delta\) in the \(\alpha_2\beta_2\gamma_2\eta\text{ Torpedo californica nAChR) 30 Å above the level of the membrane. Each nAChR subunit’s transmembrane domain is made up of a loose bundle of four \(\alpha\) helices (M1–M4), with amino acids from each M2 helix contributing to the lumen of the ion channel and M4 located most peripherally and in greatest contact with lipid. The structure of nAChR transmembrane domain suggests the existence of pockets within each subunit helix bundle and at subunit interfaces that are
potential binding sites for general anesthetics and other allosteric modulators.

In the absence of agonist, the nAChR exists predominantly in a resting, closed channel conformation with low affinity for agonist that is in equilibrium with a nonconducting desensitized state with highest affinity for agonist. Agonist binding induces conformational transitions, within a fraction of a millisecond, to an open channel state (Maenchen and Steinbach, 1998), and desensitization occurs in two kinetically distinct phases over times from 0.1 to 100 s (Hess et al., 1983).

Mutational analyses identified positions within the M2 helices that can enhance or reduce the potency of aliphatic alcohols as open-state inhibitors (Zhou et al., 2000; Wenningmann et al., 2001; Borghese et al., 2002). Because these mutations often affect ACh gating in the absence of anesthetics, and they include substitutions at positions oriented toward the lumens of the ion channel and toward the interior of each subunit helix bundle or the interfaces with another subunit, it is difficult to determine whether the positions contribute directly to anesthetic binding or are involved in allosteric modulation of gating.

Photoaffinity labeling provides a method to directly identify amino acids contributing to a drug binding site in a protein without any prior assumptions about the location of the site (Kotzyba-Hibert et al., 1995; Vodovozova, 2007). State-dependent drug binding sites have been mapped by photolabeling T. californica nAChRs in the absence of agonist (resting state), equilibrated with agonist (desensitized state), and by the use of rapid-mixing and freeze-quench techniques (Arevalo et al., 2005), in the conformations transiently stabilized by agonist (for review, see Mourot et al., 2006).

\( R(+)\)-Etomidate, one of the most potent general anesthetics used clinically, acts at micromolar concentrations as an anesthetic and as a potentiator of the responses to submaximal concentrations of GABA, whereas at concentrations above 10 \( \mu M \), it inhibits nAChRs. Aziomitodate is a photoreactive etiotope analog that is equipotent with etomidate as a general anesthetic and as a positive allosteric modulator of GABA\(_A\)R subunits (Husain et al., 2003; Liao et al., 2005). Photolabeling studies with \( ^{[3]H}\)aziomitodate identified a binding site in the GABA\(_A\)R transmembrane domain at the interface between the \( \beta \) and \( \alpha \) subunits (Li et al., 2006). In the T. californica nAChR, aziomitodate is equipotent with etomidate as a noncompetitive antagonist, binding with 10-fold higher affinity to the nAChR in the desensitized state than the resting state and photolabeling amino acids near the extra-cellular end of the ion channel domain only in the desensitized state (position M2–M20: Glu-262 and Gln-276) (Ziebell et al., 2004). However, it is not known where (or whether) it interacts with nAChRs in the open channel state.

To provide a further definition of the diversity of binding sites and state dependence of binding of a single general anesthetic in a “Cys-loop” receptor, we use rapid perfusion electrophysiological techniques to characterize aziomitodate’s state dependence and kinetics of inhibition, and we use time-resolved photolabeling to identify the nAChR binding sites occupied after exposure to \( ^{[3]H}\)aziomitodate and agonist for 50 ms (open state) compared with the desensitized state.

### Materials and Methods

**Materials.** nAChR-rich membranes were isolated from the electric organs of T. californica rays (Aquatic Research Consultants, San Pedro, CA) as described previously (Middleton and Cohen, 1991). The membranes, which contained 1 to 2 nmol of \(^{[3]}\)Hacetylecholine (ACh) binding sites/mg of protein, were stored at \(-80^\circ\)C in 38% sucrose.0.02% Na\(_2\)Azomitodate and \(^{[3]}\)Haziomitodate (16 Ci/ mmol; stored at a concentration of 14 \( \mu M \) in methanol at \(-80^\circ\)C) were synthesized as described previously (Husain et al., 2003). Endoprotease Lys-C (EndoLys-C) was from Roche Applied Sciences (Indianapolis, IN), N-tosyl-l-phenylalanine chloromethyl ketone-treated trypsin was from Worthington Biochemical (Lakewood, NJ), and Staphylococcus aureus endopeptidase Glu-C (V8 protease) was from MP Biomedicals (Solon, OH).

**Animal Use.** Female Xenopus laevis frogs (Xenopus One, Dexter, MI) were housed in a veterinarian-supervised environment in accordance with local and federal guidelines and with the approval of the institutional research animal care committee. Oocytes were harvested via minilaparotomy from frogs anesthetized with 0.1% tricaine methanesulfonate.

**Oocyte Expression of T. californica nAChRs.** In vitro transcription of T. californica nAChR subunit-specific cRNAs and the protocols for the isolation and injection of X. laevis oocytes were carried out as described previously (Sullivan and Cohen, 2000). Isolated follicle-free oocytes were injected with 0.5 to 1 ng of subunit RNAs in a molar ratio of 2/0.5/0.5 and incubated at 18°C for 48 to 72 h before measurement of ACh responses. Under our experimental conditions, when responses for intact oocytes were measured by two-electrode voltage clamp, the maximal currents elicited by ACh were typically 1 to 2 \( \mu A \). Oocytes were placed in 1 M N-methyl-d-glucamine for 5 to 20 min and then stripped manually of their vitelline membranes before isolation of detached patches.

