IDENTIFICATION OF PROPOFOL BINDING SITES IN A NICOTINIC ACETYLCHOLINE RECEPTOR WITH A PHOTOREACTIVE PROPOFOL ANALOG*

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*Running Title: Propofol nAChR Binding Sites

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Background: Propofol, a general anesthetic, potentiates GABAA receptors and inhibits nAChRs by binding to unknown sites.

Results: Photoaffinity labeling with a photoreactive propofol analog is used to identify propofol binding sites in a muscle-type nAChR.

Conclusion: Propofol binds to an intrasubunit site in the nAChR transmembrane domain.

Significance: This study helps define the diversity of allosteric modulator binding sites in Cys-loop neurotransmitter receptors.

SUMMARY
Propofol, a widely used intravenous general anesthetic, acts at anesthetic concentrations as a positive allosteric modulator of inhibitory γ-aminobutyric acid receptors (GABAAR), and this interaction is a major determinant of propofol’s anesthetic potency (1-3). In contrast, propofol inhibits excitatory nicotinic acetylcholine receptors (nAChR) (4,5), which are also members of the Cys-loop superfamily of pentameric ligand gated ion channels, as well as GLIC, a prokaryotic homolog that is a proton-gated cation channel (6). Identification of propofol binding sites in GABAAR and nAChRs is necessary to determine whether propofol binds to equivalent or distinct sites in Cys-loop receptors when it produces opposing effects as a positive or negative allosteric modulator.

Propofol, a potent intravenous general anesthetic, acts as a positive allosteric modulator of inhibitory γ-aminobutyric acid receptors (GABAAR), and this interaction is a major determinant of propofol’s anesthetic potency (1-3). In contrast, propofol inhibits excitatory nicotinic acetylcholine receptors (nAChR) (4,5), which are also members of the Cys-loop superfamily of pentameric ligand gated ion channels, as well as GLIC, a prokaryotic homolog that is a proton-gated cation channel (6). Identification of propofol binding sites in GABAAR and nAChRs is necessary to determine whether propofol binds to equivalent or distinct sites in Cys-loop receptors when it produces opposing effects as a positive or negative allosteric modulator.
N-terminal extracellular domain, a transmembrane domain made up of a loose bundle of four transmembrane helices (M1-M4), and an intracellular domain formed by the primary structure between the M3 and M4 helices. The M2 helices from each of the five subunits form the ion channel, and the M1, M3, and M4 helices form an outer ring partly exposed to lipid.

While there has been no direct identification of propofol binding sites in a GABA<sub>A</sub>R or nAChR, within GLIC crystals, propofol binds within an intrasubunit pocket formed by the four transmembrane helices (6). Within GABA<sub>A</sub>R, photoreactive derivatives of etomidate, another intravenous anesthetic, identified an intersubunit etomidate binding site in the transmembrane domain at the interface between β and α subunits, the same interface that contains the GABA binding site in the extracellular domain (13,14). Within the Torpedo nAChR, etomidate analogs have been shown to bind to three distinct sites in the transmembrane domain: the ion channel, the γ-α subunit interface, and the δ subunit helix bundle (15,16). Each of these sites are potential binding pockets for propofol, a potent inhibitor of the Torpedo nAChR expressed in oocytes (IC<sub>50</sub> = 7 μM (17)).

In this report we use AziPm (2-isopropyl-5-[3-(trifluoromethyl)-3H-diazirin-3-yl]phenol), a recently developed photoreactive propofol analog (18), to identify propofol binding sites in the Torpedo nAChR. AziPm acts as a tadpole anesthetic similar in potency to propofol, and it potentiates GABA<sub>A</sub>R responses at anesthetic concentrations (18). While AziPm is related structurally to propofol (Fig. 1), it incorporates a photoreactive 3-trifluoromethyl 3-phenyl diazirine group which can react with most amino acid side chains, including aliphatics (19). AziPm also is related in structure to TID (Fig. 1), a potent Torpedo nAChR inhibitor (20) that has been shown by photoaffinity labeling to bind in a state dependent manner to sites in the ion channel (21) and in the δ subunit helix bundle (22,23). Based upon competition radioligand binding assays, we find that propofol binds to the nAChR with higher affinity in the desensitized state than in the resting state, while AziPm binds with higher affinity in the resting state. Based upon the amino acids photolabeled by [3H]AziPm, we show that it binds to three sites in the nAChR transmembrane domain: within the δ subunit helix bundle, in the ion channel, and at the γ-α interface. Propofol binds to the site within the δ helix bundle, as shown by the full inhibition of [3H]AziPm photolabeling there. Propofol acts as an allosteric inhibitor of [3H]AziPm photolabeling in the ion channel and potentiates [3H]AziPm photolabeling at the γ-α interface.

**EXPERIMENTAL PROCEDURES**

**Materials.** Torpedo nAChR-rich membranes, purified from Torpedo californica electric organs (Aquatic Research Consultants, San Pedro, CA) as described (24), contained from 1.5-1.7 nmol [3H]ACh binding sites/mg protein. Non-radioactive AziPm was synthesized as described (18), and [3H]AziPm (10 Ci/mmole) was prepared by custom tritiation at AmBios (Newington, CT). [3H]Phencyclidine ([3H]PCP; 27 Ci/mmole) was from PerkinElmer Life Sciences; [3H]tetracaine (30 Ci/mmole) was from AmBios; and [3H]ACh (1.9 Ci/mmole) was synthesized from choline and [3H]acetic anhydride. Staphylococcus aureus Glutamic-C endopeptidase (V8 protease) and Lysobacter enzymogenes endoproteinase Lys-C (EndoLys-C) were from MP Biomedicals and Roche Diagnostics, respectively, and TPCK-treated trypsin was from Worthington Biochemical Corp.

**Radioligand Binding Assays.** The equilibrium binding of [3H]tetracaine, [3H]PCP and [3H]ACh to Torpedo nAChR-rich membranes in Torpedo physiological saline (TPS; 250 mM NaCl, 5 mM KCl, 3 mM CaCl<sub>2</sub>, 2 mM, MgCl<sub>2</sub>, 5 mM sodium phosphate, pH 7.0) was assayed by centrifugation. Propofol and AziPm were prepared as 1 M stock solutions in methanol, and methanol was present at a final concentration of 0.5% in all binding assays. All propofol dilutions were in glass containers. Membrane suspensions were pre-equilibrated at 4°C for 30 min with radioligand, incubated with various concentrations of propofol, AziPm, or other drugs for 1 hr at room temperature (4°C for [3H]ACh), and then centrifuged at 18,000×g for 60 min. After removal of the supernatants, membrane pellets were resuspended overnight in 0.1 ml of 10% SDS, and pellet and supernatant 3H were determined by liquid
scintillation counting. For [3H]ACh binding assays, membrane suspensions (40 nM Ach sites, 1 ml per sample) were pretreated with 0.1 mM diisopropylphosphofluoridate before incubation with [3H]ACh (9 nM) and other drugs. Membrane suspensions (~750 nM ACh sites, 0.2 ml per data point) were incubated with [3H]tetracaine (18 nM), in the presence of a non-desensitizing competitive antagonist (2 µM α-bungarotoxin (α-BgTx)), or [3H]PCP (4 nM), in the presence of agonist (1 mM Carb), and varying concentrations of propofol or AziPm. Non-specific binding of [3H]ACh, [3H]tetracaine, or [3H]PCP was determined in the presence of 1 mM Carb, 0.1 mM tetracaine, or 0.1 mM PCP, respectively. The total and nonspecific binding were 6,450 ± 8 cpm and 96 ± 8 cpm for [3H]ACh; 16,418 ± 524 cpm and 7,050 ± 469 cpm for [3H]tetracaine; and 11,875 ± 1,872 cpm and 2,007 ± 390 cpm for [3H]PCP.

