Specificity of Intersubunit General Anesthetic-binding Sites in the Transmembrane Domain of the Human α1β3γ2 γ-Aminobutyric Acid Type A (GABA_A) Receptor

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David C. Chiara, Selwyn S. Jayakar, Xiaojuan Zhou, Xi Zhang, Pavel Y. Savechenkov, Karol S. Bruzik, Keith W. Miller, and Jonathan B. Cohen

From the Departments of Neurobiology and Biological Chemistry and Molecular Pharmacology, Harvard Medical School,Boston, Massachusetts 02115, the Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Boston, Massachusetts 02114, and the Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, Chicago, Illinois 60612

Background: General anesthetics are a diverse chemical structure potentiate GABA_A receptors by binding to unknown sites.

Results: A photoreactive barbiturate identifies intersubunit-binding sites distinct from, but homologous to, sites identified by photoreactive etomidate analogs.

Conclusion: Propofol, barbiturates, and etomidate analogs bind with variable selectivities to two classes of sites.

Significance: This study helps define the diversity of GABA_A receptor general anesthetic-binding sites.

GABA type A receptors (GABA_A receptor), the brain’s major inhibitory neurotransmitter receptors, are targets for many general anesthetics, including volatile anesthetics, etomidate, propofol, and barbiturates. How such structurally diverse agents can act similarly as positive allosteric modulators of GABA_A receptors remains unclear. Previously, photoreactive etomidate analogs identified two equivalent anesthetic-binding sites in the transmembrane domain. The selectivity of these homologous intersubunit sites is sufficient for anesthetic action and that this explains to some degree the puzzling specificity of intersubunit general anesthetic-binding sites.

General anesthetics of diverse structures, including volatile anesthetics, propofol, etomidate, barbiturates, steroids, and alcohols, potentiate inhibitory GABA type A receptors (GABA_A receptor) in vitro with a pharmacology and concentration dependence that suggest this receptor is a major contributor to the anesthetic state (1–3). The importance of GABA_A receptors for anesthesia in vivo was demonstrated by the decreased sensitivity of "knock-in" mice bearing a single substitution at position 15 in the GABA_A receptor β3 subunit transmembrane helix 2 (β3M2–15′), a substitution that reduced GABA_A receptor sensitivity to propofol and etomidate in vitro (4). These mice had greatly reduced sensitivity to the immobilizing and hypnotic anesthetic effects of etomidate, propofol, and pentobarbital, with little change in sensitivity to volatile or steroid anesthetics (5–7).

The locations of anesthetic sensitivity determinants in GABA_A receptors have been predicted by use of homology models derived from the structures of other members of the Cys-loop superfamily of pentameric ligand-gated ion channels, the nicotinic acetylcholine receptor (nAChR) (8), the prokaryotic homologs ELIC (9) and GLIC (10), and an invertebrate glutamate-gated chloride channel (11). Each subunit contains an 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. In an (α)2(β)2γ2GABA_A receptor, the transmitter-binding sites are in the extracellular domain at the β+′–α−′ site, with amino acids from the β and α subunits forming the principal (+) and complementary (−) surfaces of the binding pocket, respectively (Fig. 1). The benzo-
diazepine-binding site is at an equivalent position at the $\alpha^+\gamma^-$ subunit interface (12, 13). In the transmembrane domain, M2 helices from each subunit associate around a central axis to form the ion channel, and amino acids from the M1 and M3 helices of adjacent subunits contribute to the subunit interfaces. The etomidate-binding sites, identified by photoaffinity labeling of amino acids in $\beta$M3 and $\alpha$M1, are in the two $\beta^+\alpha^-$ subunit interfaces about 50 Å below the agonist sites (14, 15).