**Patch-Clamp Electrophysiology.** Macroscopic currents were recorded from excised outside-out patches at room temperature (21–23°C). Fire-polished micropipettes for creating patches were pulled (P-97 Flaming/Brown Micropipette Puller; Sutter Instrument Co., Novato, CA) to a resistance of 1 to 3 M\(_\Omega\), then 150 ms in buffer, then 150 ms in ACh, then 150 ms in aziemitodate, and then returned to channel A. For each aziomitodate or ACh concentration series, measurements were performed in order of increasing concentration. The amount of time spent in each step...
varied between experiments and is noted in the text and figure legends. For azidotemide preincubation experiments, a patch was moved from buffer (channel A) to channel C containing azidotemide (10 μM) for a variable amount of time and then to channel D containing 300 μM ACh and azidotemide. Recordings were averages of four to six sweeps, with 20 to 30 s between trials and ACh control measurements taken after each condition was tested.

Three protocols were used to determine the effects of azidotemide on the kinetics of desensitization of the nAChR response. 1) The onset and recovery from agonist-induced desensitization was determined by shifting a patch from buffer to 300 μM ACh for 2 s, at which time the patch was returned to buffer, with the recovery of the response monitored at intervals by returning the patch briefly (50 ms to 300 μM ACh). 2) To determine whether azidotemide enhanced the kinetics of desensitization, the patch was moved from buffer to 300 μM ACh and 10 μM azidotemide (channel D) for 2 s and then the patch was returned to buffer, with the recovery of the response monitored at intervals by return of the patch to ACh and azidotemide (channel D) for 50 ms. 3) To test for the recovery from desensitization in the presence of ACh, after exposure to ACh + azidotemide (channel D) for 2 s, the patch was moved to 10 μM azidotemide alone (channel C) and then returned at intervals to ACh + azidotemide for 50 ms.

Data Analysis. Agonist dose response curves were fit to the equation $I = I_{\text{max}}/[1 + (K_{\text{agonist}})^n]$, where $I$ and $I_{\text{max}}$ are the current at agonist concentration $x$ and the maximum current, respectively, $K_{\text{agonist}}$ is the agonist concentration for half-maximal response, and $n$ is the Hill coefficient. The concentration dependence of drug inhibition of ACh-induced currents was fit to the equation $I = I_0/[1 + (a/IC_{50})]$, where $I_0$ is the current in the presence of inhibitor concentration $x$, $I_{\text{rec}}$ is the current in the absence of inhibitor, and $IC_{50}$ is the inhibitor concentration producing 50% inhibition. SigmaPlot (Systat Software, Inc., San Jose, CA) was used for the nonlinear least-squares fit of the data, and the standard errors of the parameter fits are indicated. The observed decline of the response from the peak initial response either in the presence of ACh alone (desensitization) or of ACh and azidotemide was fit to $I = I_0 + (I_{\text{pk}} - I_0)\exp^{-kt}$, where $t$ is time, $I_0$ is the residual response at long times, $I_{\text{pk}}$ is the peak initial current, and $k$ is the rate constant. The kinetics of recovery of the response after exposure to ACh with or without azidotemide for seconds were fit to the equation $I = I_{\text{rec}} - a\exp^{-bt}$, where $I_{\text{rec}}$ is the current after full recovery, $a$ is the amplitude of the observed slow recovery on the time scale of seconds, and $b$ is the rate constant (see Fig. 3A). The amplitude of the rapid phase of recovery, $a_1$, is equal to $I_{\text{rec}} - (a_0 + I_{\text{f}})$.

Photolabeling nAChR-Rich Membranes. For photolabeling studies, [3H]azidotemide was isotopically diluted to 1.6 Ci/mmol with an appropriate volume of nonradioactive azidotemide at 1 mg/ml in methanol, which was removed via evaporation immediately before the addition of T. californica physiological saline (250 mM NaCl, 5 mM KCl, 3 mM CaCl2, 2 mM MgCl2, and 5 mM NaPO4, pH 7.0) with or without T. californica nAChR-rich membranes resuspended at 4 mg of protein/ml. The freeze-clamp apparatus and concentrated to 10% SDS. Aliquots (100 μl) were thawed individually in 0.2 ml of elution buffer (100 mM NH4HCO3, 2.5 mM dithiothreitol, and 0.1% SDS, pH 8.4). The bands containing the α subunit were excised and layered onto a second, 15% acrylamide gel for limited digestion in “gel” with S. aureus V8 protease (White and Cohen, 1988). After electrophoresis, that gel was stained with GelCode Blue (Pierce), and the proteolytic fragments of 20 (αV8-20), 18 (αV8-18), and 10 kDa (αV8-10) were excised and eluted. The eluates were filtered and concentrated to <400 μl by centrifugal filtration. nAChR subunits or subunit fragments were acetone precipitated in 75% acetone (12 h at −20°C), and the pellets were resuspended in 100 to 200 μl of digestion buffer (15 mM Tris, 0.5 mM EDTA, 0.1% SDS, pH 8.1). Protease digests of αV8–20 and αV8–10 were fractionated directly by reversed-phase HPLC, whereas digests of the β and δ subunits were fractionated on 1.5-mm Tricine SDS-PAGE gels (Schägger and Von Jagow, 1987), which were then cut into 5-mm bands and the material eluted to identify the bands containing 3H, which were then concentrated and fractionated by reversed-phase HPLC.

The αV8–20 fragment and δ subunit were digested with EndoLys-C (0.75 U in 10 μl of distilled water per sample) for 2 weeks at 25°C. For digestion with trypsin, the β subunit and the αV8–10 fragment in reassembly buffer were diluted 5-fold with 100 mM NH4HCO3, 0.5% Genapol C-100 (CalBiochem, San Diego, CA), pH 8.1, and trypsin (100 μg in 20 mM CaCl2 at 1/10 the volume of the material to be digested) was then added for digestion at 25°C overnight (β subunit) or for 3 days (αV8–10).

Reversed-Phase HPLC. Samples were fractionated using a Brownlee Aquapore BU-300 7 μm 100 × 2.1-mm column with a NEWGUARD RP-2 7 μm guard column on an Agilent 1100 binary system (Agilent Technologies, Santa Clara, CA) with an in-line solvent degasser, a column compartment (temperature held at 40°C), and attached UV absorbance (215 nm) and fluorescence detectors. The aqueous phase was 0.08% trifluoroacetic acid, the organic phase was 60% acetonitrile, 40% isopropanol, and 0.05% trifluoroacetic acid, the flow rate was 200 μl/min, and the gradient used is included on HPLC trace figures as a dashed line. Fraction volumes were 500 μl, of which 10% was assayed for 3H.