The concentration dependent inhibition of [3H]ACh, [3H]tetracaine, and [3H]PCP binding by propofol or AziPm was fit by nonlinear least squares (Sigma Plot) to Equation 1:

\[
f(x) = \frac{(B_0-B_\infty)}{(1 + (x/IC_{50})^{nH})} + B_\infty \quad \text{Eq. 1}
\]

where \( f(x) \) is the total radioligand binding (in cpm) at competitor concentration \( x \), \( B_0 \) is the total radioligand bound in the absence of competitor, \( B_\infty \) is the residual radioligand binding at high concentrations of competitor, \( IC_{50} \) is the drug concentration inhibiting radioligand binding by 50%, and \( n_H \) is the Hill coefficient. For all fits with the exception of the propofol inhibition of [3H]PCP binding, \( B_\infty \) was set equal to the non-specific radioligand binding (\( B_{ns} \)) determined in the presence of 1 mM Carb, 0.1 mM tetracaine, or 0.1 mM PCP.

**Photolabeling nAChR-rich membranes.** Experiments were performed using ~100 µg or 10 mg protein per condition for analytical and preparative scale photolabelings. For all experiments Torpedo nAChR-rich membranes were resuspended at 2 mg protein/ml in TPS supplemented with 1 mM oxidized glutathione, an aqueous scavenger. [3H]AziPm was dried down under an argon stream to remove methanol and then resuspended for 30 min at a final concentration of ~1 µM in the nAChR-rich membrane suspension before addition of other drugs. This was followed by an additional 30 min incubation on ice. Membrane suspensions were irradiated on ice for 30 min using a 365nm UV lamp (Model EN-16, Spectronics Corp, Westbury, NJ) at a distance of 0.5 cm. Preparative scale photolabeling was performed under three paired experimental conditions to identify photolabeled amino acids: (1) in the resting state vs. desensitized states (±Carb); (2) in the absence vs. presence of 300 µM propofol (without agonist); and (3) in the desensitized state (+ Carb), in the absence vs. presence of propofol.

**SDS-Polyacrylamide Gel Electrophoresis and Proteolytic Digestions.** For photolabeling experiments on an analytical scale, nAChR subunits were resolved after photolysis by Tris-glycine SDS-PAGE on 8% acrylamide, 0.32% bisacrylamide gels which were stained with Coomassie Blue R-250. Samples were run in duplicate on two gels, one of which was prepared for fluorography using Amplify (Amersham Biosciences), and the other for measurement of 3H incorporation into individual subunits quantified by liquid scintillation counting of excised gel bands.

After photolabeling on a preparative scale, membranes were pelleted by centrifugation and then resuspended in sample buffer for electrophoresis. The β, γ and δ subunit bands were excised from the Coomassie Blue-stained gels and recovered by passive elution, concentrated to <400 µl by centrifugal filtration (Vivaspin 15 Mr 5,000 concentrators, Vivascience inc., Edgewood, NY), and then acetone-precipitated and resuspended in 100-200 µl of digestion buffer (12 mM Tris, 0.5 mM EDTA, 0.1% SDS, pH 8.6). The nAChR α subunit gel band was excised from the stained gel, soaked in overlay buffer (125 mM Tris, 5% sucrose (w/v), 0.1% SDS (w/v), 1 mM dithiothreitol, pH 6.8), and then placed in the wells of a 15% acrylamide mapping gel for in-gel digestion with 100 µg of S.aureus V8 protease to generate four subunit fragments (αV8-20 (20kDa), αV8-18 (18kDa), αV8-10 (10kDa) and αV8-4 (4kDa)) that were visualized by GelCode® Blue stain (Pierce) (25). After electrophoresis, the fragments were eluted from the excised gel bands and resuspended in digestion buffer.

**Isolation of [3H]AziPm-labeled nAChR**
subunit fragments. Aliquots of α V8-20 (~30 µg) and δ subunit (~150 µg) were digested with EndoLys-C (0.5 units/sample) for 2 weeks at room temperature. Aliquots of α V8-10 (~30 µg) and β subunit (~150 µg) were diluted with 0.5% Genapol in 50 mM NH₄HCO₃ buffer, pH 8.1, to reduce SDS concentration to <0.02 % and then digested with trypsin (50 µg/sample) in the presence of 0.4 mM CaCl₂ for 16 hrs (β subunit) or 2 days (α V8-10). The trypsin and EndoLys-C digests of β and δ subunits, respectively, were fractionated on small pore (16.5% T, 6%C) Tricine SDS-PAGE gels (26), and fragments beginning at the N-termini of βM2, δM1, and δM2 were isolated by reversed phase HPLC (rpHPLC) fractionation of materials of ~10 kDa and 10-14 kDa eluted from the β and δ subunit digests, respectively (21, 22). Fragments beginning at the N-termini of αM2 and αM4 were isolated by direct rpHPLC fractionation of the EndoLys-C and trypsin digests of α V8-20 and α V8-10 (27).

To characterize [³H]AziPm incorporation in subunit M2-M3 loops and M3 helices, aliquots of β, γ and δ subunits (~150 µg) were digested with V8 protease (100 µg) for 3 days. The digests were fractionated by rpHPLC and material recovered from the hydrophobic fractions was sequenced as described below (28).

rpHPLC and sequence analysis. nAChR subunit proteolytic fragments were fractionated on an Agilent 1100 binary reversed-phase HPLC system using a C4-Aquapore 7-µm (100 x 2.1 mm) column with an upstream C2 guard column, at 40°C. Peptide elution was monitored by absorbance at 215 nm. Aqueous trifluoroacetic acid (0.08%) was used as solvent A and acetonitrile (60%), 2-propanol (40%) and trifluoroacetic acid (0.05%) as solvent B. A non-linear elution gradient increasing from 25% to 100% solvent B in 80 min was used at a flow rate of 200 µl/min, with fractions of 0.5 ml collected.

N-terminal sequencing was performed on an Applied Biosystems Procise 492 protein sequencer, with 1/6 of the material from each cycle used to quantify the amount of PTH amino acid derivative obtained and 5/6 collected to measure the ³H released. Pooled HPLC fractions containing ³H labeled peptides were drop-loaded at 45°C onto Applied Biosystems Micro TFA glass fiber filters, except for fractions containing αM4 or δM1 which, due to their low sequencing efficiency when loaded on glass fiber filters, were loaded onto PVDF filters using ProSorb® sample preparation cartridges. All filters were then treated with Biobrene® Plus. To chemically isolate during sequence analysis the nAChR subunit fragments beginning at βThr-273, γThr-276, or δThr-281 within the M2-M3 loops (28), sequencing filters were treated with o-phthalaldehyde (OPA) prior to cycle 6 of Edman degradation, which contains a proline. OPA reacts with primary amines, but not with proline, a secondary amine. This treatment prevents further sequencing of other fragments on the filter that do not contain a proline at that cycle (24, 29).

The efficiency of photolabeling of amino acid residues within a sequenced fragment was calculated from the increase in ³H release at that cycle of Edman degradation (cpmₓ – cpmₓ₋₁) and the increased mass of the PTH-amino acid released in cycle x. The masses of the released PTH-amino acids were quantified from their peak heights relative to the peak heights of PTH-amino acid standards and then fit by non-linear least square (SigmaPlot 11) to equation 2:

\[ f(x) = I_o \times R^x \]  
(Eq. 2)

where \( f(x) \) is the mass of the peptide residue in cycle x (in pmol), \( I_o \) is the initial amount of peptide (in pmol), and \( R \) is the average repetitive yield, a parameter dependent upon the amount of sample wash off and the efficiency of peptide bond cleavage at each cycle of Edman degradation. PTH-derivatives whose amounts cannot be accurately measured (Cys, His, Ser, Trp and Arg) were omitted from this fit.