We reported recently that the $R$-enantiomer of 5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl)barbituric acid ($m$TFD-MPAB) is an extremely potent, photoreactive barbiturate that rivals etomidate in potency and stereoselectivity (16). Here, we report that $R$-[3H]$m$TFD-MPAB photolabels new anesthetic-binding sites in human $\alpha_1\beta_3\gamma_2$ GABA$_A$Rs at the $\alpha^+\beta^-$ and $\gamma^+\beta^-$ subunit interfaces. These sites are distinct but homologous to the $R$-[3H]azietomidate sites at the two $\beta^+\alpha^-$ interfaces, as all are located at the same depth in the transmembrane domain. $R$-[3H]$m$TFD-MPAB and $R$-[3H]azietomidate are highly selective for their own sites. We used the ability of derivatives of etomidate, propofol, and barbituric acid to inhibit photolabeling to determine their relative affinities for these two classes of sites. Our results begin to explain how such diverse structures can exert the same action on GABA$_A$Rs.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]Muscimol (26 or 36 Ci/mmol) was from PerkinElmer Life Sciences. The detergents $n$-dodecyl $\beta$-d-maltopyranoside and CHAPS were from Anatrace-Affymetrix (Anagrade quality). Soybean asolectin was from Sigma. $R$- and $S$-MPAB (5-allyl-1-methyl-5-phenyl-barbituric acid), $R$-(-), and $S$-(-)$m$TFD-MPAB (5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl)barbituric acid), and [3H] $R$-$m$TFD-MPAB (38 Ci/mmol) were synthesized as described (16). Racemic MPAB and MPPB (5-propyl-1-methyl-5-phenylbarbituric acid) were synthesized by phenylation of diethyl allylmaltpyranoside and CHAPS were from Anatrace-Affymetrix. Endoproteinase Lys-C (EndoLys-C) were from Worthington. Proteinase Glu-C (EndoGlu-C) and endoproteinase Asolectin (EndoAsolectin) were from Calbiochem. 2,6-di-$p$-butylphenol from Acros, and 2,6-di-tert-butyphenol from Chiron. *Saccharomyces aureus* endoproteinase Glu-C (EndoGlu-C) and *Lysobacter enzymogenes* endoproteinase Lys-C (EndoLys-C) were from Worthington and Roche Applied Science, respectively.

**Purification of Human $\alpha_1\beta_3\gamma_2$ GABA$_A$R—$\alpha_1\beta_3\gamma_2$ GABA$_A$Rs with a FLAG epitope at the N terminus of the $\alpha_1$ subunit were expressed in a tetracycline-inducible, stably transfected HEK293S cell line and purified on an anti-FLAG affinity resin with modifications of procedures used to purify a previously characterized tetracycline-inducible FLAG-$\alpha_1\beta_3$ GABA$_A$R (15, 20). Membranes harvested from 60 15-cm plates (4–8 nmol of [3H]muscimol-binding sites) were solubilized in 30 mm $n$-dodecyl $\beta$-d-maltopyranoside (instead of 2.5 mm) for 2.5 h at 4 °C, and the wash and elution buffers contained 5 mm CHAPS, 0.2 mm asolectin, or 10 mm CHAPS, 0.86 mm asolectin (instead of 13 mm cholate, 0.86 mm asolectin). Aliquots from pooled elution fractions were characterized for [3H]muscimol-binding sites and modulation by etomidate. Individual preparations, starting from membranes containing 2–4 nmol of [3H]muscimol-binding sites (15–20 pmol/mg of protein), typically resulted in 0.5–1.5 nmol of purified receptor (30–60 pmol binding sites) in 15–25 ml of elution buffer. Aliquots of purified GABA$_A$Rs were frozen and stored at −80 °C.

**Radioligand Binding Assays**—[3H]Muscimol binding to purified GABA$_A$Rs was measured by filtration after precipitation with polyethylene glycol (14). The total concentration of sites was determined at 500 nM [3H]muscimol and with 1 mM GABA to determine nonspecific binding. Anesthetic modulation of 2–3 nM [3H]muscimol binding was measured as described (15, 20), except that samples were incubated for 60 min at room temperature before addition of polyethylene glycol and γ-globulins and then filtered after a 30-min incubation at room temperature. The modulation results are presented as the percentage of the specifically bound [3H]muscimol over that without modulators.

**GABA$_A$R Photolabeling**—Purified GABA$_A$Rs in elution buffer was photolabeled on an analytical scale (40–80–μl aliquots containing ~3 pmol of [3H]muscimol sites) to characterize photolabeling at the subunit level and to quantify the effects of nonradioactive anesthetics (or agonist) on photolabeling. To identify photolabeled amino acids, GABA$_A$Rs were photolabeled on a preparative scale (1.5–2.5–ml aliquots containing ~90 pmol of [3H]muscimol sites). Appropriate amounts of $R$-[3H]$m$TFD-MPAB or $R$-[3H]azietomidate were transferred to glass tubes, and solvent (methanol) was evaporated under an argon stream. Freshly thawed GABA$_A$Rs in elution buffer was added to the tube, and the radioligand was resuspended at 4 °C with gentle vortexing for 30 min to a final concentration of 0.5–1 μM (~1 μCi per analytical sample and 25–45 μCi per preparative sample). Drugs of interest were added to the aliquots, and samples were incubated for 30 min. Samples were then placed in the wells of a 96-well plastic microtiter plate (analytical photolabeling) or in a plastic 3.5-cm Petri dish (preparative photolabeling) and irradiated on ice for 30 min with a 365-nm lamp (Spectroline 280L) at a distance of ~1 cm. Sam-
For conditions when an anesthetic only inhibited GABA_A R photolabeling, the data were fit to a single site model for competitive inhibition, as shown in Equation 1,