Sequence Analysis. N-terminal sequencing was performed on a Procise 492 protein sequencer (Applied Biosystems, Foster City, CA), modified such that one sixth of the PTH-amino acid solution from each cycle was injected into the amino acid analyzer and the other 5/6 of the sample was sent to a fraction collector for 3H-quantitation.
plied Biosystems) at 45°C, with BioBrene Plus (Applied Biosystems) added after loading to avoid heat-induced peptide bond cleavage at tryptophan (Chiara et al., 2003). Fractions containing αM or βM, which are not sequenced efficiently on glass fiber filters, were loaded for sequencing onto ProSorb polyvinylidene difluoride filter cartridges (Applied Biosystems).

The amount of released PTH derivative in each cycle, which was determined by comparison with standards from the background-corrected peak height using the model 610A Data Analysis Program (Applied Biosystems), is shown in the figures along with the 3H released in each cycle. To determine the amount of peptide in each sample, the background-subtracted number of pmol detected (f(x)) in cycle x was fit to the equation \( f(x) = I_0 \times R^x \), and the equation was solved for \( I_0 \) (initial peptide amount) and \( R \) (average repetitive yield).

![Fig. 1. Responses to ACh measured by rapid perfusion of T. californica nAChRs in detached patches. A, representative macroscopic current responses when an "outside-out" patch at a holding potential of −50 mV was exposed to varying concentrations of ACh for 100 ms. Each trace is the average of four sweeps, with 20 s between each sweep. B, the concentration dependence of the peak ACh responses, with data from two patches normalized to the response to 300 μM ACh and error bars indicating the range. The response was fit by \( K_{\text{app}} = 43 \pm 4 \mu M \) and \( n_H = 1.9 \pm 0.2 \).

**Results**

**nAChR Inhibition by Azietomidate.** When ACh responses were measured by rapid perfusion of nAChRs in detached, “outside-out” patches held at −50 mV, the concentration dependence of the ACh response was characterized by a \( K_{\text{app}} \) of 40 μM and a Hill coefficient, \( n_H \), of 2 (Fig. 1). Representative current traces are shown in Fig. 1A for T. californica nAChRs exposed to ACh concentrations from 10 μM to 1 mM. ACh at 300 μM produced a maximal response in several milliseconds, and the current response then decreased by ~20% in 100 ms. ACh at 1 mM produced a similar initial peak response, which was followed by a rapid decline of ~10%, presumably as a result of channel block, and then by a further decline at a rate similar to that seen at 300 μM ACh.

![Fig. 2. Azietomidate inhibition of T. californica nAChRs. Azietomidate inhibition of ACh responses of T. californica nAChRs expressed in X. laevis oocytes was measured by rapid perfusion of outside-out patches detached from oocytes. A, representative macroscopic current traces from a single patch exposed for 800 ms to 300 μM ACh complicated with increasing concentrations of azietomidate. For each concentration, the current traces are the average of four repetitions, with 10 s between each sweep. Each trace was normalized to the peak current response for 300 μM ACh applied before each exposure to azietomidate. After exposure to 30 μM azietomidate and 10 s recovery, the peak ACh current was ~90% of the previous control, and \( n_{\text{des}} \) was decreased by ~20% compared with the value before exposure to any azietomidate. B, the concentration dependence of azietomidate inhibition. Responses were measured by the net charge transfer during 800-ms exposure to ACh and azietomidate, normalized for each patch to the value for ACh alone. Data are the mean ± S.E.M. from four patches, and the calculated \( I_{\text{C}_{\text{50}}} \) was 5.6 ± 0.2 μM. C, dependence of the kinetics of inhibition on azietomidate concentration. For each concentration of azietomidate, the observed decline of the response at time \( t \) from the peak was fit to a single exponential: \( I_t = I_0 + (I_{\text{pk}} - I_0) \exp(-kt) \). \( I_0 \) is the residual response at long times, \( I_{\text{pk}} \) is the peak initial current, and \( k \) is the rate constant. Inhibition rates (mean ± S.E.M., three patches) are plotted against azietomidate concentration. Linear least-squares regression gives a slope of 5.0 ± 0.2 × 10^{-3} M^{-1} s^{-1}. D, effect of azietomidate preincubation on the inhibition of T. californica nAChRs in excised patches. The responses of nAChRs exposed to 300 μM ACh and 10 μM azietomidate are shown for a single patch. Before each ACh application, the patch was perfused with 10 μM azietomidate for 0, 5, or 10 s. The calculated net charge transfer during the 1-s response was 2.0 nC for the control in the absence of azietomidate, and 1.1, 0.54, and 0.48 nC after 0, 5, and 10 s of preincubation, respectively.**
To test the effects of azietomidate, nAChRs were exposed simultaneously for 800 ms to 300 μM ACh and azietomidate at concentrations varying from 1 to 30 μM (Fig. 2). For 300 μM ACh alone, the decline from the peak current response (agonist-induced desensitization) was well fit by a single exponential characterized by \( \tau \) of \( \sim 450 \pm 75 \) ms \((n = 5)\), with the current responses decreasing by \( \sim 75\% \) after 800 ms. Exposure to increasing concentrations of azietomidate reduced the peak initial responses by less than 5%, but there was a concentration-dependent increase in the rate and extent of the decline of the response from the peak. In the presence of 10 μM azietomidate, ACh responses were inhibited by \( \sim 50\% \) after \( \sim 60 \) ms and by \( \sim 98\% \) by 800 ms. Etomidate over a similar concentration range had the same effect on ACh responses (data not shown). When the concentration dependence of inhibition was quantified by measuring the net charge transfer during the 800-ms exposure period, the IC\(_{50}\) for azietomidate inhibition was \( 6 \pm 2 \) μM \((n = 4)\) (Fig. 2B), whereas for etomidate, the IC\(_{50}\) was \( 7 \pm 4 \) μM \((n = 2)\).

