Numerous Classes of General Anesthetics Inhibit Etomidate Binding to \( \gamma \)-Aminobutyric Acid Type A (GABA\(_A\)) Receptors

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Enhancement of \( \gamma \)-aminobutyric acid type A receptor (GABA\(_A\))-mediated inhibition is a property of most general anesthetics and a candidate for a molecular mechanism of anesthesia. Intravenous anesthetics, including etomidate, propofol, barbiturates, and neuroactive steroids, as well as volatile anesthetics and long-chain alcohols, all enhance GABA\(_A\)R function at anesthetic concentrations. The implied existence of a receptor site for anesthetics on the GABA\(_A\)R protein was supported by identification, using photoaffinity labeling, of a binding site for etomidate within the GABA\(_A\)R transmembrane domain at the \( \beta\)-\( \alpha \) subunit interface; the etomidate analog \([^{3}H]\)azietomidate photolabeled in a pharmacologically specific manner two amino acids, \( \alpha1\)Met-236 in the M1 helix and \( \beta\)Met-286 in the M3 helix (Li, G. D., Chiara, D. C., Sawyer, G. W., Husain, S. S., Olsen, R. W., and Cohen, J. B. (2006) J. Neurosci. 26, 11599–11605). Here, we use \([^{3}H]\)azietomidate photolabeling of bovine brain GABA\(_A\)R to determine whether other structural classes of anesthetics interact with the etomidate binding site. Photolabeling was inhibited by anesthetic concentrations of propofol, barbiturates, and the volatile agent isoflurane, at low millimolar concentrations, but not by octanol or ethanol. Inhibition by barbiturates, which was pharmacologically specific and stereospecific, and by propofol was only partial, consistent with allosteric interactions, whereas isoflurane inhibition was nearly complete, apparently competitive. Protein sequencing showed that propofol inhibited to the same extent the photolabeling of \( \alpha1\)Met-236 and \( \beta\)Met-286. These results indicate that several classes of general anesthetics modulate etomidate binding to the GABA\(_A\)R: isoflurane binds directly to the site with millimolar affinity, whereas propofol and barbiturates inhibit binding but do not bind in a mutually exclusive manner with etomidate.

\( \gamma \)-Aminobutyric acid type A receptors (GABA\(_A\)Rs)\(^3\) are major mediators of brain inhibitory neurotransmission and participate in most circuits and behavioral pathways relevant to normal and pathological function. Their regulation may underlie many psychiatric/neurological disorders. GABA\(_A\)Rs are subject to modulation by endogenous neurosteroids, as well as myriad clinically important central nervous system drugs, including general anesthetics, benzodiazepines, and ethanol (1–3). The mechanism of GABA\(_A\)R modulation by these different classes of drugs is of major interest, including the localization of their binding sites in the receptor.

Each GABA\(_A\)R is made up of five homologous subunits that associate around a central axis that forms the ion channel. Each subunit consists of a large extracellular N-terminal domain, a transmembrane domain made up of a loose bundle of four transmembrane \( \alpha \)-helices, and a cytoplasmic domain made up of the amino acids located in the primary structure between the M3 and M4 helices. In an \( \alpha \)-\( \beta\)-\( \gamma \) GABA\(_A\)R, the neurotransmitter-binding sites are located in the extracellular domain at the interfaces between the \( \beta \) and \( \alpha \) subunits, two sites per receptor, whereas the benzodiazepine sites are at an equivalent position at the \( \alpha \)-\( \gamma \) subunit interface, one per receptor (4).

Most general anesthetics enhance GABA\(_A\)R responses \textit{in vitro} at concentrations that produce immobilization \textit{in vivo}, suggesting a link between the GABA\(_A\)R and anesthesia (5, 6). The expression of receptors containing chimeric subunits combining sequences from subunits conferring different anesthetic sensitivities led to the identification of two residues that determine the sensitivity of the GABA\(_A\)R to volatile agents and alcohols (7). These residues, located in the transmembrane domains of both \( \alpha \) and \( \beta \) subunits, one in M2 and the other in M3, were hypothesized to contact a single water-filled intrasubunit pocket (2, 8). The M2 residue in \( \beta \) subunits, e.g. \( \beta3\)Asn-265, is also implicated in the \textit{in vivo} action of intravenous anesthetics, including etomidate, propofol, and barbiturates, but not anesthetic steroids (9, 10). This single residue, when mutated in a knock-in mouse, eliminated the immobilization (\( \beta3\)N265M (11)) or sedative/hypnotic (\( \beta2\)N265S (12)) actions of etomidate.

