A Cysteine Substitution Probes β3H267 Interactions with Propofol and Other Potent Anesthetics in α1β3γ2L γ-Aminobutyric Acid Type A Receptors

Alex T. Stern, B.S., Stuart A. Forman, M.D., Ph.D.

ABSTRACT

Background: Anesthetic contact residues in γ-aminobutyric acid type A (GABAA) receptors have been identified using photolabels, including two propofol derivatives. O-propofol diazirine labels H267 in β3 and α1β3 receptors, whereas m-azi-propofol labels other residues in intersubunit clefts of α1β3. Neither label has been studied in αβγ receptors, the most common isoform in mammalian brain. In αβγ receptors, other anesthetic derivatives photolabel m-azi-propofol-labeled residues, but not βH267. The authors’ structural homology model of α1β3γ2L receptors suggests that βH267 may abut some of these sites.

Methods: Substituted cysteine modification–protection was used to test β3H267C interactions with four potent anesthetics: propofol, etomidate, alphaxalone, and R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirinylphenyl) barbituric acid (mTFD-MPAB). The authors expressed α1β3γ2L or α1β3H267Cγ2L GABA receptors in Xenopus oocytes. The authors used voltage clamp electrophysiology to assess receptor sensitivity to γ-aminobutyric acid (GABA) and anesthetics and to compare p-chloromercuribenzenesulfonate modification rates with GABA versus GABA plus anesthetics.

Results: Enhancement of low GABA (eliciting 5% of maximum) responses by equihypnotic concentrations of all four anesthetics was similar in α1β3γ2L and α1β3H267Cγ2L receptors (n > 3). Direct activation of α1β3H267Cγ2L receptors, but not α1β3γ2L, by mTFD-MPAB and propofol was significantly greater than the other anesthetics. Modification of β3H267C by p-chloromercuribenzenesulfonate (n > 4) was rapid and accelerated by GABA. Only mTFD-MPAB slowed β3H267C modification (approximately twofold; P = 0.011).

Conclusions: β3H267 in α1β3γ2L GABA receptors contacts mTFD-MPAB, but not propofol. The study results suggest that β3H267 is near the periphery of one or both transmembrane intersubunit (α+/β− and γ+/β−) pockets where both mTFD-MPAB and propofol bind. (Anesthesiology 2016; 124:89-100)

PROPOFOL, etomidate, barbiturates, and alphaxalone enhance γ-aminobutyric acid type A (GABA) receptor gating, contributing to sedation, hypnosis, and immobilization.1–3 GABA receptors are pentameric ligand-gated ion channels (pLGICs). The most common subtypes in mammalian brain contain two α, two β, and one γ subunits arranged as shown in figure 1.4–5 Each subunit has an N-terminal extracellular domain and a four-helix (M1 to M4) transmembrane domain (TMD). Subunit interfacial surfaces are designated “plus (+)” (M3 side) or “minus (−)” (M1 side).4 Current structural homology models of αβγ receptors, based on crystallized homomeric pLGICs from bacteria, nematodes, and humans β3, are similar.6–11 Anesthetic-binding residues in GABA receptors (fig. 1, C and D) have been identified using both photolabel derivatives (fig. 2 and table 1) and substituted cysteine modification–protection (table 1). Two propofol derivatives, m-azi-propofol (azi-Pm) and e-propofol diazirine (e-PD), photolabel distinct residues.12–14 In α1β3 receptors, azi-Pm labels residues in β3-M3 (β3M286), α1-M1 (α1M236), and β3-M1 (β3M227).13 These residues are also labeled in αβγ receptors by either azi-etomidate or the potent barbiturate R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirinylphenyl) barbituric acid (mTFD-MPAB) (fig 1, C and D, and table 1).15,16 Propofol inhibits photolabeling by azi-Pm,
azi-etomidate, or mTFD-MPAB.13,16,17 O-PD also inhibits azi-etomidate and mTFD-MPAB incorporation.13 However, in β3 homomers and α1β3 receptors, o-PD uniquely labels β3H267 (M2-17'), which is not labeled by other anesthetics14 (table 1). To date, neither azi-Pm nor o-PD has been studied in αβγ GABA A receptors.

Conflicting structural interpretations of propofol photo-labeling results, and particularly the role of βH267, emerge from homology model analyses. In silico docking calculations for propofol in the β3 crystal structure suggest that H267 contributes to binding sites separate from those where azi-Pm binds.22 In contrast, our α1β3γ2L homology model (fig. 1) locates βH267 near and possibly within α+β- and γ+β- pockets containing residues labeled by both azi-Pm and mTFD-MPAB.

Substituted cysteine modification–protection is sensitive to steric interactions between anesthetics and putative contact residues. Sulfhydryl-specific reagents covalently modify accessible cysteine-substituted residues, usually producing functional changes.23 Bound anesthetic may hinder chemical modification of cysteines located near or within anesthetic sites. For example, both etomidate and propofol block modification of αM236C and βM286C in α1β2/3γ2 receptors18,20,21 (table 1). This approach also has identified several nonphotolabeled anesthetic contact residues in β+/α- interfaces (fig. 1 and table 1)12,18,24 but has not been reported for anesthetic interactions with other transmembrane interface pockets.