Azietomidate at increasing concentrations enhanced the rate and extent of the decline of the ACh response. At all concentrations, that decline was well fit by a single exponential, and the rate increased linearly with azietomidate concentration (Fig. 2C). Although the kinetics of decline of the response in the presence of ACh alone reflects the kinetics of agonist-induced desensitization, the observed concentration-dependent inhibition by azietomidate could reflect either enhanced kinetics of desensitization or the kinetics of binding of azietomidate. Assuming the latter case, the observed inhibition would be consistent with an apparent bimolecular association rate constant for azietomidate of \( 5.0 \pm 0.2 \times 10^5 \) M\(^{-1}\)s\(^{-1}\) (data from three patches). Additional experiments, described below, establish that the nAChRs inhibited by azietomidate are not in the desensitized state that is stabilized by agonist.

Preincubation with azietomidate had only subtle effects on the inhibition of the ACh response (Fig. 2D). Prior exposure to 10 μM azietomidate for either 5 or 10 s resulted in a decrease of the peak initial response by \( \sim 15\% \) and a doubling in the rate of decline of the ACh response compared with simultaneous addition.

**Recovery from Inhibition.** To determine whether azietomidate stabilized the same nAChR-desensitized state as ACh, we measured the kinetics of recovery of ACh responses after exposure to 300 μM ACh alone for 2 s or to ACh and 10 μM azietomidate (Fig. 3). The general features of the ACh response expected for such a protocol are schematized in Fig. 3A, and experimental data are shown for patches exposed only to buffer during the recovery period (Fig. 3B) or exposed to 10 μM azietomidate throughout the recovery period (Fig. 3C), with the parameters characterizing the recovery kinetics in Table 1.

After exposure to either ACh or ACh and azietomidate, recovery occurred in two phases: a rapid phase (amplitude, \( a_f \)), complete within 100 ms, and a slow phase (amplitude, \( a_s \)), characterized by \( \tau_s \sim 0.7 \) s. For the ACh controls, the response decreased by 70% during the 2-s application of ACh, whereas in the presence of ACh and azietomidate, the response decreased to baseline. For the ACh controls, 80 to 85% of the recovery occurred during the slow phase. After exposure to ACh and azietomidate, the observed recovery was similar whether or not azietomidate was present during the recovery phase. In both conditions, after 100-ms recovery, the ACh response was close to that seen after desensitization by ACh alone. Thus, the rapid phase of recovery was larger after

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**Fig. 3.** Recovery of ACh responses after inhibition by azietomidate. A, a schematic defining the parameters characterizing the kinetics of recovery of ACh responses after exposure to ACh in the absence or presence of azietomidate. After exposure to a stream of ACh ± azietomidate, the patch was switched to a stream that contains buffer, with ACh sensitivity monitored by moving the patch for 50 ms into 300 μM ACh + azietomidate after 0.1, 0.3, 1.0, and 3.0 s of recovery. \( I_f \) is the peak initial response to 300 μM ACh ≥ 10 μM azietomidate; \( I_{rec} \) is the current after 1.95 s. The observed recovery was fit to a single exponential: \( I = I_{rec} - a_s \exp(-bt) \), where \( I_{rec} \) is the current after full recovery, \( a_s \) is the amplitude of the observed slow recovery on the time scale of seconds, and \( b \) is the rate constant. The amplitude of the rapid phase of recovery, \( a_f \), is equal to \( I_f - (a_s + I_f) \). B and C, experimental traces from two patches. The black traces are the ACh controls (1.95-s exposure to 300 μM ACh), followed by recovery in buffer with test pulses of 300 μM ACh, and the gray traces are the currents seen during exposure to ACh + 10 μM azietomidate, followed by recovery in buffer alone (B) or in a stream containing 10 μM azietomidate (C), with recovery tested by exposure to 300 μM ACh and 10 μM azietomidate. Traces shown in B and C are the average of four to six sweeps, each with 20 to 30 s between each sweep. The parameters characterizing the recovery kinetics are detailed in Table 1.
exposure to ACh and azietomidate than to ACh alone, whereas the slow phase occurred with an amplitude and kinetics similar to that seen for ACh alone. These results establish that when azietomidate inhibits the nAChR in the presence of ACh, it does not do so by stabilizing the ACh-induced desensitized state.

**Comparison of nAChR Inhibition by Azietomidate and QX-222.** To further characterize the state of the nAChR inhibited by azietomidate, two additional rapid perfusion protocols were used to compare nAChR inhibition by azietomidate with inhibition by QX-222, which acts as an open channel blocker characterized by rapid association and dissociation \[k_+ = 4 \times 10^7 \text{ M}^{-1}\text{s}^{-1}, \quad k_- = 1 \times 10^3 \text{s}^{-1}\] (Dilger and Vidal, 1994). In one protocol, nAChRs were exposed to ACh and QX-222 or azietomidate and then returned to buffer. After exposure to ACh and QX-222, the current was ~30% of control (ACh alone) after 30 ms, and a transient increase in current (“rebound” current) was seen after the patch was moved to buffer, which results from ion flow through open channels as QX-222 dissociates (Fig. 4A). For nAChRs exposed to 300 \(\mu\)M ACh and 10 \(\mu\)M azietomidate, the current was at ~50% of control (ACh alone) after 100 ms, and when the patch was then switched to buffer, the current decreased within milliseconds to baseline without evidence of any “rebound” current (Fig. 4C). Thus, azietomidate did not act as an open channel blocker characterized by rapid dissociation.

In a second protocol, responses were measured when the patch was shifted from ACh and QX-222 or azietomidate to ACh alone. After exposure to ACh and QX-222 for 30 ms, when the patch was returned to ACh, the current rapidly increased to the level seen for ACh alone (Fig. 4B), which indicates that QX-222 dissociates rapidly from nAChRs that had been inhibited without any increase in the fraction in the desensitized state. When the patch was moved to a stream of ACh after 30-ms exposure to ACh and azietomidate, the current neither increased to control levels as seen for QX-222 nor declined as in the continued presence of azietomidate. Rather, the current response declined at a rate similar to the control ACh response (Fig. 4D). This response suggests that the nAChRs that had bound azietomidate within 30 ms remained occupied and inhibited during the subsequent 200-ms exposure to ACh, whereas ACh binding to the nAChRs that had not bound azietomidate resulted in channel gating and then desensitization.