We synthesized a photoreactive analog of etomidate, \([^{3}H]\)azietomidate (2-\((3\)-methyl-3\-H-diazen-3-yl)ethyl 1-(phenylethyl)-1H-imidazole-5-carboxylate), which retained anesthetic activity (13). \([^{3}H]\)Azietomidate photolabeled purified bovine brain GABA\(_A\)R in a pharmacologically specific manner, photoincorporating into two residues (\( \alpha1\)Met-236 and \( \beta\)Met-286)\(^4\) (14). These amino acids are in the membrane-spanning \( \alpha1\)M1 and \( \beta3\)M3 helices, the latter being one of the identified

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\( ^{3} \)The abbreviations used are: GABA\(_A\)R, \( \gamma \)-aminobutyric acid type A receptor; HPLC, high pressure liquid chromatography; CHAPS, 3-\((3\)-cholamidopropyl\)dimethylammonio)-1-propanesulfonic acid.

\( ^{4} \)As discussed by Li et al. (14), because of the high degree of sequence conservation for \( \alpha \) subunit subtypes in the M1 region and \( \beta \) subunit subtypes in the M3 region, including protease cleavage sites, the labeled amino acids cannot be assigned to specific subunit subtypes, and therefore, they are referred to as \( \alpha\)Met-236 (using the \( \alpha1\) subunit numbering) and \( \beta\)Met-286.
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affinity determinants for etomidate and propofol (15). These photolabeling results suggested a GABA<sub> AR</sub> structural model based on homology with the nicotinic acetylcholine receptor (14, 16) that positioned both photolabeled amino acids within an intersubunit binding pocket at the β-α interface, two copies of a single class of sites per pentamer. This structural model is supported by cysteine replacement cross-linking data defining the orientation of various GABA<sub> AR</sub> transmembrane helices within and between subunits (17, 18). Neuroactive steroids enhance rather than inhibit <sup>[3]H</sup>azietomidate binding (19), indicating that the steroid gating site and etomidate modulation sites do not coincide, although other positions in αM1 and βM3, as well as in αM4, have been identified as neurosteroid sensitivity determinants (20). In this study, we use <sup>[3]H</sup>azietomidate photolabeling to determine whether etomidate binds competitively with other structural classes of general anesthetics. Our results provide evidence that isoflurane at millimolar concentrations may inhibit binding competitively, propofol and barbiturates act as allosteric inhibitors, and ethanol and octanol at anesthetic concentrations have no effect on <sup>[3]H</sup>azietomidate binding. Thus, several structural classes of general anesthetics, including propofol, barbiturates, and volatile agents, but not alcohols, interact, directly or indirectly, with the intersubunit etomidate-binding site in the transmembrane domain of GABA<sub> AR</sub> proteins.

MATERIALS AND METHODS

Solubilization and Purification of Bovine Brain GABA<sub> AR</sub>s—The GABA<sub> AR</sub> was solubilized and purified on a benzodiazepine Ro7/1986-1 affinity column as described (14). Important modifications of previous protocols that allowed an ~3000-fold purification of the GABA<sub> AR</sub> at high yield and with retention of positive allosteric modulation of <sup>[3]H</sup>muscimol binding by etomidate, propofol, pentobarbital, isoflurane, octanol, or neuroactive steroids included (i) the use of the detergent C<sub>12</sub>E<sub>9</sub> in conjunction with CHAPS in the solubilization buffer, (ii) extensive washing of the affinity resin with 10 mM CHAPS, 0.06% asolectin, 10% sucrose, and (iii) elution with clorazepate rather than flurazepam and urea. The purified protein contains a mixture of GABA<sub> AR</sub> subtypes of varying subunit composition that bind the benzodiazepine affinity column (14). <sup>[3]H</sup>Muscimol binding assays were performed as described (14).