In the current study, we tested the hypothesis that in α1β3γ2L receptors, βH267 is near propofol and mTFD-MPAB sites in α+β- and γ+β- interfaces, but not those for etomidate or alphaxalone in β+/α- interfaces.25,26 By using voltage clamp electrophysiology, we pharmacologically characterized α1β3H267Cγ2L receptors and compared rates of βH267C modification by p-chloromercuribenzenesulfonate in the absence versus presence of anesthetics. The β3H267C

---

**Fig. 1.** Anesthetic-binding sites in a structural model of α1β3γ2L γ-aminobutyric acid type A receptors. (A) A structural homology model of α1β3γ2L γ-aminobutyric acid type A receptors,12 viewed from the side. Subunits are color coded: α1 = gold, β3 = blue, and γ2 = green. The peptide chain backbones are depicted as ribbons and loops. The extracellular domain (ECD) and transmembrane domain (TMD) are labeled. Intracellular domains have been truncated to match those of the GluCl template. (B) The TMD viewed from the extracellular space, depicting the established subunit arrangement, the four-helix bundles of each subunit, and the transmembrane pockets formed at subunit interfaces. Amino acid residues thought to interact with anesthetics based on either photolabeling or cysteine modification and protection (table 1) are identified as ball-and-stick structures. The two βH267 residues (magenta) are located in the α+/β- and γ+/β- interfaces. (C) A close-up view from a perspective similar to that in A, identifying putative anesthetic contact residues in the α+/β- interface (on the left) and one of the β+/α- interfaces (on the right). (D) A close-up view of the same two transmembrane interfacial pockets from the extracellular space. A subset of the putative anesthetic contact residues, including βH267, is labeled.
Azi-ETO = azi-etomidate; Azi-Pm = m-azi-propofol; ETO = etomidate; GABA_A = γ-aminobutyric acid type A; mTFD-MPAB = R-5-allyl-1-methyl-5-[m-trifluoromethyl-diazirinylphenyl] barbituric acid; ND = no published data; α-PD = α-propofol diazirine; PRO = propofol; TDBzl-ETO = o-trifluoromethyl-diazirinylphenyl etomidate; — = negative modification or protection result.

Table 1. Anesthetic Contact Residues in GABA_A Receptors

<table>
<thead>
<tr>
<th>Residue</th>
<th>Receptor Type</th>
<th>Interfacial Sites</th>
<th>Photolabels</th>
<th>Substituted Cysteine Modification—Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1L232</td>
<td>α1 β3 γ2L</td>
<td>β+/α−</td>
<td>—</td>
<td>ETO^18</td>
</tr>
<tr>
<td>α1M236</td>
<td>α1 γ, α1β3</td>
<td>β+/α−</td>
<td>Azi-ETO^15</td>
<td>ETO, PRO^12,18</td>
</tr>
<tr>
<td>α1T237</td>
<td>α1 β3 γ2L</td>
<td>β+/α−</td>
<td>TDBzl-ETO^19</td>
<td>ND</td>
</tr>
<tr>
<td>α1239</td>
<td>α1 β3</td>
<td>β+/α−</td>
<td>Azi-Pm^13</td>
<td>ETO^18</td>
</tr>
<tr>
<td>α1529</td>
<td>α1 β3 γ2L</td>
<td>α+/β−, α+/γ−†</td>
<td>mTFD-MPAB^16</td>
<td>ND</td>
</tr>
<tr>
<td>α1Y294</td>
<td>α1 β3 γ2L</td>
<td>α+/β−, α+/γ−†</td>
<td>mTFD-MPAB^16</td>
<td>ND</td>
</tr>
<tr>
<td>β3M227</td>
<td>β3 γ2L</td>
<td>β+/α−</td>
<td>mTFD-MPAB^16</td>
<td>ND</td>
</tr>
<tr>
<td>β3N265</td>
<td>β3 γ2L</td>
<td>β+/α−</td>
<td>ETO, PRO^12†</td>
<td>§</td>
</tr>
<tr>
<td>β3H267</td>
<td>β3, α1β3</td>
<td>β+/β−, α+/β−</td>
<td>Azi-ETO^15</td>
<td>ETO, PRO^20,21</td>
</tr>
<tr>
<td>β3M286</td>
<td>α1 β3 γ2L</td>
<td>β+/α−</td>
<td>TDBzl-ETO^19</td>
<td>ND</td>
</tr>
<tr>
<td>β3F289</td>
<td>β3, α1β3</td>
<td>β+/β−</td>
<td>mTFD-MPAB^13</td>
<td>ND</td>
</tr>
<tr>
<td>β3V290</td>
<td>α1 β3 γ2L</td>
<td>β+/α−</td>
<td>TDBzl-ETO^19</td>
<td>ND</td>
</tr>
<tr>
<td>γ2S301</td>
<td>α1 β3 γ2L</td>
<td>γ+/β−</td>
<td>mTFD-MPAB^16</td>
<td>ND</td>
</tr>
</tbody>
</table>

* p-Chlormercursbenesulfonate application to α1/239C did not alter function. † To date, there is no evidence of anesthetic contact with γ-M1 helix residues, so anesthetic binding in the α/γ interface remains speculative. ‡ A binding role for γ-M1 helix was indirectly demonstrated using α1 β236C protection. § Current study. ¶ mTFD-MPAB did not photolabel [M286 or [F289 in α1 β3 γ2L, but did in α1 β3. Thus, incorporation into these residues was likely at the β+/β− interface. Azi-ETO = azi- etomidate; Azi-Pm = m-azi-propofol; ETO = etomidate; GABA_A = γ-aminobutyric acid type A; mTFD-MPAB = R-5-allyl-1-methyl-5-[m-trifluoromethyl-diazirinyl phenyl] barbituric acid; ND = no published data; α-PD = α-propofol diazirine; PRO = propofol; TDBzl-ETO = o-trifluoromethyl diazirinylphenyl etomidate; — = negative modification or protection result.
University of Illinois at Chicago, Chicago, Illinois) and prepared as a 100 mM stock in methanol. After dilution for electrophysiology studies, methanol concentration was less than 0.01%, which produced no significant modulation of either wild-type or mutant GABA_A receptors. Picrotoxin (PTX) was purchased from Sigma-Aldrich and dissolved (2 mM) in electrophysiology buffer. p-Chloromercuribenzenesulfonate acid sodium salt (pCMBS) was purchased from Toronto Research Chemicals (Canada). All other chemicals were purchased from Sigma-Aldrich.