The efficiency of photolabeling of amino acid residues (cpm/pmol) was then calculated using Equation 3:

\[ \frac{(cpm_x – cpm_{x-1})}{(5 \times I_o \times R^x)} \]  
(Eq. 3)

Molecular modeling. A model of *Torpedo californica* nAChR structure based on the cryoelectron microscopy structure of the *Torpedo marmorata* nAChR (PDB code, 2BG9 (9)) was used to dock, using the Discovery studio (Accelrys, Inc) software package, AziPm molecules within (i) the ion channel, (ii) the δ
subunit helix bundle, and (iii) the γ-α subunit interface. We docked AziPm using the CHARMm-based molecular dynamics simulated-annealing program CDOCKER. A randomly oriented AziPm molecule was seeded at the center of binding site spheres positioned between M2-6 and M2-13 within the ion channel (r = 10 Å), within the pocket formed by δM2-18, δPhe-232 and δCys-236 in the δ subunit helix bundle (r = 8 Å), or at the level of αM2-10 in the γ-α subunit interface (r = 8 Å). The CDOCKER program was set up to first seed fifty randomly distributed replicas of AziPm in the center of the spheres, and high temperature molecular dynamics was used to generate 50 random conformations for each replica. For each conformation, 400 energy-minimized docking solutions were generated using simulated annealing and full potential minimization. Connolly surface representations defined by a 1.4 Å diameter probe of the ensemble of the 15 docking solutions with the lowest CDOCKER interaction energies are shown for each of the binding sites.

RESULTS.

Propofol and AziPm inhibition of [3H]ACh, [3H]tetracaine, and [3H]PCP equilibrium binding. In initial experiments, we compared the effects of propofol and AziPm on the equilibrium binding of [3H]ACh to Torpedo nAChR-membranes (Fig. 2A). For nAChR-rich membranes in the presence of agonist at equilibrium, >99% of nAChRs are in the equilibrium desensitized state that binds ACh with highest affinity, while in the absence of agonist, ~85% of nAChRs are in the resting, closed channel state and ~15% in the desensitized state (30,31). Those conformations are interconvertible, and allosteric modulators such as PCP and proadifen increase the fraction of nAChRs in the desensitized state in the absence of agonist (stabilize the desensitized state) and enhance ACh equilibrium binding affinity (32,33).

We measured [3H]ACh binding at a concentration sufficient to occupy ~20% of sites, a condition chosen to detect either increases or decreases of ACh binding affinity. Under this condition, proadifen, a desensitizing aromatic amine non-competitive antagonist (33), maximally increased [3H]ACh binding by 30%. Propofol at concentrations above 10 µM also increased [3H]ACh binding, producing a maximal increase of 20% at 300 µM, with inhibition seen at 1 mM. In contrast, AziPm only inhibited [3H]ACh binding, with a steep concentration dependence (IC50 = 134 ± 13 µM; Hill coefficient, nH = 2.5 ± 0.5) (Fig. 2A).

We also examined the effects of propofol and AziPm on the equilibrium binding of nAChR channel blockers and found that propofol bound with higher affinity to the nAChR in the presence of agonist, while AziPm bound preferentially to the nAChR in absence of agonist. [3H]tetracaine binds in the nAChR ion channel with 30-fold higher affinity in the resting, closed channel state than in the desensitized state (34), while [3H]PCP binds to a site in the channel with 5-fold higher affinity in the nAChR desensitized state (32). Both propofol and AziPm inhibited the binding of [3H]tetracaine (Fig. 2B, measured in the presence of α-BgTx, a non-desensitizing competitive antagonist) and [3H]PCP (Fig. 2C, measured in the desensitized state in the presence of the agonist carbamylcholine (Carb)).

Propofol at high concentrations inhibited [3H]tetracaine binding by >95%, with an IC50 of 125 ± 14 µM and with a steep concentration dependence (nH = 1.6 ± 0.3) consistent with allosteric inhibition resulting from propofol’s stabilization of the nAChR in a desensitized state. Propofol at 1 mM reduced [3H]PCP binding by only 74 ± 3% (2 experiments) compared to the full inhibition produced by excess non-radioactive PCP. However, the concentration dependence was consistent with a single site model (IC50 = 47 ± 5 µM, nH = 1.09 ± 0.09). This partial inhibition of binding indicates an allosteric rather than competitive mechanism of inhibition such that when propofol is bound to the nAChR, the [3H]PCP dissociation constant increases ~4-fold.

AziPm at 100 µM decreased [3H]tetracaine and [3H]PCP binding by 90% and ~75%, respectively, but at higher concentrations AziPm increased binding of both channel blockers. This increase probably reflects a non-specific perturbation of membrane structure caused by this hydrophobic drug, since AziPm also increased [3H]tetracaine binding in the presence of 100 µM non-radioactive tetracaine, a concentration sufficient to displace all [3H]tetracaine from its high affinity site in the ion channel. For AziPm
concentrations up to 100 µM, the observed concentration dependences of inhibition of [3H]tetracaine and [3H]PCP binding were consistent with a competitive mechanism of inhibition characterized by IC50s of ~10 and ~50 µM, respectively.

[3H]AziPm photo incorporation into nAChR-rich membranes. nAChR-rich membranes were photolabeled with [3H]AziPm in the absence of any other drug or in the presence of agonist (Carb) and/or other drugs (PCP, tetracaine, or propofol). The amount of [3H]AziPm incorporation within nAChR subunits (and other membrane polypeptides) was determined by SDS-PAGE using fluorography (Fig. 3A) and 3H liquid scintillation counting (Fig. 3B).

In the absence of agonist, [3H]AziPm incorporated into each nAChR subunit, with the δ subunit labeled most efficiently. Tetracaine reduced α and β subunit photolabeling by 40% and δ subunit labeling by 60%. Since tetracaine binds within the ion channel, this inhibition suggests that [3H]AziPm photolabels residues within the ion channel. Propofol also inhibited labeling of the δ subunit by 60%, but it increased α subunit incorporation by 20% (Fig. 3B). For nAChRs in the absence of agonist, the 1,900 cpm of tetracaine- or propofol-inhibitable [3H] incorporation in the nAChR δ subunit indicated photolabeling at 25 cpm/pmol δ subunit (~0.5% of subunits).

In the presence compared to the absence of Carb, nAChR subunit photolabeling was reduced by ~20%. Propofol inhibited δ subunit photolabeling by ~50%, while PCP reduced it by only 20%. As in the absence of agonist, propofol increased α subunit labeling ~20% in the presence of Carb.

To provide an initial localization of the photolabeled amino acids within the α subunit, we characterized the distribution of [3H]AziPm incorporation within the large, non-overlapping fragments of the α subunit that can be generated by “in-gel” digestion with S. aureus V8-protease: fragments of 20 kDa (αV8-20, beginning at αSer-173 and containing ACh binding site Segment C and the M1-M3 transmembrane helices), 18 kDa (αV8-18, beginning at αThr-52 and containing ACh binding site Segments A and B), and 10 kDa (αV8-10, beginning at αAsn-339 and containing the cytoplasmic MA and transmembrane M4 helices) (9,25). For the nAChR photolabeled in the resting state, 59%, 3%, and 38% of [3H] were recovered in αV8-20, αV8-18, and αV8-10, respectively (Fig. 3C). The pharmacologically sensitive photolabeling within the α subunit was restricted to αV8-20, where tetracaine and Carb inhibited photolabeling by 60% and 40%, respectively, and propofol increased labeling by ~15%.