\[ f_I(x) = \left( f_0 - f_{\text{n}} \right) / \left( 1 + x/IC_{50} \right) + f_{\text{n}} \]  

(Eq. 1)

where \( f_I(x) \) is the \(^3\)H counts/min incorporated in a subunit at anesthetic concentration \( x \); \( f_0 \) is the subunit counts/min in the absence of inhibitor; \( f_{\text{n}} \) is the nonspecific subunit photolabeling, and \( IC_{50} \) is the total drug concentration reducing photolabeling by 50%. When a drug only enhanced photolabeling, data were fit to Equation 2,

\[ f_2(x) = \left( f_{\text{max}} - f_0 \right) / \left( 1 + EC_{50}/x \right) + f_2 \]  

(Eq. 2)

where \( f_2(x) \) is the counts/min incorporated at drug concentration \( x \); \( f_{\text{max}} \) is the maximal level of photolabeling in counts/min; \( f_0 \) is the subunit photolabeling in counts/min in the absence of drug, and \( EC_{50} \) is the total drug concentration producing 50% of maximal enhancement. If an anesthetic at low concentrations produced an enhancement of photolabeling and then inhibition at high concentrations, data were fit to a model assuming anesthetic binding to independent potentiating and inhibitory sites (Equation 3),

\[ f_3(x) = \left( f_2(x) - f_{\text{n}} \right) / \left( 1 + x/IC_{50} \right) + f_{\text{n}} \]  

(Eq. 3)

**Chemical and Enzymatic Fragmentation**—Aliquots isolated from gel bands enriched in either \( \alpha 1 \) or \( \beta 3 \) subunits were digested at 20°C with either 100 µg of endoproteinase Glu-C (EndoGlu-C, Worthington) for 2 days or 0.5 units of endoproteinase Lys-C (EndoLys-C, Roche Applied Science) for 2 weeks. For chemical cleavage at the C terminus of methionines, samples immobilized on PVDF sequencing filters were treated with cyanogen bromide as described (21, 22). For chemical cleavage at the C terminus of tryptophans, samples on PVDF filters were treated with BNPS-skatole as described (23), except that after precipitation of the excess BNPS-skatole, the digestion solution was loaded onto a second PVDF filter, and material on the two filters was sequenced simultaneously (15).

**HPLC Purification and Protein Microsequencing**—Reversed-phase HPLC was performed as described (24) on an Agilent 1100 binary pump system using a Brownlee Aquapore BU-300 column. Samples were eluted at 0.2 ml/min with increasing concentrations of 60% isopropanol, 40% acetonitrile, 0.05% TFA. Elution of peptides was monitored by the absorbance at 215 nm and by liquid scintillation counting of a 10% aliquot of each 0.5-ml fraction.

Samples were sequenced on an Applied Biosystems Procise 492 protein sequencer modified to collect two-thirds of each cycle for PTH-derivative detection/quantification and one-third for \(^3\)H determination by liquid scintillation counting. For direct sequencing of intact subunits or subunit digests containing SDS, samples were loaded onto Prosort PVDF filters (Applied Biosystems) following the manufacturer’s instructions. HPLC fractions for sequence analysis were drop-loaded at 45°C onto TFA-treated glass fiber filters that were then treated with Biobrene™. For selected samples, the sequencer was paused after the designated cycles, and the sample filter was treated with o-phthalaldehyde (OPA) before resuming.
sequencing as follows: (i) to block all free N termini before treatment of the filter with cyanogen bromide or (ii) to chemically isolate for further sequencing only those fragments containing a proline in the designated cycle. OPA reacts with primary amines, but not with secondary amines, and treatment with OPA prevents further sequencing of fragments not containing a proline at that cycle, thereby confirming that any subsequent peak of $^3\text{H}$ release originated from the proline-containing peptide (25, 26). PTH-derivatives were quantitated by peak areas in counts per min/pmol (cpm/pmol) was calculated by applying Equation 5, in which $E(x) = 2 \times (\text{cpm}_x - \text{cpm}_{x-1})/I_0 \times R^x$ (Eq. 5), where $\text{cpm}_x$ is the counts/min in cycle $x$.