**Molecular Biology**

Complementary DNAs for human GABA_A receptor α1, β3, and γ2L subunits were cloned into pCDNA3.1 vectors (Invitrogen, USA). A mutation encoding β3H267C was created with oligonucleotide-directed mutagenesis by using a QuikChange kit (Agilent Technologies, USA). Several clones from the mutagenesis reaction were subjected to DNA sequencing through the entire β3 coding region to confirm the presence of the intended mutation and absence of stray mutations. A single mutant clone was selected for further use.

**Oocyte Electrophysiology**

Messenger RNA synthesis and Xenopus oocyte expression were performed as we have described.28 Electrophysiology experiments were conducted at room temperature (21° to 23°C). Oocytes were voltage clamped at ~50 mV and signals were low-pass filtered at 1 kHz (Model OC-725B; Warner Instruments, USA). Electrophysiological signals were digitized at 200 Hz (iWorx RA834; iWorx Systems Inc., USA) and recorded digitally on a personal computer running Lab-scribe v3 software (iWorx Systems Inc.). Oocyte superfusion in a custom-built flow chamber was software controlled through the iWorx RA834 interface to solenoid switches (ALA-VM8; ALA Scientific Associates, USA) and a submersible dead-volume manifold. Fivefold data reduction and further low-pass (10 Hz) digital filtering (using Clampfit) were performed as we have described.28

Electrophysiological signals were low-pass filtered at 1 kHz (Model OC-725B; Warner Instruments, USA). Electrophysiological signals were digitized at 200 Hz (iWorx RA834; iWorx Systems Inc., USA) and recorded digitally on a personal computer running Lab-scribe v3 software (iWorx Systems Inc.). Oocyte superfusion in a custom-built flow chamber was software controlled through the iWorx RA834 interface to solenoid switches (ALA-VM8; ALA Scientific Associates, USA) and a submersible dead-volume manifold. Fivefold data reduction and further low-pass (10 Hz) digital filtering (using Clampfit) were performed as we have described.28

**Cysteine Modification with pCMBS**

Voltage-clamped oocytes expressing GABA_A receptors were repetitively activated with alternating EC5 and 1 mM GABA pulses every 5 min until at least three sequential sets of responses were constant (± 5%). Oocytes were then exposed to pCMBS (alone, with GABA, or with GABA + anesthetic) for 5 to 12 s followed by 5-min ND96 wash. In oocytes expressing wild-type α1β3γ2L receptors, exposure to pCMBS (1 mM × 60 s, followed by a 5-min wash in ND96 buffer) produced no significant changes in currents stimulated with low (EC5 = 4 μM) or 1 mM GABA. We tested a range of pCMBS concentrations on oocytes expressing α1β3H267Cγ2L receptors. Exposure to 1 μM pCMBS for 10 s resulted in an approximately fivefold increase in response to low GABA (EC5 = 4 μM) relative to saturating GABA (1 mM). In most oocytes, the change in response ratio (I_max/I_min) was associated with increased response to 3 μM GABA and a modest reduction in response to 1 mM GABA. Repeated 10-s exposures to 1 μM pCMBS did not produce further change in response ratio, suggesting that β3H267C modification was complete after a single exposure. For experiments comparing the apparent initial covalent modification rates in α1β3H267Cγ2L receptors, we used a much lower pCMBS concentration (0.1 μM), which produced a similar change in response ratio as 1 μM pCMBS.

**Electrophysiological Data Analysis**

Analyses for agonist concentration–responses and propofol-induced left shift followed our approach described elsewhere.28,30 Peak GABA-stimulated currents were normalized to maximal GABA responses, and GABA concentration–response data for individual oocytes in the absence and presence of propofol were fitted with logistic functions using nonlinear least squares (GraphPad Prism version 5; GraphPad Software, Inc., USA):

\[
I_{\text{Agonin}} = \frac{I_{\text{max}} - I_{\text{min}}}{1 + 10^{\log(GABA/EC50)\text{-log}[\text{Agonist}]_{\text{mic}}} + I_{\text{min}}} \tag{1}
\]

where EC50 is the half-maximal activating concentration and nH is Hill slope.

EC50 shift ratio was calculated from the difference in log(GABA EC50) values [Δ(log(EC50))] measured in the presence of 5 μM propofol versus control.