**TABLE 1**

Kinetics of recovery of ACh responses after inhibition by azietomidate

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<th></th>
<th>(I_0)</th>
<th>(I_f)</th>
<th>(I_{rec})</th>
<th>(a_s)</th>
<th>(\tau_s)</th>
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<td>115 ± 1</td>
<td>770 ± 20</td>
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<td>2410.02</td>
<td>228 ± 2</td>
<td>161 ± 4</td>
<td>470 ± 30</td>
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**Fig. 4.** Comparison of nAChR inhibition by the open channel blocker QX-222 (100 \(\mu\)M, A and B) and by azietomidate (10 \(\mu\)M, C and D). *T. californica* nAChRs were exposed simultaneously to 300 \(\mu\)M ACh and inhib- itor according to four different protocols that are numbered on the traces: 1) 800 ms of ACh, before return to buffer; 2) 800 ms of ACh + inhibitor, before return to buffer; 3) 30 ms of ACh + QX-222 (A) or 100 ms of ACh + azietomidate (C), before return to buffer; or 4) 30 ms of ACh + QX-222 (B) or ACh + azietomidate (D), before return to ACh alone for 170 ms. In each panel, the bar above the current traces indicates the duration of exposure to ACh and QX-222 or azietomidate before switching the patch to a stream of buffer (A and C) or ACh (B and D). A and B are all from a single patch, as are those from C and D. Each trace is an average of four sweeps, with a 20- to 120-s buffer wash between each trial.
[3H]Azietomidate Photolabeling of nAChRs Frozen after 50-ms Exposure to Agonist. For nAChRs exposed to an agonist concentration producing a maximal response, the electrophysiological analyses established that there was little desensitization within 50 ms and that simultaneous exposure to 10 μM azietomidate inhibited the response by ~50%, with no evidence that the inhibition was associated with stabilization of nAChRs in the slowly reversible desensitized state stabilized by agonist. Therefore, to characterize [3H]azietomidate photolabeling when a maximal fraction of nAChRs are in the open state, nAChRs were frozen for photolabeling 50 ms after exposure to [3H]azietomidate at a final concentration of 10 μM and with the agonist carbamylcholine (Carb) at a concentration (10 mM), which produces a maximal response for native T. californica nAChRs in sealed vesicles (Forman et al., 1987). This photolabeling condition will be referred to as “Open,” although these nAChRs will be distributed in the multiple conformational states transiently stabilized by agonist (open, “preopen” (Lape et al., 2008), and fast-desensitized). In parallel, nAChRs pre-equilibrated with 10 mM Carb were photolabeled when frozen after 50 ms exposure to 10 μM [3H]azietomidate and 10 mM Carb (Des, agonist desensitized state). Table 2 summarizes for comparison purposes the efficiencies (cpm/mol) of [3H]azietomidate photolabeling of nAChR amino acids in each of the photolabeling conditions.

Photolabeled amino acids in the α subunit transmembrane domain were identified by isolating labeled fragments from proteolytic digests of two large subunit fragments that are produced by digestion “in gel” with V8 protease (White and Cohen, 1988): a 10-kDa fragment (αV8–10, beginning at αAsn-339 and containing αM4) and a 20-kDa fragment (αV8–20, beginning at αSer-162/173 and containing M1-M3). When the trypsin digest of αV8–10 was fractionated by reversed-phase HPLC (Fig. 5A), ~80% of [3H] was recovered in or near the flow-through fractions, and ~20% was recovered in a broad hydrophobic peak (70–95% organic) that is known to contain the fragments spanning αM4. Sequence analysis of the pool of the hydrophobic peak (Fig. 5B) revealed the presence of a primary sequence beginning at αSer-388, extending through αLys-400 and αM4, and a secondary sequence beginning at αTyr-401. The major peak of [3H] release was in cycle 3, with a smaller peak of release in cycle 12. The [3H] release in cycle 3 resulted from labeling of αGlu-290 in the primary sequence (Open, 1.2 cpm/pmol; Des, 0.6 cpm/pmol), because labeling of αAla-403 in the secondary sequence would have also resulted in a peak of [3H] release in cycle 16 from the primary sequence, which was not seen. The [3H] release in cycle 12 probably resulted from labeling of αCys-412 in the secondary sequence (Open, 0.9 cpm/pmol; Des, 0.5 cpm/pmol), because there were also low peaks of [3H] release in cycle 25. Because [3H]azietomidate photolabels αGlu-390, at least some of the [3H] in the HPLC flow-through fractions of Fig. 5A must result from the presence of labeled αGlu-390 in the hydrophilic peptides also generated when αV8–10 is cleaved at αGlu-400 to produce the fragment beginning at αTyr-401. However, additional studies are required to determine whether [3H]azietomidate photolabels other amino acids between αAsn-339 and αGlu-387 that would also be contained within the hydrophilic fragments produced by trypsin cleavage of αV8–10.

To identify the amino acids photolabeled in αV8–20, digestion with EndoLys-C was used, which generates fragments beginning at αHis–186 (containing αM1) and at αMet-242 (containing M2 and M3), which can be resolved by reversed-phase HPLC (Ziebell et al., 2004). When the EndoLys-C digests of αV8–20 were fractionated by reversed-phase HPLC (Fig. 5C), ~20% of [3H] was recovered in a hydrophobic peak centered at 90% organic, which by sequence analysis contained the fragment beginning at αMet-242 as the primary sequence and a single peak of [3H] release in cycle 20 corresponding to labeling of αGlu-262 (Open, 25 cpm/pmol; Des, 13 cpm/pmol) (Fig. 5D).