Propofol inhibition of [3H]AziPm photolabeling in the δ subunit. To further characterize the effects of propofol on [3H]AziPm photo incorporation, we examined the concentration dependence of propofol inhibition of δ subunit [3H] incorporation in nAChRs photolabeled in the absence and presence of Carb (Fig. 4). In the presence of agonist, propofol inhibition of [3H]AziPm δ subunit labeling was consistent with a simple, single site model (nH = 1) with an IC50 of 37 ± 8 µM that was similar to the IC50 of 47 µM for the inhibition of [3H]PCP binding in the desensitized state (Fig. 2C). In the absence of agonist, propofol inhibited [3H]AziPm δ subunit photolabeling with a concentration dependence (IC50 = 140 ± 30 µM, nH = 2) similar to that seen for propofol inhibition of [3H]tetracaine binding in the ion channel (IC50 = 125 ± 14 µM, nH = 1.6 ± 0.3) (Fig. 2B).

[3H]AziPm and propofol bind in the nAChR δ subunit helix bundle in the desensitized state. In the presence of agonist, propofol inhibited δ subunit photolabeling to a greater extent than PCP, suggesting that propofol inhibits [3H]AziPm photolabeling of a site other than the ion channel. The δ subunit helix bundle pocket was a likely candidate since [125I]TID, a drug related in structure to AziPm (Fig. 1), binds within this pocket in the nAChR open and desensitized states, photolabeling amino acids in δM1 (δPhe-232 and δCys-236), δM2 (δThr-274 (δM2-18’)), and δM2-δM3 loop (δIle-288) (22,23). To determine whether there was propofol-inhibitable [3H]AziPm photolabeling of this site, we isolated and sequenced fragments beginning near the N-termini of δM2 and δM1 from nAChRs photolabeled in the presence of Carb ± 300µM propofol. Sequence analysis of the δM2 fragment (Fig. 5A) revealed peaks of [3H] release in cycles 18 and 21, which indicated photolabeling of δThr-274 and
\( \delta \text{Arg-277} \), residues positioned within the \( \delta \) subunit helix bundle (9). Propofol inhibited labeling of these residues by 80% and 75%, respectively. N-terminal sequencing through \( \delta \text{M1} \) (Fig. 5B) revealed peaks of \(^3\text{H}\) release in cycles 27 and 31, consistent with photolabeling of \( \delta \text{Phe-232} \) and \( \delta \text{Cys-236} \). Propofol inhibited labeling of these residues by 95% and 40%, respectively (Fig. 5B).

When photolabeling was performed in the absence of Carb, no \(^3\text{H}\) incorporation was detected in \( \delta \text{Phe-232} \), \( \delta \text{Cys-236} \), or \( \delta \text{Thr-274} \), indicating that if labeling occurred, it was at <5% the level seen in the presence of Carb (Fig. 5C). We also characterized \(^3\text{H}\)\text{AziPm} photolabeling in the \( \delta \text{M2-M3} \) loop by isolating and sequencing a fragment beginning at \( \delta \text{Thr-281} \). In the presence of Carb, photolabeling of \( \delta \text{Ile-288} \), if it occurred, was at less than 3% the level of \( \delta \text{Phe-232} \) (data not shown).

\(^3\text{H}\)\text{AziPm} photoincorporation in \( \alpha \text{M2} \). When nACHRs were photolabeled in the absence of agonist, \(^3\text{H}\)\text{AziPm} incorporation in the \( \delta \) subunit was inhibited by both propofol and tetracaine. However, propofol enhanced and tetracaine inhibited \( \alpha \) subunit photolabeling (Fig. 3).

Furthermore, the propofol-enhanced photolabeling in the \( \alpha \) subunit was within \( \alpha \text{V8-20} \), the 20 kDa fragment that contains the M1-M3 transmembrane helices. Sequence analysis of an \( \alpha \) subunit fragment beginning at \( \alpha \text{Met-243} \) (the N-terminus of \( \alpha \text{M2} \)), isolated from \( \alpha \text{V8-20} \), revealed peaks of \(^3\text{H}\) release in cycles 6 and 10 that established photolabeling of \( \alpha \text{Ser-248} \) (\( \alpha \text{M2-6} \)) and \( \alpha \text{Ser-252} \) (\( \alpha \text{M2-10} \)) (Fig. 6A). Propofol reduced by 65% the labeling of \( \alpha \text{M2-6} \), a position lining the lumen of the ion channel, while it increased by four-fold the photolabeling of \( \alpha \text{M2-10} \), a position near the \( \gamma-\alpha \) and \( \beta-\alpha \) subunit interfaces (15,16). Sequence analysis of this \( \alpha \text{Met-243} \) fragment isolated from nACHRs photolabeled in the presence of agonist (Fig. 6B) established that \(^3\text{H}\)\text{AziPm} also photolabeled \( \alpha \text{M2-6} \) and \( \alpha \text{M2-10} \) in the desensitized state, but at lower efficiency than in the resting state.

\(^3\text{H}\)\text{AziPm} photoincorporation in the M2 ion channel domain is inhibited by agonist (nACHR desensitization) or by propofol. \(^3\text{H}\)\text{AziPm} photoincorporation into \( \alpha \text{M2-6} \) provided evidence that AziPm binds within the ion channel and that this binding is sensitive to agonist and to propofol. To further characterize \(^3\text{H}\)\text{AziPm} photolabeling within the ion channel, fragments beginning at the N-termini of \( \beta \text{M2} \) (\( \beta \text{Met-249} \)) and \( \delta \text{M2} \) (\( \delta \text{Met-257} \)) were also isolated and sequenced from nACHRs photolabeled with \(^3\text{H}\)\text{AziPm}: (i) in the absence and presence of Carb, to compare photolabeling in the resting and desensitized states; and (ii) in the absence and presence of 300 \( \mu \text{M} \) propofol. When the fragment beginning at \( \beta \text{Met-249} \) was sequenced, the peaks of \(^3\text{H}\) release in cycles 6 and 13 of Edman degradation established labeling of \( \beta \text{Ser-254} \) (\( \beta \text{M2-6} \)) and \( \beta \text{Val-261} \) (\( \beta \text{M2-13} \)) that was inhibited >90% by agonist (Fig. 7A) and ~90% by propofol (Fig. 7B). When the fragment beginning at \( \delta \text{Met-257} \) was sequenced, the peak of \(^3\text{H}\) release in cycle 13 established labeling of \( \delta \text{Val-269} \) (\( \delta \text{M2-13} \)) that agonist inhibited by >90% (Fig. 7C) and propofol inhibited by 85% (Fig. 7D). Since positions \( \alpha \text{M2-2}, \alpha \text{M2-6}, \alpha \text{M2-9}, \alpha \text{M2-13}, \alpha \text{M2-16} \), and \( \alpha \text{M2-20} \) line the lumen of the ion channel (in all nACHR subunits and Cys-loop receptors (8)), our results demonstrate that \(^3\text{H}\)\text{AziPm} binds in the ion channel in the resting state.

\(^3\text{H}\)\text{AziPm} photolabeling in \( \beta \text{M3}, \gamma \text{M3} \) and \( \delta \text{M3} \). The propofol-enhanced photoincorporation at \( \alpha \text{M2-10} \) provided evidence that \(^3\text{H}\)\text{AziPm}, but not propofol, binds to a site at the \( \gamma-\alpha \) interface where photoreactive etomidate analogs bind and photolabel \( \alpha \text{M2-10} \) as well as amino acids in \( \gamma \text{M3} \) (\( \gamma \text{Met-295}, \gamma \text{Met-299} \)) (15,16). In addition, the M3 helix of each subunit contains lipid-exposed residues photolabeled by the hydrophobic probe, \(^{125}\text{I}\)TID (i.e., \( \gamma \text{Phe-292}, \gamma \text{Leu-296}, \) and \( \gamma \text{Asn-300} \) (35)). To determine whether \(^3\text{H}\)\text{AziPm} photolabeled \( \gamma \text{Met-295}, \gamma \text{Met-299}, \) or other amino acids in \( \gamma \text{M3} \), we isolated and sequenced a fragment beginning at \( \gamma \text{Thr-276} \) from nACHRs photolabeled in the absence or presence of Carb (Fig. 8A). There was no evidence of labeling of \( \gamma \text{Met-295} \) or \( \gamma \text{Met-299} \). However, there was a small peak of \(^3\text{H}\) release in cycle 25, which indicated labeling of \( \gamma \text{Asn-300} \) at similar efficiency in the absence and presence of agonist. When nACHR was photolabeled in the absence or presence of 300 \( \mu \text{M} \) propofol, the efficiency of photolabeling of \( \gamma \text{Asn-300} \) differed by <10% (Table 1). We also sequenced the corresponding
fragments from the δ and the β subunits that begin at δThr-281 and βLeu-275 and contain δM3 and βM3. For the δ (Fig. 8B) and β subunit (not shown) fragments, there were peaks of 3H release in cycle 25, which indicated incorporation at δAsn-305 and βLeu-297, the positions in δM3 and βM3 equivalent to γAsn-300.