Photoaffinity Labeling of Purified GABA<sub> AR</sub>—The GABA<sub> AR</sub> was photolabeled on an analytical scale (~6 pmol of <sup>[3]H</sup>muscimol-binding sites/sample) to examine the concentration dependence of anesthetic modulation of <sup>[3]H</sup>azietomidate incorporation, as determined by SDS-PAGE, and photolabeling was carried out on a preparative scale (95 pmol of <sup>[3]H</sup>muscimol-binding sites/sample) to determine whether propofol inhibited <sup>[3]H</sup>azietomidate photolabeling of αM-Met-236 or βMet-286 or caused labeling of other amino acids. Effects of propofol, which has an aqueous solubility of 0.9 mM (21), were examined at concentrations up to 0.2 mM. A stock solution of propofol was prepared at 200 mM in dimethyl sulfoxide, which was present at a final concentration of 0.1% (v/v) in each irradiated sample. The peak <sup>[3]H</sup>muscimol binding fraction (5 ml) from each affinity column elution was used for labeling without further dialysis or concentration. An aliquot of GABA<sub> AR</sub> (~40 nM <sup>[3]H</sup>muscimol-binding sites, 2.5 ml for preparative labeling, or 0.14 ml for analytical labeling) was equilibrated with <sup>[3]H</sup>azietomidate (final concentration, 0.7 μM for preparative labeling or 1 μM for analytical labeling) ± additional drugs in the presence of 1 mM GABA and 10 mM clorazepate (a benzodiazepine agonist), incubated on ice for 1 h in the dark (4 °C), and irradiated (30 min, 365 nm). After photolabeling, the total protein was precipitated with methanol/chloroform, solubilized in sample loading buffer, and fractionated by SDS-PAGE. The resulting gel lanes were cut into 3-mm slices, and <sup>3</sup>H incorporation was determined either directly by liquid scintillation counting (analytical labeling) or after elution from each slice into 1 ml of elution buffer (14). Aliquots of the eluted bands were assayed for <sup>3</sup>H and pooled for proteolytic digestion.

Enzymatic Digestion, Reversed-phase High Pressure Liquid Chromatography (HPLC), and Protein Microsequencing—Digestion of <sup>[3]H</sup>azietomidate-photolabeled GABA<sub> AR</sub> subunits with endoproteinase Lys-C, reversed-phase HPLC fractionation of the digests, and peptide microsequencing were each performed as described (14).

RESULTS

Numerous Chemical Classes of General Anesthetics Modulate <sup>[3]H</sup>Muscimol Binding to Purified Bovine Brain GABA<sub> AR</sub>—Chemical structures of the compounds studied are shown in Fig. 1. Propofol, pentobarbital, isoflurane, and n-octanol each allosterically enhanced the equilibrium binding affinity of
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FIGURE 2. Concentration-dependent enhancement of [3H]muscimol binding to purified bovine GABA<sub>R</sub> by propofol (A), pentobarbital (B), isoflurane (C), and n-octanol (D). Plotted are the percentage changes of specific [3H]muscimol binding (5 nM) (mean ± S.E.) in the presence of increasing concentrations of compounds. Binding assays were performed as described (14).

[3H]muscimol for affinity-purified bovine brain GABA<sub>R</sub>R in detergent solution (Fig. 2), as reported previously for etomidate (14) and for barbiturates using membranes from brain homogenates (22, 23).

Some General Anesthetics Inhibit GABA<sub>R</sub> Photolabeling by [3H]Azietomidate—When purified bovine brain GABA<sub>R</sub>R photolabeled with [3H]azietomidate in the absence or presence of 200 nM nonradioactive etomidate was fractionated by SDS-PAGE, the [3H] incorporated into GABA<sub>R</sub>R subunit polypeptides of ∼50–55 kDa was inhibitable by >90% in the presence of etomidate and was shown to result from labeling of α1Met-236<sup>4</sup> (or the homologous methionine in α2, 3, or 5) in α1M and βMet-286<sup>4</sup> in βM3 (14). To test for the effects of other classes of anesthetics, we photolabeled the GABA<sub>R</sub>R with [3H]azietomidate in the presence of the drugs at varying concentrations, always in the presence of 1 mM GABA and the benzodiazepine clorazepate.

Propofol, which has in vivo and GABA<sub>R</sub>-enhancing actions and potency similar to etomidate (5, 6), produced a concentration-dependent inhibition of [3H]azietomidate photolabeling of the purified GABA<sub>R</sub>R, as indicated by SDS-PAGE (Fig. 3, A and B). At high concentrations, propofol reduced photolabeling maximally by ∼50%, whereas in a parallel sample, 200 nM etomidate inhibited photolabeling by ∼90%, as seen previously (14). Propofol produced a half-maximal inhibition at a concentration (IC<sub>50</sub>) of ∼10 μM, the same concentration that produced half-maximal potentiation of [3H]muscimol binding to the purified GABA<sub>R</sub>R (Fig. 2A) but ∼5-fold higher than the EC<sub>50</sub> for GABA<sub>R</sub> potentiation as measured by electrophysiological recording (24). The maximal inhibition by propofol appeared to reach a plateau in four independent photolabeling experiments using [3H]azietomidate at concentrations between 0.7 and 3 μM (data not shown).