**β3H267C in α1β3γ2L GABA_A Receptors**

GABA enhancement were assessed in both wild-type and α1β3H267Cγ2L receptors by using equipotent anesthetic concentrations (2 × EC50 for loss of righting reflexes in Xenopus tadpoles = 2.5 μM alphaxalone, 5 μM propofol, 3.2 μM etomidate, and 8 μM mTFD-MPAB). The eliciting 5% of maximal response (EC5) GABA concentration was identified for individual oocytes by testing GABA concentrations ranging from 2 to 4 μM. After establishing stable EC5 and 1 mM responses, oocyte currents were recorded during exposure to first anesthetic alone for 30 s, followed by anesthetic combined with EC5 GABA for another 15 to 30 s.
lower pCMBS concentration of 10 nM. Two or three 5- to 12-s applications of 10 nM pCMBS (each followed by 5-min ND96 wash) typically resulted in less than a doubling of \( I_{\text{pCMBS}}/I_{\text{max}} \), that is, less than 20% of the change associated with complete modification. After repeated exposures to 10 nM pCMBS, each oocyte was also exposed to 1 μM pCMBS for 10 s to assess \( I_{E/C}/I_{\text{max}} \) after full modification.

To test for anesthetic protection (inhibition of β3H267C modification), apparent modification rates with pCMBS plus 1 mM GABA were compared with rates with pCMBS plus 1 mM GABA and anesthetic. The GABA-bound receptor was chosen as the index condition because GABA binding enhances the affinity of receptors for anesthetics, thereby increasing anesthetic site occupancy. The anesthetic concentrations used in protection studies were 10 μM alphaxalone; 10 and 30 μM etomidate; 5, 10, and 30 μM propofol; and 8 and 16 μM mTFD-MPAB. These anesthetic concentrations enhance the activation of both wild-type and mutant GABA\(_{A}\) receptors at least 10-fold (see Results), and estimates of etomidate and propofol affinities for GABA-bound receptors suggest that over 90% of anesthetic sites are occupied under these conditions. For modification rate analysis, \( I_{\text{pCMBS}}/I_{\text{max}} \) rate ratios were normalized to the premodification control and plotted against cumulative pCMBS exposure in units of nanomolar × seconds. Normalized response ratios were fitted by linear least squares to determine the apparent initial modification rate (slope, in \( \text{M}^{-1} \text{s}^{-1} \)). We fitted modification rates for both individual oocytes and for combined response ratio data from groups of oocytes for each condition. These resulted in slightly different mean and standard error values, due to differential data weighting, without affecting our overall conclusions.

**Molecular Structural Modeling**

We used a structural model for the α1β3γ2 GABA\(_{A}\) receptor based on GluCl bound to ivermectin (Protein Data Bank 3RH5), which we have described in a prior publication. The optimized structure was visualized and analyzed by using University of California San Francisco Chimera v1.10, San Francisco, California. Optimized molecular structure models for the anesthetic drugs were built and analyzed using AvoGadro v1.1.1.

**Statistical Analysis**

Oocytes were obtained from at least two frogs and randomly selected for each experiment. Blinding was not used during experiments or analysis. Group sizes (n > 3 for functional characterization; n = 4 for modification rate comparisons) were based on prior experience with these techniques. Additional control modification experiments (with GABA plus pCMBS) were performed with each set of protection studies. Results are reported as mean ± standard error unless otherwise noted. Statistical analyses were performed by using Prism 5.02 (GraphPad Software, Inc.). Statistical comparisons of anesthetic direct activation and GABA enhancement in both wild-type and α1β3H267Cγ2L receptors were based on two-way ANOVA and pairwise Bonferroni posttests. Apparent pCMBS modification rates measured under multiple conditions (i.e., sets of individual oocyte results) were compared by using Kruskal–Wallis with Dunn multiple comparison test. Other pairwise comparisons were performed by using Student’s t tests or Mann–Whitney test. Statistical significance was inferred at \( P \) value less than 0.05.

**Results**

*Xenopus* oocytes injected with mRNA mixtures encoding α1, wt β3 or β3H267C, and γ2L GABA\(_{A}\) receptor subunits were studied by using two-electrode voltage clamp. In wild-type control experiments, α1β3γ2L receptors produced GABA-dependent currents with \( EC_{50} \) averaging 31 μM (data not shown; n = 3; 95% CI, 18 to 49 μM), consistent with previous reports. Propofol (5 μM) produced a 12-fold GABA \( EC_{50} \) shift in wild-type receptors (data not shown; \( n = 3; 95\% \text{ CI}, 6.3-\text{to 23-fold} \)).

Voltage-clamped oocytes expressing α1β3H267Cγ2L receptors produced inward currents in response to GABA, in a concentration-dependent and reversible manner (fig. 3A). The fitted GABA \( EC_{50} \) value for α1β3H267Cγ2L receptors was 25 μM (\( n = 3; 95\% \text{ CI}, 19 \text{ to 32 μM} \)), similar to wild type. Coapplication of GABA with propofol (5 μM) enhanced currents elicted by GABA concentrations less than 100 μM (fig. 3B), producing a 15-fold (95% CI, 7.7- to 30-fold) leftward shift in the averaged concentration–response curve (fig. 3C) to 1.6 μM (\( n = 3; 95\% \text{ CI}, 0.83 \text{ to 3.2 μM} \)). Again, this result does not significantly differ from wild type, indicating that mutant receptors retain near-normal sensitivity to propofol. In oocytes expressing α1β3H267Cγ2L receptors with maximal peak currents over 5 μA, PTX (2 mM) applied in the absence of GABA did not alter basal leak currents (fig. 3D; \( n = 3 \)), indicating that spontaneous receptor activation is below the detection threshold (approximately 5 nA or 0.1% of maximal peak). Currents elicited with 1 mM GABA were not enhanced by propofol, indicating that high GABA concentrations activated approximately 100% of α1β3H267Cγ2L receptors (fig. 3D). We have previously estimated that spontaneous activation of wild-type receptors has a probability below 0.01% and that maximal GABA efficacy in wild-type receptors is approximately 85%.