[3H]AziPm photolabeling within αM4. Within the nAChR transmembrane domain, the M4 helices have the greatest exposure to lipid (9), and photolabeling with [125I]TID identified 5 amino acids forming a strip on the αM4 helix that are at the lipid interface (35). We characterized [3H]AziPm labeling within αM4 by sequencing a fragment beginning at αTyr-401 isolated from tryptic digests of αV8-10. Peaks of 3H release in cycles 12 and 18 of Edman degradation (Fig. 8C) established agonist-insensitive labeling of αCys-412 and αCys-418, the amino acids photolabeled most efficiently by [125I]TID. When we sequenced fragments isolated from nAChRs photolabeled in the presence of 300 µM propofol, we found that [3H]AziPm photolabeling of αCys-412 and αCys-418 was also insensitive to propofol (Table 1).

**DISCUSSION**

Propofol has been reported to be a potent inhibitor of α4β2 neuronal (IC50 = 5 µM) and Torpedo nAChRs (IC50 = 7 µM), but not mouse muscle nAChR (IC50 = 45 µM) (5,17). In this study, we identify the Torpedo nAChR binding sites for [3H]AziPm, a novel photoactive propofol analog, and we use inhibition of [3H]AziPm photolabeling to identify propofol binding sites. We summarize in Table 1 the photoincorporation efficiency (cpm/pmol) and pharmacological specificity for the labeled amino acids. Based upon the locations of the photolabeled residues in the structure of the nAChR (Fig. 9), we find that [3H]AziPm binds to three distinct sites within the nAChR transmembrane domain: (1) within the δ subunit helix bundle in the desensitized state (photolabeling δM2-18' and δPhe-232/δCys-236 in δM1); (2) in the ion channel in the resting state (photolabeling αM2-6', βM2-6' and βM2-13', and δM2-13'); and (3) at the γ-α and/or β-α transmembrane interface (photolabeling of αM2-10'). Within the nAChR structural model, computational docking calculations predict that AziPm can bind to each of the three sites, and the predicted binding modes are shown in Fig. 9 in Connolly surface representation.

[3H]AziPm and propofol bind in the δ subunit helix bundle pocket. An intrasubunit drug binding site in the nAChR transmembrane domain at the extracellular end of the δ subunit helix bundle was first identified by the agonist-dependent and isoflurane-inhibitable photolabeling of δTyr-228 by the volatile anesthetic, [14C]halothane (volume, 85 Å3) (36). Similar to [3H]AziPm, [125I]TID (volume, 150 Å3), a potent nAChR inhibitor with structural similarity to propofol (volume, 180 Å3) and AziPm (volume, 178 Å3) (Fig. 1), photolabeled in the presence of agonist, but not in the absence, δM2-18' (δThr-274) and δPhe-232/δCys-236 in δM1(22,23). [125I]TID also photolabeled δIle-288 in the δM2-M3 loop, which was not photolabeled by [3H]AziPm. Importantly, time-resolved photolabeling studies established that [125I]TID labeling of this site was strongly state-dependent, being much more efficient in the transient, open channel and desensitized states than in the equilibrium desensitized state (22,37). It remains to be determined whether this also holds true for [3H]AziPm. This site was not photolabeled by a larger hydrophobic drug, the photoactive etomidate analog [3H]TDBzl-etomidate (vol., 285 Å3) (15), or by the positively charged inhibitor [3H]chlorpromazine (38). The inhibition of [3H]AziPm photolabeling of δThr-274 (δM2-18') and δPhe-232 by ~85% in the presence of propofol provides strong evidence that propofol binds to this site in the nAChR in the desensitized state. Since [3H]AziPm (or other drugs) only photolabel the δ subunit helix bundle in the presence of agonist, we could not determine whether propofol binds to this site in the absence of agonist.

We estimated propofol’s affinity for this intrasubunit binding site by determining the concentration dependence of propofol inhibition of [3H]AziPm photoincorporation in the δ subunit (IC50 = 37 ± 8 µM, nH = 1.08 ± 0.2). This determination was possible because under our photolabeling conditions, in the nAChR desensitized state [3H]AziPm photolabels amino acids in the δ subunit helix bundle pocket (δM2-10').
18', δPhe-232) at >10-fold the efficiency of amino acids in the ion channel domain (δM2-13') (Table 1). Surprisingly, propofol inhibition of [3H]PCP binding in the ion channel was characterized by essentially the same concentration dependence (IC50 = 47 ± 5 µM, nH = 1.09 ± 0.09), mediated by an allosteric mechanism, consistent with an ~4-fold reduction of [3H]PCP affinity. While it is possible that propofol allosterically inhibits [3H]PCP binding by occupying a site in the ion channel with the same affinity as for the site in the δ subunit helix bundle, a more plausible interpretation would suggest that propofol occupying the site in the δ helix bundle results in an allosteric inhibition of [3H]PCP binding in the ion channel. Consistent with this interpretation, for the nAChR in the desensitized state, non-radioactive TID acts as a competitive inhibitor of [3H]PCP binding (20), and PCP fully inhibits [125I]TID photolabeling at positions M2-2' and -6' in the ion channel. At the same time, PCP acts as an allosteric inhibitor of [125I]TID photolabeling in the δ subunit helix bundle, reducing photolabeling by ~40% (23).

Does propofol bind in the nAChR ion channel? AziPm binds in the ion channel and propofol inhibits [3H]AziPm channel photolabeling. However, this inhibition occurs by an allosteric, rather than a competitive, mechanism. Based upon equilibrium radioligand binding assays, propofol binds to the Torpedo nAChR preferentially in the desensitized state, while AziPm binds preferentially in the resting, closed channel state (Fig. 2). Unlike AziPm, propofol was a more potent inhibitor of channel blocker binding in the desensitized state (+agonist, [3H]PCP, IC50 = 50 µM) than in the absence of agonist ([3H]tetracaine, IC50 = 125 µM). Propofol inhibited [3H]tetracaine binding with a steep concentration dependence (nH = 1.6 ± 0.3), and at concentrations >10 µM, propofol increased [3H]ACh binding. These binding properties indicate that in the absence of agonist propofol acts as a desensitizing allosteric nAChR inhibitor. Since [3H]AziPm channel photolabeling is reduced by >90% in the desensitized state (+Carb), propofol inhibition of [3H]AziPm channel photolabeling is an expected consequence of propofol-induced nAChR desensitization.

The concentration dependence of propofol inhibition of ion channel photolabeling in the absence of agonist, which was determined from the inhibition δ subunit photolabeling, provides further evidence that the inhibition is a consequence of nAChR desensitization. In the absence of agonist, propofol inhibited δ subunit photolabeling (IC50 = 140 ± 30 µM, nH = 2) only at concentrations where it enhanced nAChR desensitization, as judged by [3H]ACh binding, and with the same concentration dependence as it inhibited [3H]tetracaine binding (IC50 = 125 ± 14 µM, nH = 1.6 ± 0.3). These steep concentration dependences indicate that inhibition was not simply competitive and are consistent with allosteric inhibition as a result of nAChR desensitization. Since, as noted above, propofol binding in the δ subunit helix bundle in the desensitized state inhibits allosterically [3H]PCP binding in the ion channel, it is likely that propofol binding in the δ subunit helix bundle pocket in the absence of agonist enhances nAChR desensitization and inhibits allosterically [3H]AziPm channel photolabeling.