To determine whether propofol inhibited [3H]azietomidate photolabeling of αMet-236 and/or βMet-286, samples enriched in α or β subunit, isolated by SDS-PAGE (supplemental Figs. S1 and S2), were digested with endoproteinase Lys-C and fractionated by reversed-phase HPLC (supplemental Fig. S3). Edman sequence analyses of fractions enriched in α1M (Fig. 3C) and βM3 (Fig. 3D) confirmed that αMet-236 and βMet-286 were photolabeled by [3H]azietomidate in the absence of propofol and established that propofol at a saturating concentration (150 μM) inhibited photolabeling at each position by 60–75%, without resulting in photolabeling of other amino acids within α1M or βM3.

Barbiturates inhibited [3H]azietomidate photolabeling in a pharmacologically specific and stereospecific manner (Fig. 4). Pentobarbital at concentrations of 10–1000 μM produced a concentration-dependent reduction of [3H]azietomidate photolabeling, reaching a maximal inhibition of ∼40%, with an IC<sub>50</sub> of ∼200 μM (Fig. 4A), close to the concentration producing half-maximal potentiation of [3H]muscimol binding (Fig. 2B) but 4-fold higher than the EC<sub>50</sub> for GABA<sub>R</sub> potentiation measured electrophysiologically (25). Phenobarbital, which has 20-fold lower potency than pentobarbital as a GABA<sub>R</sub> potentiator (23), reduced [3H]azietomidate photoincorporation only at concentrations above 1 mM, with ∼25% inhibition at 10 mM, the highest concentration studied (Fig. 4B). The stereoisomeric pair (+) and (−)-N-methylphenobarbital (mephobarbital) produce central nervous system depression and GABA<sub>R</sub>-enhancing activity only for the (−)-isomer (23). (−)-Mepobarbital reduced [3H]azietomidate photolabeling maximally by ∼30%, with an IC<sub>50</sub> of ∼30 μM (Fig. 4C), whereas (+)-mepobarbital at concentrations up to 1 mM did not alter [3H]azietomidate photoincorporation (Fig. 4D).

Isoflurane, a volatile anesthetic, is known to enhance GABA<sub>R</sub> function (2, 26). As shown in Fig. 5 (A and B), isoflu- raned inhibited GABA<sub>R</sub> subunit labeling by >80% at 10 mM, with an IC<sub>50</sub> of 2 mM (Fig. 5B), a concentration within a factor of 2 of that enhancing [3H]muscimol binding (Fig. 2C) but ∼5-fold higher than the electrophysiological EC<sub>50</sub> for GABA<sub>R</sub> potentiation (27, 28). In contrast, neither ethanol at a concentration as high as 170 mM (Fig. 5C) nor n-octanol up to 1 mM (Fig. 5D) inhibited [3H]azietomidate photolabeling, whereas octanol at 1 mM enhanced [3H]muscimol binding to the purified receptor by ∼80% (Fig. 2D).