We extended our study of anesthetic interactions at β3H267 to three other potent anesthetics that modulate GABA\(_{A}\) receptors: etomidate, alphaxalone, and mTFD-MPAB. By using voltage clamp electrophysiology, we compared the effects of equipotent drug concentrations (2 × the \( EC_{50} \) for loss of righting reflexes in tadpoles) in both wild-type α1β3γ2L (fig. 4A) and α1β3H267Cγ2L (fig. 4B) GABA\(_{A}\) receptors. In current recordings where oocytes were first exposed to anesthetic for 30 s followed by anesthetic + EC5 GABA, we found that 5 μM propofol, 3.2 μM etomidate, 2.5 μM alphaxalone, and 8 μM mTFD-MPAB produced indistinguishable (approximately 10-fold) enhancing effects.
effects on EC5 GABA responses in both α1β3γ2L and α1β3H267Cγ2L receptors (fig. 4C). These studies also revealed that both propofol and mTFD-MPAB directly activated α1β3H267Cγ2L receptors significantly more than the other anesthetics and also far more than these drugs activated wild-type receptors (fig. 4D).

After applying pCMBS (1 μM for 10 s) to voltage-clamped oocytes expressing α1β3H267Cγ2L receptors, followed by 5-min wash in electrophysiology buffer, we observed a fivefold increase in the response to 3 μM GABA (approximate EC5) relative to the 1 mM GABA response (an

Fig. 3. Functional characterization of α1β3H267Cγ2L γ-aminobutyric acid (GABA) type A receptors. (A) Traces are currents measured from a single voltage-clamped oocyte expressing α1β3H267Cγ2L GABA type A receptors. Bars over the traces identify GABA concentration (μM) and period of exposure. (B) Traces are recorded from the same oocyte as in A, activated with various GABA concentrations combined with 5 μM propofol (PRO). (C) Combined GABA concentration–responses from three oocytes in the absence and presence of propofol. Normalized data were fitted with equation 1 (see Materials and Methods). Fitted GABA EC50 values are 25 μM (approximate EC5) relative to the 1 mM GABA response (an activations with various GABA concentrations). The first trace depicts response to 1 mM GABA, the second to EC5 GABA (ranging from 3 to 6 μM), and the third shows current elicited during exposure to anesthetic (at 2 × EC50 for loss of righting reflexes in tadpoles, indicated in micromolar) and then anesthetic plus EC5 GABA. Anesthetic concentrations are indicated in micromolar. (B) The traces are from oocytes expressing α1β3H267Cγ2L receptors, studied as described for A. (C) A scatter plot showing all EC5 enhancement results with α1β3H267Cγ2L receptors, tested with a different anesthetic drug (alphaxalone [ALF], etomidate [ETO], R-5-allyl-1-methyl-5-[m-trifluoromethyl]-diazirinyl[phenyl] barbituric acid [MPAB], and propofol [PRO]). The first trace depicts response to 1 mM GABA, the second to EC5 GABA (ranging from 3 to 6 μM), and the third shows current elicited during exposure to anesthetic (at 2 × EC50 for loss of righting reflexes in tadpoles, indicated in micromolar) and then anesthetic plus EC5 GABA. Anesthetic concentrations are indicated in micromolar. (D) A scatter plot showing all direct activation results with α1β3H267Cγ2L receptors, studied as described for A. (D) A scatter plot showing all direct activation results with α1β3H267Cγ2L receptors, studied as described for A. (D) A scatter plot showing all direct activation results with α1β3H267Cγ2L receptors, studied as described for A. (D) A scatter plot showing all direct activation results with α1β3H267Cγ2L receptors, studied as described for A.
Fig. 5. Modification of α1β3H267Cγ2L γ-aminobutyric acid (GABA) type A (GABA$_A$) receptors with p-chloromercuribenzenesulfonate (pCMBS). The panels on the left show examples of voltage clamp current traces during modification under four different conditions. Colored traces are responses to 3 μM GABA, and black traces are responses to 1 mM GABA. Arrows indicate modification exposures, which were followed by 5-min wash. The starred arrows indicate exposure to 1 μM pCMBS for 10 s. The panels on the right show the corresponding initial linear rate analyses for combined normalized response $I_{3\mu M}/I_{1mM}$ ratios from all oocytes used for each condition. Points represent the ratio of $I_{3\mu M}/I_{1mM}$ normalized to the premodification control, and plotted against cumulative pCMBS exposure. Points in the upper right portion of the panel represent response ratios after modification with 1 μM pCMBS. (A) Modification in the absence of GABA. Traces are recorded from one voltage-clamped oocyte expressing α1β3H267Cγ2L GABA$_A$ receptors before and after sequential 10-s exposures to 10 nM pCMBS. (B) Initial modification rate analysis for combined data from all oocytes modified with pCMBS alone (n = 5). The line through the first four points
example is shown in fig. 5A). Repeated exposure to 1 μM pCMBS (with postexposure wash) did not further increase the normalized response ratio (I_{3H267C}/I_{mM}), indicating that a single 10-s exposure fully and irreversibly modified all receptors. In contrast, when oocytes expressing α1β3y2L receptors were exposed to 1 mM pCMBS for up to 60 s, no changes were observed in spontaneous leak or current responses to low and high GABA concentrations (n = 3; data not shown). Therefore, the effect of pCMBS on α1β3H267Cγ2L function was due to covalent bond formation at β3H267C.