To identify the amino acids photolabeled in the δ subunit transmembrane domain, the photolabeled δ subunits were digested with EndoLys-C, which generates fragments of 10 to 14 kDa, one beginning at δPhe-206 and containing δM1 and another beginning at δMet-257 at the N terminus of δM2 and extending through δM3, that can be separated by reversed-phase HPLC after fractionation of the digests by Tricine-SDS PAGE (Arevalo et al., 2005). When the materials from these gel bands (Fig. 6A) were pooled and fractionated by reversed-phase HPLC (Fig. 6B), ~60% of [3H] was recovered in a peak at ~75% organic that contained the fragment beginning at δMet-257 as the primary sequence, and ~10% of [3H] was recovered in fractions eluting at ~60% organic that contained the fragment beginning at δPhe-206. For the fragment beginning at δMet-257, there was a major peak of [3H] release in cycle 20 (Fig. 6C), consistent with photolabeling of δM2–20 (δGln-276; Open, 1 cpm/pmol; Des, 0.3 cpm/pmol). For the fragment beginning at δPhe-206 (Fig. 6D), a single peak of [3H] release in cycle 31 indicated labeling within δM1 of δCys-236 (Open, 0.7 cpm/pmol; Des, 0.2 cpm/pmol).

Frozen-State Photolabeling of nAChRs Equilibrated with [3H]Azietomidate. To compare with our previous results obtained with nAChRs in the desensitized state photolabeled in solution (Ziebell et al., 2004), we photolabeled nAChRs in the frozen state that had been equilibrated in
Fig. 5. Photolabeling in the nAChR α subunit transmembrane domain after 50-ms exposure to agonist and [3H]azietomidate. nAChR-rich membranes (12 mg/condition) equilibrated without (Open, filled symbols) or with (Des, open symbols) 10 mM Carb were exposed to 10 μM [3H]azietomidate + 10 mM Carb for 50 ms before freezing and photolabeling. After photolabeling, V8 protease digests of the α subunit were fractionated by SDS-PAGE and visualized by Coomassie blue stain, and material was eluted from the 10 kDa (V8–10) and 20 kDa bands (V8–20). The left panels are reversed-phase HPLC fractionations of (A) trypsin digests of V8–10 samples (11,000 cpm injected each condition; recovery 90%) and (C) EndoLys-C digests of V8–20 (F, 17,300 cpm injected; E, 9600 cpm injected; 70% recoveries). Also included are the absorbance at 215 nm (dotted line) and the HPLC gradient (% organic). The right panels are [3H] and PTH-amino acids released during sequence analysis of nAChR subunit fragments beginning near the amino terminus of M4 (pools of HPLC fractions 26–31 from A) (B), and M2 (pools of HPLC fractions 29–31 from C) (D). The primary amino acid sequences are shown above each panel. B, each sample contained α subunit fragments beginning at Tyr-401 (Des, I0 = 23 pmol; Open, I0 = 16 pmol, not shown) and at Ser-388 (Des, I0 = 46 pmol; Open, I0 = 28 pmol, not shown). The major peak of [3H] release in cycle 3 indicated labeling of αGlu-390, and the minor peaks of [3H] release in cycles 12 and 25 indicated labeling of Cys-412 within the fragments beginning at Tyr-401 and Ser-388, respectively. D, each sample contained as the primary sequence the fragment beginning at Met-243 at the N terminus of M2 [Open and Des (□), I0 = 2.9 pmol]. The peak of [3H] release in cycle 20 indicated labeling of αGlu-262. The pool of HPLC fractions 32 to 35 from C contained fragments beginning at Ser-173 (9 pmol, each condition) and at Met-243 (7 pmol, each condition) and a single peak of [3H] release in cycle 20.

Fig. 6. Photolabeling in the nAChR α subunit transmembrane domain after 50-ms exposure to agonist and [3H]azietomidate. nAChR β subunits isolated from the labeling described in Fig. 5 were digested with EndoLys-C and fractionated by Tricine SDS-PAGE. A, the distribution of [3H] eluted from 5-mm bands of the gel (Open, 26,800 cpm loaded, 14,600 recovered; Des, 17,400 cpm loaded, 10,200 cpm recovered). The mobilities of the molecular mass markers are indicated above the graph. B, reversed-phase HPLC fractionation of material eluted from gel bands 7–9 (F, 6350 cpm injected, 6000 recovered; E, 4060 cpm injected, 3330 cpm recovered). Also included are the absorbance at 215 nm for the Des sample (solid line) and the HPLC gradient in percent organic phase (dashed line). The right panels are [3H] and PTH-amino acids released during sequence analysis of nAChR subunit fragments beginning at the amino terminus of M2 (pools of HPLC fractions 26–29) (C) and M1 (pools of HPLC fractions 22–24) (D). C, the primary sequence began at Met-257 at the N terminus of M2 (Open, I0 = 61 pmol, not shown; Des, I0 = 60 pmol) and secondary sequences began at Asn-437 (Open, 21 pmol; Des, 20 pmol) and Phe-206 (Open, 3.7 pmol; Des, 2.8 pmol). The peak of [3H] release in cycle 20 was consistent with labeling at Gln-276 from the primary sequence detected. D, the primary sequence began at Phe-206 before M1 (Open, I0 = 30 pmol, not shown; Des, I0 = 29 pmol). The peak of [3H] release in cycle 31 indicated labeling of αCys-236.
solution with [3H]azietomidate (18 μM) in the presence of a high concentration of 10 mM Carb, and then mixed with 10 mM Carb and frozen after 50-ms exposure. Membranes equilibrated with [3H]azietomidate in the absence of agonist and then frozen after 50-ms exposure to Carb were also photolabeled in parallel.

To identify the amino acids photolabeled within αM2, the fragment beginning at αMet-242 at the N terminus of αM2 was isolated by reversed-phase HPLC from an EndoLys-C digest of αV8–20. Sequence analysis of that fragment (Fig. 7A) revealed a single major peak of 3H release in cycle 20, corresponding to the labeling of αGlu-262. For nAChRs pre-equilibrated with [3H]azietomidate and Carb (desensitized state), αGlu-262 was labeled at 40 cpm/pmol, whereas for the samples pre-equilibrated with [3H]azietomidate alone and then exposed to Carb for 50 ms, αGlu-262 was labeled at 70 cpm/pmol.