DISCUSSION

In this study, we photolabeled bovine brain GABA<sub>R</sub>Rs with the photoreactive etomidate analog [3H]azietomidate to determine whether other structural classes of general anesthetics bind to the same site as etomidate. Azietomidate, like etomidate, displays stereospecificity as an anesthetic in modulating GABA<sub>R</sub> function and in binding to GABA<sub>R</sub>Rs in vitro (13). [3H]Azietomidate photoincorporates in a pharmacologically specific manner into two amino acids in bovine brain GABA<sub>R</sub>R, α1Met-236<sup>4</sup> within α1M and βMet-286<sup>4</sup> within βM3, with photolabeling of those amino acids enhanced in the presence of GABA and inhibited by etomidate (14). These two amino acids were proposed to be located in a drug-binding pocket at the interface between the β and α subunits, a conclusion that has been supported by cysteine substitution cross-linking studies (18) that define the relative orientations of the α1M and βM3 helices and by mutagenesis studies that establish that α1Met-236 and βMet-286 are important determinants of GABA<sub>R</sub>}
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**FIGURE 3.** Propofol inhibits [^3H]azietomidate photolabeling of the GABA_\text{AR}. A, the GABA_\text{AR} was photolabeled with 1 \mu M [^3H]azietomidate in the presence of 1 mM GABA, 10 mM clorazepate, and propofol at 0.5 (i), 1 \mu M (\Delta), 10 \mu M (\triangle), 100 \mu M (\triangledown), and 200 \mu M (\bigcirc), and covalent \(^3\)H incorporation was analyzed by SDS-PAGE. B, shown is the concentration dependence of propofol inhibition, determined by normalizing the \(^3\)H in the GABA_\text{AR} subunit gel bands in the presence of propofol to that in the absence of propofol. The data points are the average and range from two independent photolabeling experiments. The solid lines are fits of the data to a logistic function. C, in the same experiment, 200 \mu M etomidate inhibited photolabeling by \(~90\%\). Error bars indicate S.E. C and D, propofol inhibits [^3H]azietomidate photolabeling of the GABA_\text{AR} at both \alpha_1\text{Met}-236 (\alpha M1) and \beta_3\text{Met}-286 (\beta M3). \(^3\)H, 0, and phenylthiohydantoin-amino acids 0, released during Edman sequencing of fragments enriched in m1 (C) and \beta M3 (D) were isolated by SDS-PAGE and reversed-phase HPLC from affinity-purified GABA_\text{AR} photolabeled with 0.7 \mu M [^3H]azietomidate in the presence of 1 mM GABA and 10 mM clorazepate with 0, or without (D) 150 \mu M propofol (supplemental Figs. S1–S3). C, sequence analysis of photolabeling in \alpha M1. The peptide beginning at \alpha_1\text{Met}-223 (–propofol, initial amount (\text{I}_0) = 0.6 pmol, repetitive yield (\text{R}) = 91\%; +propofol, \text{I}_0 = 1.6 pmol, \text{R} = 83\%) was the only peptide that persisted after treatment with p-thalaldehyde prior to cycle 11 to prevent sequencing of any peptide not containing a proline at this cycle. Release of \(^3\)H in cycle 14 established photolabeling of \alpha_1\text{Met}-236 (–propofol) at 140 cpm/pmol (95–210 cpm/pmol) that was reduced by propofol to 35 cpm/pmol (22–56 cpm/pmol). These ranges were calculated using the standard errors from the fits for \text{R} and \text{I}_0. D, sequence analysis of photolabeling in \beta M3. The peptide beginning at \beta\text{Met}-286 was present in both samples (–propofol, \text{I}_0 = 0.7 pmol, \text{R} = 93\%; +propofol, \text{I}_0 = 0.5 pmol, \text{R} = 96\%), and the peak of release of \(^3\)H in cycle 7 indicates photolabeling of \beta_3\text{Met}-286 (–propofol) at 50 cpm/pmol (33–79 cpm/pmol) that was reduced by propofol to 19 cpm/pmol (10–39 cpm/pmol). E, shown is the alignment of subtypes of \alpha or \beta subunits in the regions of M1 or M3 (both in gray), respectively, illustrating the high sequence conservation in these regions. In boldface are the labeled Met residues as well as the conserved Pro residue in cycle 11 of Edman degradation (C).

EC_{50} for potentiation of [^3H]muscimol binding by propofol, pentobarbital, or isoflurane was within a factor of 2 of the IC_{50} seen for the inhibition of [^3H]azietomidate photolabeling of the same receptor preparation. In each case, that concentration was \(~5–10\)-fold higher than the EC_{50} typically reported for the anesthetic potentiation of GABA_\text{A} responses and closer to the EC_{50} reported for direct anesthetic gating of the GABA_\text{A}R in the absence of GABA (24, 25, 27, 28). However, concentrations of etomidate up to 100 \mu M produce a progressive decrease in GABA EC_{50} without any evidence of saturation (32), and the
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Data were well fit by an allosteric model with each GABA<sub>A</sub>R containing two equivalent binding sites contributing to potentiation and to direct gating. Each α<sub>β</sub>β<sub>γ</sub> GABA<sub>A</sub>R contains at the β-α interfaces two equivalent binding sites for [3H]azetomidate and etomidate, and these sites were proposed to mediate etomidate’s two effects, enhancement of GABA and direct channel gating efficacies (14, 19).

The partial inhibition of azetomidate labeling by propofol is compelling evidence against a direct competitive interaction (mutually exclusive binding), as long as we exclude the possibility that there is a population of receptors photolabeled by azetomidate that are insensitive to propofol, a hypothetical situation for which there is no experimental evidence (see below). Although a mechanism of allosteric inhibition can account for the observed partial reduction of [3H]azetomidate photolabeling seen in the presence of high concentrations of propofol or barbiturates, alternative explanations must also be considered.