In oocytes expressing α1β3H267Cγ2L receptors, initial pCMBS modification rates were assessed using repeated 5- to 12-s exposures to 10 nM pCMBS. At this concentration, the I_{3H267C}/I_{mM} response ratio increased by approximately 40% after a cumulative 30 s of exposure (fig. 5B). Linear fits to the normalized response ratios plotted against cumulative pCMBS exposure for all oocytes (fig. 5B; n = 5) indicated an apparent slope of (mean ± SEM) 1.3 ± 0.19 × 10^6 M^{-1} s^{-1}. The average of individual oocyte modification rates (mean ± SEM) was similar (1.3 ± 0.24 × 10^6 M^{-1} s^{-1}). When pCMBS was coapplied with 1 mM GABA (e.g., fig. 5C), the apparent rate of modification (all oocytes; n = 9) increased to 3.6 ± 0.25 × 10^6 M^{-1} s^{-1} (fig. 5D). The average of individual oocyte modification rates with GABA was 3.9 ± 0.58 × 10^6 M^{-1} s^{-1}, threefold higher (P = 0.0078; Mann–Whitney test) than the rate without GABA. The maximal change in normalized response ratio remained approximately fivefold after coapplication of 1 μM pCMBS with GABA (fig. 5D). Coaplication of pCMBS with 1 mM GABA plus 10 μM propofol (e.g., fig. 5E) resulted in an apparent rate of modification (all oocytes; n = 5) of 3.0 ± 0.47 × 10^6 M^{-1} s^{-1} (fig. 5F). The individual oocyte modification rates with GABA + propofol (3.5 ± 0.69 × 10^6 M^{-1} s^{-1}) and the overall effect of modification were similar to those in the presence of GABA alone. Additional protection experiments using 30 μM propofol (data not shown; n = 5) also indicated no reduction in the modification rate.

We also tested whether etomidate, alphaxalone, or mTFD-MPAB alter the rate of pCMBS modification in GABA-activated α1β3H267Cγ2L receptors, applying the same approach used for propofol. Etomidate (10 and 30 μM) and alphaxalone (10 μM) produced no changes, whereas mTFD-MPAB (8 μM; fig. 5, G and H) reduced the average modification rate approximately twofold (fig. 6; P = 0.011; Mann–Whitney test). Attempts to study protection using higher (16 μM) mTFD-MPAB concentrations were complicated by very slow washout producing residual direct activation and desensitization of α1β3H267Cγ2L receptors, resulting in widely varying apparent modification rates in repeated experiments.

**Discussion**

In a α1β3y2L background, we investigated β3H267C effects on anesthetic sensitivity and tested whether bound anesthetics protect this cysteine from modification by pCMBS. Although βH267 was photolabeled by ω-PD in β3 and α1β3 receptors, we found that in α1β3y2L, propofol did not protect β3H267C from chemical modification. In similar studies with etomidate, alphaxalone, and mTFD-MPAB, only mTFD-MPAB reduced the rate of β3H267C modification. This suggests that β3H267 is near at least one of the two mTFD-MPAB “β−” sites in α1β3y2L, as predicted by our structural homology model (fig. 1D).13,16 Our negative β3H267C protection results with etomidate and

**Fig. 5. (Continued),** has a fitted slope of 1.3 ± 0.19 × 10^6 M^{-1} s^{-1}. Maximal normalized response ratio = 5.4 ± 0.25 (n = 5; mean ± SEM). (C) Modification in the presence of GABA. Current responses from a single oocyte during sequential 10-s exposures to 10 nM pCMBS plus 1 mM GABA. (D) Initial modification rate analysis for all oocytes modified with pCMBS plus GABA (n = 9). The fitted linear slope is 3.6 ± 0.25 × 10^6 M^{-1} s^{-1}. Maximal normalized response ratio = 5.2 ± 0.24 (n = 8; mean ± SEM). (E) Modification in the presence of GABA and propofol. Current responses from one oocyte before and after sequential 10-s exposures to 10 nM pCMBS plus 1 mM GABA plus 10 μM propofol (PRO). (F) Initial modification rate analysis for all oocytes modified with pCMBS plus GABA and propofol (n = 5). The fitted linear slope is 3.0 ± 0.47 × 10^6 M^{-1} s^{-1}. Maximal normalized response ratio = 5.3 ± 0.27 (n = 5; mean ± SEM). (G) Modification in the presence of GABA and R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirinylphenyl) barbituric acid (mTFD-MPAB). Current responses from one oocyte before and after sequential 10-s exposures to 10 nM pCMBS plus 1 mM GABA plus 8 μM mTFD-MPAB. (H) Initial modification rate analysis for all oocytes modified with pCMBS plus GABA and MPAB (n = 7). The fitted linear slope is 1.4 ± 0.22 × 10^6 M^{-1} s^{-1}. Maximal normalized response ratio = 5.2 ± 0.18 (n = 5; mean ± SEM).
alphaxalone are also consistent with prior evidence that these anesthetics bind in β+/α− interfaces.15,19,25,26 Earlier studies showed that BH267 mutations influence GABA\textsubscript{A} receptor modulation by both Zn\textsuperscript{2+} and protom.5,35–35 We also found that the BH267C mutation selectively sensitized receptors to activation by both mTFD-MPAB and propofol, linking BH267 to channel gating and the nearby α+/β− and γ+/β− sites where these anesthetics bind. The absence of BH267C effects on receptor agonism by GABA, etomidate, and alphaxalone rules out global allosteric effects of the mutation. Indeed, the anesthetic specificity of both pharmacological effects (fig. 4) and biochemical protection (fig. 6) indicates local interactions of \( \text{BH267} \) with the “βα−” anesthetic sites.