[3H]AziPm photolabeling of αM2-10'. The presence of an anesthetic binding site at the nAChR γ-α interface was first identified by photolabeling with photoreactive etomidate analogs, [3H]TDBzl-etomidate and [3H]TFD-etomidate, that photolabeled αM2-10' and γMet-295/γMet-299 in γM3 (15,16). Just as propofol enhanced [3H]AziPm photolabeling of αM2-10 in the absence of agonist, [3H]TDBzl-etomidate photolabeling of αM2-10' was enhanced in the presence of the desensitizing channel blockers PCP or propofen. The photoreactive binding site was located at the γ-α interface based upon the photolabeling of γMet-295/γMet-299. In the absence of photolabeling of similar amino acids in γM3 or βM3, we do not know whether [3H]AziPm binds at the γ-α or β-α interface (or both). In contrast to [3H]AziPm, [125I]TID did not photolabel αM2-10', γMet-295 or γMet-299, although it photolabeled γAsn-300 and other amino acids in the M3 helices that are exposed to lipid (35).

Propofol binding sites in Cys-loop receptors. Propofol acts as an inhibitor of muscle and neuronal nAChRs and of GLIC, and our
results identify an intrasubunit propofol binding site in the Torpedo AChR transmembrane domain equivalent to the site identified in GLIC (6). It remains to be determined whether propofol binds to an equivalent intrasubunit binding site in the GABA$_A$ receptor, where it acts as a positive allosteric modulator. For the Torpedo nAChR, the positive allosteric modulator TDBzl-etomidate does not bind within the pocket formed by the $\delta$-subunit helix bundle. Instead, it binds to an intersubunit site at the interface between $\gamma$ and $\alpha$ subunit (15). However, for the homopentameric $\alpha$7 nAChR, mutational analyses provide evidence for an intrasubunit binding site for positive allosteric modulators within the helix bundle pocket (39,40).

General anesthetics of diverse chemical structure act as GABA$_A$R positive allosteric modulators, and the results of early mutational analyses were interpreted in terms of structural models that predicted intrasubunit binding sites for alcohols and volatile anesthetics, as well as intravenous anesthetics including propofol and etomidate (41). However, improved GABA$_A$R homology models indicate that many of the positions determining anesthetic sensitivity do not project within an intrasubunit pocket (42). In addition, photoreactive etomidate analogs identify an intersubunit etomidate binding site at the interface between $\beta$ and $\alpha$ subunits that contain the GABA binding site in the extracellular domain (13,14), and in crystals of GluCl, a homopentameric invertebrate glutamate-gated chloride channel, the positive allosteric modulator ivermectin is bound at this intersubunit binding site (10). With the availability of [$^3$H]AziPm, which reacts broadly with aliphatic and nucleophilic side chains and identifies propofol binding sites in the Torpedo nAChR, and other recently developed propofol analogs (15), it may soon be possible to identify propofol binding sites in GABA$_A$Rs.

REFERENCES


FOOTNOTES

*This research was supported by US Public Health Service Grants GM-58448 (To J.B.C. and K.W.M.) and GM-55876 (RGE). We thank Drs. David Chiara and Ayman Hamouda for helpful comments on the manuscript.

1Abbreviations: GABA, γ-aminobutyric acid; GABA_A, γ-aminobutyric acid type-A receptor; nAChR, nicotinic acetylcholine receptor; Azip, 2-isopropyl-5-[3-(trifluoromethyl)-3H-diazirin-3-yl]phenol; TID, 3-(trifluoromethyl)-3-(m-iodophenyl)diazirine; α-BgTx, α-bungarotoxin; Carb, carbamylcholine; PCP, phencyclidine; azietomidate, 2-(3-methyl-3H-diaziren-3-yl)ethyl 1-(1-phenylethyl)-1H-imidazole-5-carboxylate; TDBz1-etomidate, 4-[3-(trifluoromethyl)-3H-diazirin-3-yl]benzyl 1-(1-phenylethyl)-1H-imidazole-5-carboxylate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EndoLys-C, Lysobacter enzymogenes endoproteinase Lys-C; V8 protease, S. aureus Glutamic-C
endopeptidase; rpHPLC, reversed phase high performance liquid chromatography; OPA, o- phthalaldehyde; BNPS-skatol, 3-bromo-3-methyl-2-(2-nitrophenylthio)-3H-indole.

The $^3$H release profiles for the δPhe-206 fragments (+Carb) in panels B and C have major increases in $^3$H release in cycle 27, establishing the photolabeling of δPhe-232. The detected $^3$H releases in cycles 28 (or 29) are also well above the background release in cycle 26. However, because each cycle of Edman degradation occurs at $<100\%$ efficiency, some or all of the $^3$H release collected in cycle 28 must also originate from the photolabeling of δPhe-232. For the data of panel B, the $^3$H releases observed in cycles 28 and 29 compared to cycle 27 are consistent with photolabeling of δPhe-232 and an $\sim 97\%$ average efficiency of peptide bond cleavage at each cycle of Edman degradation.
FIGURE LEGENDS

FIGURE 1. Structures of propofol, its photoactive analog, AziPm, and TID.

FIGURE 2. Propofol and AziPm differentially modulate the equilibrium binding of $[^{3}H]$ACh and the channel blockers $[^{3}H]$tetracaine and $[^{3}H]$phenacyclidine (PCP). Propofol (●) and AziPm (○) modulation of the equilibrium binding by Torpedo nAChR of: A, $[^{3}H]$ACh; B, $[^{3}H]$tetracaine (+α-BgTx); and C, $[^{3}H]$PCP (+Carb, desensitized state). Binding was determined by centrifugation assay; non-specific binding ($B_{ns}$) was determined in the presence of excess competitor (A, 1 mM Carb; B, 100 µM tetracaine; or C, 100 µM PCP). The specific radioligand binding at propofol or AziPm concentration $x$, $(B_{x} - B_{ns})$, normalized to the specific binding in the absence of propofol or AziPm ($B_{0} - B_{ns}$), is plotted. A, Propofol at 100 µM potentiates $[^{3}H]$ACh binding by 20%, similar to the potentiation by proadifen (Δ). AziPm at 1 mM inhibited specific binding by >95% binding (IC$_{50}$ = 134 ± 10 µM, n$_{H}$ = 2.5 ± 0.5). B, Propofol inhibited specific binding of $[^{3}H]$tetracaine by >95%, but with a steep concentration dependence (IC$_{50}$ = 125 ± 14 µM, n$_{H}$ = 1.6 ± 0.3). At concentrations up to 100 µM, AziPm produced a dose-dependent inhibition of binding, with a non-specific increase in binding seen at higher concentrations in either the absence (○) or presence (●) of 100 µM non-radioactive tetracaine. For AziPm concentrations ≤ 100 µM, the concentration dependence of inhibition (solid curve) was consistent with full inhibition at higher concentrations (IC$_{50}$ = 12 ± 1 µM, n$_{H}$ = 0.94 ± 0.07). C, Propofol maximally reduced $[^{3}H]$PCP specific binding by ~75%. The concentration dependence of inhibition was consistent with a Hill coefficient of 1 (IC$_{50}$ = 47 ± 5 µM; B$_{\infty}$ = 24 ± 2 %, n$_{H}$ = 1.09 ± 0.09). As seen for $[^{3}H]$tetracaine, at concentrations above 100 µM AziPm increased $[^{3}H]$PCP binding. At AziPm concentrations ≤ 100 µM, the concentration dependence of inhibition (solid curve) was consistent with full inhibition at higher concentrations (IC$_{50}$ = 52 ± 6 µM, n$_{H}$ = 1).