(i) Although photoincorporated [3H]azetomidate is no longer in reversible equilibrium with the competing anesthetics, this cannot account for the partial reduction in photolabeling because under our labeling conditions [3H]azetomidate is incorporated into only ~2% of receptors (14). In addition, the fact that etomidate and isoflurane at high concentrations can produce full inhibition establishes that the extent of inhibition must reflect site occupancy by reversibly bound [3H]azetomidate. (ii) Because the purified GABA<sub>A</sub>R preparation from bovine brain used for photolabeling is a heterogeneous population of GABA<sub>A</sub>Rs that bind to the benzodiazepine affinity column, i.e. receptors of variable subunit composition containing a γ subunit, it is possible that propofol and barbiturates bind only to a subset of the GABA<sub>A</sub>R subunit combinations that bind [3H]azetomidate and etomidate. This is a very unlikely explanation because in vitro studies testing anesthetic sensitivities of various subunit combinations indicate that there is no evidence for a receptor subunit population that is sensitive to etomidate but insensitive to propofol. The subunit combinations most sensitive to etomidate are also sensitive to propofol and barbiturates (25, 33), and none of the receptors most widely expressed in brain are insensitive to either of those anesthetics (10).

All structural classes of general anesthetics are positive allosteric modulators of [3H]muscimol binding and agonist benzodiazepine binding, as well as negative modulators of bicuculline binding and benzodiazepine inverse agonist binding and of the channel blocker ligands like t-[35S]butylbicyclophosphorothionate (22, 23, 34–36). Thus, one might expect the negative interactions between the binding of the different positive modulatory anesthetics (inhibition of etomidate binding by propofol, barbiturates, and isoflurane) to be competitive rather than allosteric. However, our photolabeling studies were carried out in the presence of GABA and benzodiazepines, and the weak energetic coupling that can account for our data may result because etomidate and propofol, for example, actually bind in close proximity within the binding pocket at the interface between the β and α subunits. Clearly, further studies using photoreactive analogs of propofol or other anesthetics are required to directly identify their binding sites.

Extensive mutational analyses have identified GABA<sub>A</sub>R amino acids in each of the transmembrane helices (6, 20, 37) of the α and β subunits that make important energetic contributions to the actions of anesthetics and that may potentially contribute directly to the structure of the anesthetic-binding sites. For reference purposes, those positions can be identified by using a common nomenclature that numbers the transmembrane helices from their N-terminal ends (4, 16). βM2-15 is a major determinant of the potency and efficacy of etomidate, propofol, and pentobarbital as GABA<sub>A</sub>R modulators in vitro and as anesthetics in vivo (9, 10). βM2-15 was not photolabeled...
by [3H]azetomidate, but in our GABA$_4R$ structural model (14, 19), it is located at the border of the etomidate-binding pocket, accessible from the $\beta$-a interface and from the interior of the $\beta$ subunit helix bundle. $\beta$M3-4, the position in the $\beta$ subunit photolabeled by [3H]azetomidate, is a sensitivity determinant for propofol (26), and different-sized analogues of propofol suggested that this residue plays a role consistent with a binding site (24). Furthermore, propofol reduces the rate of reaction of a sulfhydryl-reactive reagent with cysteine-substituted $\beta$M3-4 but not $\beta$M2-15 (29). Although this result suggested that propofol may bind in close proximity to $\beta$M3-4, the 50% reduction of the reaction rate produced by propofol could also result from an allosteric effect, just as the rate of modification of the cysteine at $\beta$M3-4 was increased 3-fold in the presence of GABA compared with its absence. These studies also suggest the possibility that $\beta$M2-15 may contribute indirectly to anesthetic action, i.e. by allosteric conformational coupling, rather than by the presence in a binding pocket for etomidate, propofol, and barbiturates.

Extensive mutational analyses provide strong evidence that volatile anesthetics and alcohols interact with a site distinct from the etomidate site identified by photolabeling. $\alpha$M2-15 and $\alpha$M3-4 were the positions first identified as important sensitivity determinants for volatile anesthetics and alcohols (7), and a series of mutations at $\alpha$M2-15 suggest that the size of the amino acid side chain affects anesthetic action, consistent with a competitive interaction. Further studies will be required to determine whether propofol binds with lower affinity to the etomidate site than to other sites in the GABA$_4R$R and/or whether isoflurane occupancy of the etomidate site provides an equivalent or less of an energetic contribution to gating than the binding of etomidate (or the binding of isoflurane at the other sites in the GABA$_4R$).

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