Consistent with our observations, a prior study of \( \alpha_1 \text{BH267C} \gamma_2 \) also reported enhanced channel gating after pCMSB modification.36 The pCMSB modification rate at BH267C (approximately 4 \times 10\textsuperscript{6} M\textsuperscript{−1} s\textsuperscript{−1} with GABA) was approximately 10-fold faster than other TMD cysteine substitutions we have examined.12,18,21 The rapid modification of BH267C indicates a relatively high degree of probe and water exposure for a TMD side chain but remains far slower than pCMSB reactions with free cysteine in bulk water at pH 7.5 (estimated near 10\textsuperscript{8} M\textsuperscript{−1} s\textsuperscript{−1}).37 GABA increased the rate of modification, indicating GABA-dependent structural rearrangements near BH267. The dynamic structural changes in the GABA\textsubscript{A} receptor TMD that accompany channel activation and desensitization remain uncertain although comparisons of crystallized GluCl structures9,10 and biophysical studies of bacterial pLGICs38 in different states suggest that the extracellular ends of M2 and M3 helices tilt away from the pore, possibly expanding intersubunit pockets and their water content.

The interpretation of our new results must consider limitations of photolabeling, cysteine modification–protection, and structural models of heteromeric GABA\textsubscript{A} receptors. Photolabeling is an unbiased method for identifying ligand contact loci. Photolabels must be structurally and pharmacologically similar to the “parent” drug of interest. Also required are sufficient target protein quantity and purity, efficient and stable photo-adduct formation, and a sensitive method for identifying incorporation sites. Limitations include the potential for photolabeling sites other than those where the parent drug acts and for selective photochemical reactions with amino acids that may not exist in drug-binding sites. The BH267C residue was identified as the sole contact in β3 and α1β3 receptors photolabeled with o-PD using mass spectroscopic proteonomic analysis.14 Subsequently, Jayakar et al.13 reported that o-PD displaced azi-etomidate and mTFD-MPAB labeling in α1β3 receptors, implying that o-PD interacts with residues other than BH267 in heteromeric receptors (that contain a β3–β3 interface). Thus, o-PD photolabeling may have missed other contact residues due to technical limitations. Photolabeling results for azi-Pm and o-PD may also reflect different orientations of photoactive groups at ring positions 2 and 6 (presumably near BH267) relative to positions 3 and 5 when bound in the same site with steric constraints. Indeed, modifications at these propofol ring positions also produce distinct effects on drug potency or efficacy.39 Similarly, etomidate’s photolabel derivatives15,19 have identified only a portion of its currently known contact residues. Others were identified in αβγ receptors using cysteine modification–protection.

The substituted cysteine modification–protection strategy uses an unmodified ligand and sulphhydryl-selective chemistry to test the interactions at putative contact residues. Important considerations for this method include the following: (1) ligand binding is retained in the cysteine-substituted mutant receptor, (2) ligand occupies a large fraction of its sites during protection experiments, and (3) a similar mixture of receptor states is present during modification in both the absence and presence of ligand. In our current experiments, evidence indicates that all these conditions were met. Modulation of α1β3BH267Cγ2L receptors by propofol and the other anesthetics was similar to that in wild-type GABA\textsubscript{A} receptors (fig. 4C), indicating minimal changes in affinity\textsuperscript{40} binding. By using high GABA concentrations, we established conditions where nearly all α1β3BH267Cγ2L receptors were either in open or desensitized states that have high anesthetic affinity relative to resting/closed receptors. Propofol modestly slows GABA\textsubscript{A} receptor desensitization without altering its extent,40 implying that both open and desensitized receptors bind propofol with similar affinities. Thus, similar receptor state mixtures were present during modification with or without anesthetics. Our prior estimate of the propofol dissociation constant for GABA-bound α1β2γ2L receptors (K\textsubscript{D} = \( \approx 1 \mu\text{M} \))31 suggests that 10 μM propofol occupies approximately 83% of sites and 30 μM propofol occupies approximately 93% of sites. Photolabeling inhibition also indicates that propofol binds to both etomidate and mTFD-MPAB sites with similar affinities.16

We studied propofol interactions with BH267 in α1β3γ2L, and our results do not address β3 and α1β3 receptors that were photolabeled with o-PD.14 Propofol contact might occur only within β/β interfaces that are absent in αβγ receptors. Even if propofol contacts BH267 in wild-type receptors, the histidine-to-cysteine mutation reduces side chain size and may also alter orientation, reducing contact in the mutant. Given that in β3 homomers H267 is positioned between the intersubunit cleft and the ion channel,6 it is conceivable that propofol binds near BH267 but does not effectively protect the sulphhydryl group from pCMSB in the receptor pore. However, our “positive control” finding that mTFD-MPAB protects BH267 suggests that this is unlikely and that the technique worked as intended. Moreover, a recent study of BH267W effects in β3 and α1β3 also found no evidence for propofol interactions with this residue.41

Photolabeling has established that in α1β3 receptors, propofol, azi-Pm, o-PD, and mTFD-MPAB compete for binding sites in α+/β− and β+/β− interfaces.13,16 Considering these data together with our current results suggests that
A. T. Stern and S. A. Forman