FIGURE 3. Photoincorporation of $[^{3}H]$AziPm into Torpedo nAChR–rich membranes (A & B) and within nAChR α subunit fragments (C). A, Torpedo nAChR-rich membranes were photolabeled with 1 µM $[^{3}H]$AziPm in the absence (lane 1) or presence of other drugs (lane 2, 100 µM tetracaine; lane 3, 100 µM PCP; lane 4, 300 µM propofol; lane 5, 1 mM Carb; lane 6, 1 mM Carb and 100 µM PCP; and lane 7, 1 mM Carb and 300 µM propofol). Subunits were resolved by SDS-PAGE (representative Coomassie Blue stain shown in lane 8) and $^3$H incorporation into subunits was determined by fluorography (lane 1-7). The mobilities of the α, β, γ and δ nAChR subunits, rapsyn (Rsn) and the Na$^+$/K$^+$-ATPase α subunit ($\alpha_{NaK}$) are indicated. B, Gel bands containing nAChR α, β, γ and δ nAChR subunits and α$_{NaK}$ were excised from duplicate gels, and $^3$H incorporation was determined by liquid scintillation counting. For each gel band the average cpm ± SD are plotted. C, nAChR α subunits were isolated from nAChR-rich membranes (400 µg protein/~650 pmol ACh binding sites) photolabeled with $[^{3}H]$AziPm in the absence of other drugs (Control) or in the presence of tetracaine (100 µM), propofol (300 µM), or Carb (1 mM). The isolated α subunit gel bands were loaded onto a second 15% polyacrylamide gel for “in-gel” digestion with V8-protease to produce four subunit fragments that were visualized by staining the gel with Gel-Code Blue (Pierce). The $^3$H incorporation within the excised gel bands was determined by liquid scintillation counting. The locations of the 4 subunit fragments within the α subunit primary structure are indicated above.

FIGURE 4. Comparison of the propofol inhibition of $[^{3}H]$AziPm photoincorporation in the δ subunit in the absence or presence of agonist. Torpedo nAChR-rich membranes were photolabeled with 1.5 µM $[^{3}H]$AziPm in the absence (■) or presence (○) of Carb. Aliquots were also photolabeled in the presence of 0.1 mM tetracaine (−Carb) or 0.1 mM PCP (+Carb). After photolabeling, nAChR subunits were resolved by SDS-PAGE, and $^3$H incorporation in the excised δ subunits was determined by
liquid scintillation counting. For each condition, the normalized subunit photolabeling, \((\overline{\delta}_n - \overline{\delta}_{na})/\overline{\delta}_n\times 100\), was calculated from two gels with duplicate samples (mean ± SD), where \(\overline{\delta}_n\) is the \(\delta\) subunit \(^3\)H cpm at propofol concentration \(x\), \(\overline{\delta}_0\) the \(^3\)H cpm in the absence of propofol, \(\overline{\delta}_{na}\) is the \(^3\)H cpm incorporated in the presence of 100 \(\mu\)M tetracaine (–Carb) or 300 \(\mu\)M propofol (+Carb). In the absence of Carb, \(\overline{\delta}_n\) and \(\overline{\delta}_{na}\) were 4295 ± 50 cpm and 1300 ± 50 cpm, respectively. In the presence of Carb, \(\overline{\delta}_n\) and \(\overline{\delta}_{na}\) were 3010 ± 15 cpm and 1170 ± 10 cpm (with 2060 ± 15 cpm incorporated in the presence of 100 \(\mu\)M PCP). The concentration dependence of propofol inhibition of \(^{[3]}\)Htetracaine binding (IC\(_{50}\) = 125 \(\mu\)M, \(n = 1.6\), from Fig. 2B) is plotted as the solid line, and the dotted line is the concentration dependence of inhibition of \(^{[3]}\)HPCP binding (+Carb, IC\(_{50}\) = 47 \(\mu\)M, \(n_h = 1.09\), from Fig. 2C). For the direct fit of the concentration dependence of propofol inhibition of \(\delta\) subunit photolabeling (+Carb): IC\(_{50}\) = 37 \(\mu\)M ± 8, \(n_h = 1.06 ± 0.2\).

**FIGURE 5.** \(^{[3]}\)H|AziPm photolabeling within the \(\delta\) subunit helix bundle is propofol-inhibitable and agonist-dependent. Quantification of \(^{[3]}\)H (●, △, ▲) and PTH-amino acids (□) released during sequence analysis of *Torpedo* nAChR subunit fragments containing \(\delta\)M2 (A) and \(\delta\)M1 (B & C) isolated by SDS-PAGE and rpHPLC from EndoLys-C digests of nAChRs photolabeled +Carb (△), +Carb + 300 \(\mu\)M propofol (▲), or without agonist or propofol (●). A & B. For nAChRs photolabeled +Carb + 300 \(\mu\)M propofol, upon sequencing the fragment beginning at \(\delta\)Met-257 (A, \(I_o = 34\) pmol, both conditions), the major peak of \(^{[3]}\)H release (+Carb (△) in cycle 18 indicates photolabeling of \(\delta\)Thr274 (\(\delta\)M2-18', 2.3 cpm/pmol) that propofol inhibited by 80%. The smaller peaks of \(^{[3]}\)H release in cycles 13 and 21 indicate photolabeling of \(\delta\)Val-269 (\(\delta\)M2-13', 0.2 cpm/pmol) and \(\delta\)Arg277 (0.4 cpm/pmol) that propofol inhibited by 50% and 35%, respectively. B. The peaks of \(^{[3]}\)H release in cycles 27 and 31 seen when the fragment beginning at \(\delta\)Phe-206 was sequenced (\(I_o = 37\) pmol, both conditions, with OPA treatment at cycle 20 (\(\delta\)Pro-225)) indicate photolabeling of \(\delta\)Phe-232 (1.7 cpm/pmol) and \(\delta\)Cys-236 (0.46 cpm/pmol) that propofol inhibited by 95% and 40%, respectively. C. When fractions enriched in the \(\delta\)Phe-206 fragment were sequenced from nAChRs photolabeled in the absence (●) or presence (△) of Carb, the fragment beginning at \(\delta\)Phe-206 was the primary sequence (\(I_o = 7\) pmol, both conditions (□), with a secondary sequence beginning at \(\delta\)Met-257 (\(I_o = 0.5\) pmol, not shown). For the +Carb sample, the peaks of \(^{[3]}\)H release in cycles 27 and 31 indicate photolabeling of \(\delta\)Phe-232 (10 cpm/pmol) and \(\delta\)Cys-236 (3.2 cpm/pmol) that was reduced by >90% in the absence of Carb. For the –Carb sample, the peak of \(^{[3]}\)H release in cycle 13 resulted from photolabeling of \(\delta\)Val-269 (\(\delta\)M2-13') in the secondary sequence that was reduced by >90% in the presence of agonist (see Fig. 7).