To identify the amino acids photolabeled in δM2, the fragment beginning at δMet-257 at the N terminus of δM2 was purified by reversed-phase HPLC after fractionation of the δ subunit digest by Tricine-SDS PAGE. Sequence analysis of the fragment beginning at δMet-257 (Fig. 7B) revealed a single peak of 3H release in cycle 20, consistent with labeling of δGln-276 at 1 cpm/pmol for the sample photolabeled in the desensitized state, and at 2 cpm/pmol for the sample pre-equilibrated with [3H]azietomidate alone. Although for nAChRs photolabeled in solution δM2–2 and δM2–6 were labeled at 10 and 5% the level of δM2–20, for nAChRs photolabeled in the frozen state, the labeling of those positions, if it occurred, was at less than 5% the labeling of δM2–20.

We also characterized [3H]azietomidate photolabeling within βM2, which had not been characterized previously. Digestion of the β subunit with trypsin generates a ~10-kDa fragment beginning at βMet-249, the N terminus of βM2, that can be isolated by Tricine-SDS-PAGE and HPLC purification (White and Cohen, 1992). For that fragment, which was sequenced only from nAChRs photolabeled after equilibration with [3H]azietomidate in the absence of Carb, there was also only a single peak of 3H release in cycle 20, consistent with labeling of βAsp-268 at 4 cpm/pmol (i.e., at a similar efficiency as βGln-276 and at ~5% the efficiency of αGlu-262).

Discussion

We use rapid-perfusion electrophysiological techniques to establish that azietomidate acts primarily as an nAChR open-state inhibitor without enhancing the kinetics of desensitization. Full inhibition by 10 μM azietomidate develops within a second in the presence of ACh without any increase in the fraction of nAChRs in the desensitized state beyond that seen for ACh alone, and after removal of ACh, azietomidate did not stabilize nAChRs in the desensitized state. Because substantial inhibition develops after exposure to agonist and 10 μM azietomidate for 50 ms, these conditions were chosen for time-resolved photolabeling. Further studies, requiring a combination of single channel and rapid perfusion analyses, will be required to determine whether nAChR inhibition results from physical occlusion of the ion channel or from stabilization of the nAChR in a novel, nonconducting state.

The observed concentration-dependent increase in the rate of inhibition by azietomidate indicates that its apparent rate constant for open-state inhibition (k_, 5 × 10^5 M^-1 s^-1) is ~100-fold lower than that of the positively charged QX-222 (Dilger and Vidal, 1994) and ~10-fold lower than that of octanol and pentobarbital (Forman et al., 1995; Dilger et al., 1997). One factor that may account for azietomidate’s low apparent rate constant is that it has a substantially higher lipid solubility (octanol/water partition coefficient, P = 3600) than octanol (P = 1400) or pentobarbital (P = 130). If the aqueous concentration determines the rate of inhibition, our calculation underestimates the value of k_. Slow onset and recovery from inhibition are also reported for propofol (P = 4300; Dilger et al., 1994).

nAChR Photolabeling by [3H]Azietomidate. In the nAChR structure (Fig. 8), the photolabeled amino acids are distributed in four different structural domains: 1) at the extracellular end of the ion channel (αM2–20, βM2–20, and δM2–20); 2) within the δ subunit helix bundle (δCys-236 in δM1); 3) in the cytoplasmic basket formed by the MA helices that precede the M4 helices in the primary structure (αGlu-390); and 4) at the lipid interface (αCys-412 in αM4).
included in Fig. 8 are Connolly surface representations (see Materials and Methods) of predicted azietomidate binding pockets within the ion channel near M2–20 (white), in the δ subunit helix bundle (blue), and in the nAChR cytoplasmic basket domain (yellow). Table 2 compares the efficiencies of [3H]azietomidate incorporation in these structural domains. Although αGlu-262 is the residue photolabeled most efficiently, and it is plausible that binding to the extracellular end of the ion channel accounts for the functional inhibition of the nAChR, occupancy at the other sites could also contribute to inhibition. Additional photolabeling over a range of azietomidate concentrations would be required to determine the relative occupancy of each site.

The time-resolved photolabeling data in Table 2 show that the accessibility at the extracellular end of the ion channel and at the other sites are similar in the state(s) transiently stabilized by agonist and in the equilibrium desensitized state, because the same amino acids in each domain are photolabeled in both states at efficiencies that differ by less than 3-fold. However, [3H]azietomidate cannot access the ion channel in the resting state (Ziebell et al., 2004). The other sites were not characterized in that study.

Photolabeling in the nAChR Ion Channel. Photolabeling of the nAChR with [3H]azietomidate and other aliphatic diazirines has established that the activated intermediates react most efficiently with nucleophilic side chains (Glu, Asp, Tyr) (Pratt et al., 2000; Ziebell et al., 2004; Hamouda et al., 2006). Thus, the pattern of labeling of the glutamates and aspartates at the outer end of the channel (Table 2) likely delineates the binding pocket and orientation of azietomidate. That all of the M2–20, but none of the M2–24 (βGlu-272, δGlu-280) residues examined were photolabeled suggests that the pocket extends down the channel, which is lined by hydrophobic residues for ~15 Å (Figs. 8, B and C). The homologous region in GABAA Rs is lined with polar residues, providing a rationale for the much higher concentrations of azietomidate required to inhibit GABAA Rs. Orientation may also explain why αGlu-262 is labeled >10-fold more efficiently than βM2–20 (βAsp-268), because there is no obvious structural or photochemical explanation. However, we cannot rule out the possibility that the unlabelled acidic side chains at position M2–24, which are oriented toward the channel in the resting state structure, have moved in the photolabeled conformations. Azioctanol also photolabels αM2–20 (αGlu-262) in the desensitized state (Pratt et al., 2000), and mutational analyses associate irreversible nAChR inhibition with reaction at αGlu-262 (Forman et al., 2007).

All of the above results are consistent with the preferred binding site for azietomidate being at the extracellular end of the ion channel. An alternative interpretation, based upon photolabeling studies of the nAChR in solution (Ziebell et al., 2004), was that the reactive amino acids at the extracellular end of the channel reacted with photoactivated azietomidate as it diffused into or out of an equilibrium binding site at the cytoplasmic end of the ion channel. This possibility was based upon the photolabeling of amino acids at the cytoplasmic end of the ion channel (Ser/Thr at positions M2–2 and M2–6 of the α and δ subunits) at ~1% the efficiency of αGlu-262 (Fig. 6C). In the frozen state photolabeling, however, the equilibrium binding is trapped and there would be no ongoing transient diffusion past M2–20.