Fig. 7. β3H267 and other α+/β- anesthetic contact residues line a contiguous pocket. (A) A portion of our α1β3γ2L structural homology model is shown with peptide backbone as ribbons and side chains depicted as spherical shells (hydrogens are hidden). The view is from the extracellular space, off-axis, through a planar cut (atoms cut by this plane appear hollow). The peptide backbones of transmembrane helices are highlighted and labeled. The side chain of β3H267 is in magenta, and other side chains known to contribute to anesthetic binding are shaded in green and labeled. Other side chain atoms are color coded (gray = carbon, red = oxygen; blue = nitrogen; and yellow = sulfur). Some side chains (β3L223, β3Q224, β3H267) is located near the periphery of at least one of the mTFD-MPAB sites in α1β3γ2L and further from subregions of the β- pockets that interact with both mTFD-MPAB and propofol. In our structural homology model, contiguous cavities extend from β3H267 to residues photolabeled by mTFD-MPAB and azi-Pm (fig. 7, A and B), including α1S270, another residue thought to interact with anesthetics.42,43 The model-derived distances from the β3H267 imidazole to α1S270, α1A291, and α1Y294 range from 7.0 to 12.7 Å, whereas distance from β3H267 to γ2S301 is 12.8 Å. The largest projection length of R-mTFD-MPAB is 10.9 Å, whereas that of propofol is 7.6 Å. Thus, mTFD-MPAB is large enough to bind near α1A291 or γ2S301 and impede pCMBS access to β3H267C, whereas propofol is smaller and may fail to obstruct this interaction. To fully reconcile photolabeling with our protection results, we also posit that azi-Pm and α-PD both occupy β- sites overlapping those for propofol and mTFD-MPAB, yet photolabel different residues because of constrained binding orientations.

Some alternative GABA₆ receptor structural models do not contain a contiguous pocket linking β3H267 with the residues labeled by mTFD-MPAB and azi-Pm. Franks42 conducted docking calculations for propofol in the β3 crystal structure that shows two separated pockets (fig. 7C) and found these consistent with α-PD photolabeling of β3 homomers.5 Jayakar et al.13 also describe an α1β3 model based on Gloeobacter violaceus ligand-gated ion channel where β3H267 forms part of a pocket adjacent to the ion channel and separated by intruding side chains from inter-subunit residues photolabeled by azi-Pm. The accuracy of structural models vis-à-vis the various functional states of α1β3γ2L and other GABA₆ receptors remains speculative. Small helix rotations or side chain rearrangements in the models shown in figure 7, B and C, could alter the shape and
contiguity of the depicted pockets. Our current protection results favors a structure for α1β3γ2L receptors with “β−” anesthetic-binding pockets contiguously linking the α-PD-, azi-Pm-, and mTFD-MPAB-photolabeled residues.

Analysis of other β3H267 mutations in α1β3γ2L may provide further insights into its roles in anesthetic modulation. However, functional analysis alone may not distinguish between mutant-associated changes in anesthetic binding versus transduction.12,30 This is because anesthetics are highly efficacious agonists of GABAγ receptors, binding almost exclusively to activated and desensitized states. In contrast, cysteine modification–protection has identified likely anesthetic contact even at residues where cysteine substitution did not significantly alter sensitivity to anesthetic.18 This further highlights the importance of complementary methods to probe both functional and steric interactions between drug and receptor.

In summary, in cysteine modification–protection studies of α1β3H267Cy2L GABAγ receptors and four potent general anesthetics (propofol, etomidate, alphaxalone, and mTFD-MPAB), only mTFD-MPAB slowed β3H267C modification, indicating steric proximity. The β3H267C mutation also selectively enhanced direct agonism by both propofol and βmTFD-MPAB), only mTFD-MPAB slowed (but not etomidate or alphaxalone) bind.

Acknowledgments

The authors thank Karol Bruzik, Ph.D., and Pavel Savechenkov, Ph.D. (both from the Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, Chicago, Illinois), for providing access to R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirinylphenyl) barbituric acid (mTFD-MPAB). The authors thank Keith Miller, D.Phil. (Department of Anesthesia Critical Care & Pain Medicine, Massachusetts General Hospital, Boston, Massachusetts), and Jonathan Cohen, Ph.D. (Department of Neurobiology, Harvard Medical School, Boston, Massachusetts), for comments and suggestions on the study and article.

This work was supported by grant no. GM089745 from the National Institutes of General Medical Sciences (Bethesda, Maryland). γ-Aminobutyric acid receptor molecular graphics and distance analyses were performed with the University of California, San Francisco Chimera package (v1.10), San Francisco, California. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by grant no. NIGMS P41-GM103311).

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Forman: Department of Anesthesia, Critical Care, and Pain Medicine, Jackson 444, Massachusetts General Hospital, 55 Fruit Street, Boston, Massachusetts 02114. saforman@partners.org. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. Anesthesiology’s articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References

18. Stewart DS, Hotta M, Li GD, Desai R, Chiara DC, Olsen RW, Forman SA: Cysteine substitutions define etomidate binding...
and gating linkages in the α-M1 domain of γ-aminobutyric acid type A (GABA A) receptors. J Biol Chem 2013; 288:30373–86


24. McCracken ML, Borghese CM, Trudell JR, Harris RA: A transmembrane amino acid in the GABA A receptor β2 subunit critical for the actions of alcohols and anesthetics. J Pharmacol Exp Ther 2010; 335:600–6


43. Mascia MP, Trudell JR, Harris RA: Specific binding sites for anesthetics and β3H267C in α1β2γL GABA A Receptors