**FIGURE 6.** \(^{[3]}\)H|AziPm photolabeling within \(\alpha\)M2. Quantification of \(^{[3]}\)H (●, ○, △) and PTH-amino acids (□) released during sequence analysis of *Torpedo* nAChR subunit fragments containing \(\alpha\)M2 isolated from nAChRs photolabeled with 1 \(\mu\)M \(^{[3]}\)H|AziPm without other drugs (●), + 300 \(\mu\)M propofol (○), or + 1 \(\mu\)M Carb (△). A. For nAChRs photolabeled ± propofol, when sequencing the fragment beginning at \(\alpha\)Met-243 (\(I_o = 4.3\) pmol, both conditions), the peaks of \(^{[3]}\)H release in cycles 6 and 10 indicate photolabeling in the absence of propofol of \(\alpha\)Ser-248 (\(\alpha\)M2-6) at 14 cpm/pmol and \(\alpha\)Ser-252 (\(\alpha\)M2-10) at 8 cpm/pmol. In the presence of propofol, photolabeling of \(\alpha\)M2-6 was reduced to 5 cpm/pmol while photolabeling of \(\alpha\)M2-10 was increased to 34 cpm/pmol. B. For nAChRs photolabeled ± Carb, the fragment beginning at \(\alpha\)Met-243 was present at 4 pmol (+Carb, □) and at 8 pmol (–Carb, not plotted). For the –Carb condition, \(\alpha\)M2-6 and \(\alpha\)M2-10 were photolabeled (–Carb/+Carb) at 3.2/1.8 cpm/pmol and 6.5/5.3 cpm/pmol, respectively.

**FIGURE 7.** Agonist- and propofol-inhibitable \(^{[3]}\)H|AziPm photolabeling in \(\beta\)M2 and \(\delta\)M2. Quantification of \(^{[3]}\)H (●, △, ○) and PTH-amino acids (□) released during sequence analysis of *Torpedo* nAChR subunit fragments containing \(\beta\)M2 (A & B) and \(\delta\)M2 (C & D) isolated by SDS-PAGE and rpHPLC from trypsin or EndoLys-C digests of nAChRs photolabeled without agonist or propofol (●), +Carb (△), or + 300 \(\mu\)M propofol (○). A & C. For nAChRs photolabeled ± Carb, when sequencing the
fragment beginning at βMet-249 (A, I₀ = 11 pmol, both conditions), the peaks of ³H release in cycles 6 and 13 (–Carb) indicate photolabeling βSer-254 (βM2-6) at 7.5 cpm/pmol and βVal-261 (βM2-13) at 6.2 cpm/pmol that was reduced +Carb by >90%. When sequencing the fragment beginning at δMet-257 (C, I₀ = 20 pmol, both conditions), the peak of ³H release in cycle 13 (–Carb) indicates photolabeling of δVal-269 (δM2-13) at 48 cpm/pmol that Carb reduced by >90%. In the presence of Carb, the peak of ³H release in cycle 18 indicates photolabeling of δThr-274 (δM2-18’) at 16 cpm/pmol. B & D, For nAChRs photolabeled ± propofol, when sequencing the fragment beginning at βMet-249 (B, I₀ = 6 pmol, both conditions), the peaks of ³H release in cycles 6 and 13 (–Carb) indicate photolabeling of βM2-6 and βM2-13 at 5.2 and 4.5 cpm/pmol, respectively, that propofol reduced by ~90%. When sequencing the fragment beginning at δMet-257 (D, I₀ = 15 pmol, both conditions), the peak of ³H release in cycle 13 (–Carb) indicates photolabeling of δM2-13’ at 28 cpm/pmol that propofol reduced by 85%.

**FIGURE 8.** Agonist-independent [³H]AziPm photolabeling in nAChR M3 and M4 helices. Quantification of ³H (○,△) and PTH-amino acids (□) released during sequence analysis of Torpedo nAChR subunit fragments beginning near the N-termini of γM3 (A), δM3 (B) and αM4 (C) from Torpedo nAChR photolabeled with 1 μM [³H]AziPm in the absence (○) or presence (△) of Carb (1 mM). The γ and δ subunit fragments were isolated by rpHPLC from V8 protease digests and chemically isolated during sequence analysis by treatment OPA at cycle 6 of Edman degradation (see Methods). The α subunit fragment was isolated by rpHPLC fractionation of a trypsin digest of αV8-10. A & B, When sequencing the fragments beginning at γThr-276 (A, I₀ (–/+/Carb) = 11/17 (□) pmol) and δThr-281 (B, I₀ (–/+/Carb) = 19/40 (□) pmol), the peaks of ³H release in cycle 25 (–Carb) indicate photolabeling of δAsn-300 and δAsn-305 at 1.5 and 2.5 cpm/pmol, respectively. For the +Carb samples, γAsn-300 was photolabeled at 1.7 cpm/pmol. Due to a sequencer failure at cycle 20, data was not obtained for δAsn-305 (+Carb). C, When sequencing the fragment beginning at αTyr-401 (I₀ (–/+/Carb) = 36/44 (□) pmol), the peaks of ³H release in cycle 12 and 18 indicate photolabeling (–Carb/+Carb) of αCys-412 and αCys-418 at 6.4/6.1 and 2.7/1.5 cpm/pmol, respectively.

**FIGURE 9.** [³H]AziPm binding sites in the Torpedo nAChR transmembrane domain. A, Side view of the extracellular and transmembrane domains of the T. californica nAChR (α, gold; β, blue; γ, green; δ, magenta), based upon the T. marmorata nAChR structure (PDB code 2BG9), with Carb (red Connolly surface) in the ACh binding sites. A Connolly surface of a single AziPm molecule, colored by atom (178 Å³), is included for comparison. B, View of the Torpedo nAChR transmembrane domain from the bottom of the extracellular domain, including in Connolly surface representation the ensembles of the 15 AziPm molecules docked with lowest CDOCKER interaction energy within: (i) the δ subunit helix bundle (471 Å³), (ii) the ion channel (486 Å³), and (iii) the γ-α interface (348 Å³). The photolabeled amino acids are represented in stick format: (i) within the δ subunit helix bundle (in green, δThr-274, δPhe-232 and δCys-236); (ii) within the ion channel (in cyan, αSer-248, βSer-254, βVal-261 and δVal-269); and (iii) within the γ-α interface (in red, αSer-252). C, Enlarged side-view of the nAChR transmembrane domain with the γ and α₆ subunits and the M4 helices of the α₂, β and δ subunits removed for better visibility of the photolabeled amino acids and the AziPm binding pockets in the δ subunit helix bundle and in the ion channel.
Pharmacological specificity of [3H]AziPm photoincorporation into residues in the nAChR transmembrane domain (cpm/pmol of PTH derivative)

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
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<tr>
<td>Resting vs.</td>
<td>–Carb –Propofol</td>
<td>–Carb +Carb</td>
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<sup>a</sup> 3H incorporation (cpm/pmol of PTH derivative) in each residue was calculated from the observed 3H release and the initial and repetitive yields as described under “Experimental Procedures”. <sup>b</sup>The data are presented as mean (±range) for the results from two independent photolabeling experiments for the conditions of Experiment 2, while the data of Experiments 1 and 3 are each from single experiments with one aliquot of each sample sequenced. The cpm/pmol are the values for positions at which the peak of 3H release was >20% over the background release in the previous cycles. The upper limits of the photolabeling in the other cycles were determined from the random variation of the background release of 3H in the adjacent sequencing cycles. <sup>c</sup> For nAChRs in the desensitized state (+Carb), the substantial differences in photolabeling efficiency of amino acids in the δ subunit helix bundle in Experiment 3 compared to Experiment 1 are likely to be related to the differences seen for [125I]TID photolabeling in the fast desensitized and equilibrium desensitized states (22,37). ND, not determined.
Figure 1
Figure 2
Figure 3
Figure 5

[Diagram showing the results of Edman degradation with cycles and plots for M1 and M2 proteins.

(A) Plot showing (Δ, Δ) 3H (cpm x 10^5) against cycle of Edman degradation.

(B) Plot showing (Δ, Δ) 3H (cpm x 10^5) with a peak at cycle 23 and an arrow labeled OPA.

(C) Plot showing (Δ, Δ) 3H (cpm x 10^5) with a peak at cycle 28 and a dot indicating a significant change.]
Figure 6
Figure 7
Figure 8
Identification of Propofol Binding Sites in a Nicotinic Acetylcholine Receptor with a Photoreactive Propofol Analog

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J. Biol. Chem. published online January 8, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M112.435909

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