Photolabeling in the δ Subunit Helix Bundle. The photolabeling in the presence of agonist of δM1 Cys-236, a residue that projects within the helix bundle, suggests that [3H]azietomidate binds therein. In previous studies at 20°C, the fragment containing δM1 was photolabeled by [3H]azietomidate in nAChRs in the desensitized, but not in the resting state (Ziebell et al., 2004). Within the nAChR structure (Fig. 8, C and D), which is believed to be the closed state, the pocket at the extracellular end of the δ subunit helix bundle, capped by the M2-M3 loop, is large enough to accommodate azietomidate, but it ends ~10 Å above the level of the

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**Fig. 8.** The binding sites for [3H]azietomidate in the nAChR. Views of the T. marmorata nAChR structure (Protein Data Bank code 2BG9) (α, gold; β, blue; γ, green; δ, magenta) showing α-helical (cylinders) and β-sheet (ribbon) secondary structure. A, a perspective parallel to the membrane surface (with the γ subunit omitted). B, an expanded view of A focused on the top of the channel (γ and α, omitted). C, the transmembrane helices viewed looking down the ion channel. D, the δ subunit helix bundle looking down the M1 helix. E, the amino acid sequences of each of the M2 helices of the nAChR structure, with the amino acids highlighted in the structures indicated by the same colors. The residues labeled by [3H]azietomidate are shown in stick format, color-coded for location: ion channel, position M2–20 (αGlu-262, βAsp-269; and δGlu-276, red); the δ subunit helix bundle (δCys-236, white); αM4 (αCys-412, cyan); the cytoplasmic basket formed by the MA helices (αGlu-390, cyan). Also indicated in stick format in the M2 helices are unlabeled acidic side chains (βGlu-273 and δGlu-280, green) that project into the channel lumen and the prolines (orange) that precede those positions in each subunit. δPhe-232 (yellow), the amino acid in δM1 that is photolabeled by [125I]TID (Arevalo et al., 2005) and [3H]benzophenone (Garcia et al., 2007), is included, as are the amino acids in αM4/αM4 (αGlu-398, αAsp-407, purple) photolabeled by [3H]azicholesterol (Hamouda et al., 2006). The volumes defined by the ensemble of the 10 lowest energy orientations of azietomidate docked at the extracellular end of the ion channel (gray, 680 Å3), in the δ subunit helix bundle (blue, 570 Å3), and in the cytoplasmic basket (yellow, 970 Å3) are shown in Connolly surface representations with a single azietomidate docked in its lowest energy orientation in each pocket (see Materials and Methods).
labeled δCys-236, suggesting that activation causes the structure to change in this region.

Two other nAChR inhibitors photolabel within the δ subunit helix bundle in an agonist-dependent manner: [125I]TID photolabels δCys-236, δPhe-232, δThr-274 (M2–18), δLeu-278 (M2–22), and δIle-288 (M2-M3 loop) at least 10 times more efficiently after transient exposure to agonist than in the equilibrium desensitized state (Arevalo et al., 2005; Hamouda et al., 2008), and [3H]benzophenone photolabels δPhe-232, δPro-286, and δIle-288 at 20°C only in the presence of agonist (Garcia et al., 2007). However, the dramatic difference in [125I]TID photolabeling between the transient and equilibrium agonist-stabilized states is in contrast to the <3-fold difference seen for [3H]azietomidate photolabeling within the helix bundle. Although TID (vol. 150 Å³) and benzophenone (vol. 125 Å³) are both smaller than azietomidate (vol. 240 Å³), 4-[3-(trifluoromethyl)-3H-diazirin-3-yl]-benzyl-1-(1-phenylethyl)-1H-imidazole-5-carboxylate (vol. 284 Å³), a larger aryl diazirine, did not photolabel the δ subunit helix bundle in either resting or desensitized state (Nirthanan et al., 2008).

**Photolabeling in the nAChR Cytoplasmic Domain.** Azietomidate photolabeling of αGlu-390, within the basket formed by the MA helices that surround the cytoplasmic access to the ion channel (Fig. 8A), provides the first evidence that this region can act as a drug binding site. αGlu-390 is ~25 Å below the entry to the ion channel and oriented toward the interior of the basket near the base of the portals between each of the MA helices, where the basket dimensions are appropriate for an azietomidate binding site (Fig. 9). This is unlikely to be a random photolabeling event because azietomidate did not photolabel other negatively charged side chains in this region: αAsp-389, αGlu-391, αGlu-397, or αGlu-398. However, its affinity is likely to be lower than that of the channel because of the relative degree of photoincorporation in the two regions (Table 2). Further studies will be required to explore the site's functional significance, but we note that it is close to residues that control channel conductance in the αβ2 neuronal nAChR and the 5-HT3A receptor (Hales et al., 2006).

**General Anesthetic Actions on Cys-Loop Receptors.** Although azietomidate probably inhibits the nAChR via the ion channel site, our results provide direct evidence of the diversity of binding sites even for a single general anesthetic in a Cys-loop receptor. Anesthetic binding sites in GABAA and glycine receptors have been predicted to exist in the transmembrane domain within the pockets within the subunit helix bundles, where the volume of substituted side
chains determine the "cut-off" for the size of active anesthetics (Wick et al., 1998; Krasowski et al., 2001). [14]Chloretone (vol, 86 Å2) binds within the nAChR ß subunit helix bundle in the absence or presence of agonist, whereas [3H]azetidomate binds only in the presence of agonist, and the slightly larger [3H]-[3-(trifluoromethyl)-3H]diaizin-3-yl]benzyl-1-(1-phenyl-ethyl)-1H-imidazole-5-carboxylic acid: a derivative of the stereoselective general anesthetic etomidate for labeling ligand-gated ion channels. J. Med Chem 46:1257–1265.


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