### Molecular Modeling

Comparison of structural models for $\alpha_1\beta_3$ GABA$_A$-R constructed by homology with GLIC and GluCl (15) established that the positions of amino acids of BM3/αM1 and αM3/βM1 contributing to the $\beta^+\alpha^-$ and $\alpha^+\beta^-$ interfaces, respectively, were the same. Compared with the GLIC-derivated structure, there was an increased distance in the GluCl structure between the M3 and M1 helices where the allosteric potentiator ivemectin is bound in GluCl. Because etomidate could be docked within the more constrained intersubunit pocket of the GLIC-derived model, we constructed a $\beta_3\alpha_1\beta_3\alpha_1\gamma_2$ GABA$_A$-R homology model based on a GLIC structure (Protein Data Bank code 3P50) as described (15), with the exception that the human $\gamma_2$ subunit sequence replaced the third $\beta_3$ subunit in place of $\beta_3\alpha_1\beta_3\alpha_1$ (Fig. 15). In the GLIC structure, Tyr-263, the M3 residue homologous to GABA$_A$-R, was positioned $3.2\text{Å}$ from the diazirine as determined by a $180^\circ$ rotation of the phenyl group around the C5-phenyl bond. The Connolly surface, determined by a probe of radius 1.4 Å, for the 300 solutions defined a volume of $353\text{Å}^3$. The lowest energy solution was positioned with the diazirine carbon within $4.5\text{Å}$ from the photolabeled residues $\beta_3\text{Met}-227$ and $\alpha_1\text{Tyr}-294$, the phenyl ring stacked with $\alpha_1\text{Tyr}-294$, the N-methyl of barbituric acid within $4\text{Å}$ of $\alpha_1\text{Ser}-270$ ($\alpha_1\text{M2}^\gamma-15\gamma^\gamma$) and $\alpha_1\text{Tyr}-294$, and the C5 allyl within $4\text{Å}$ of $\beta_3\text{Ile}-264$ ($\beta_3\text{M2}^-14\gamma^\gamma$). CDOCKER interaction energies overlapped for the two orientations, with the lowest 15 solutions differing by 2 kcal/mol and all 300 solutions differing by 10 kcal/mol.

At the $\gamma^\gamma-\beta^\gamma$ interface, $R-\text{mTFD-MPAB}$ was docked in two orientations with overlapping CDOCKER interaction energies that differed by $<5\text{ kcal/mol}$. For 77 of 200 solutions (volume of 329 $\text{Å}^3$), $R-\text{mTFD-MPAB}$ was docked at the $\alpha^+\beta^-$ interface, with diazirine carbon within $5\text{Å}$ of $\beta_3\text{Met}-227$ and the NH of barbituric acid $\sim3\text{Å}$ from $\gamma_2\text{Ser}-280$ ($\gamma_2\text{M2}^\gamma-15\gamma^\gamma$). In the second solution, $R-\text{mTFD-MPAB}$ was oriented vertically within the interface, with the diazirine pointing up, $\sim5\text{Å}$ from $\gamma_2\text{Ser}-280$ and $\gamma_2\text{Ser}-301$, but 8 Å from $\beta_3\text{Met}-227$. The NH of barbituric acid was pointing down, 4 Å from $\gamma_2\text{Phe}-304$.

### RESULTS

**Photolabeling $\alpha_1\beta_3$ and $\alpha_1\beta_3\gamma_2$ GABA$_A$-Rs with $R-[^3\text{H}]\text{Azietomidate and } R-[^3\text{H}]\text{mTFD-MPAB}$—**The $\text{FLAG-}\alpha_1\beta_3\gamma_2$ GABA$_A$-R purified in asolectin/CHAPS contained $\gamma_2$ subunits, as evidenced by the ratio of $[^3\text{H}]\text{muscimol}$ to $[^3\text{H}]\text{flunitrazepam}$-binding sites (1.2 ± 0.6). Energetic coupling was preserved between anesthetic sites in the transmembrane domain and the agonist site in the extracellular domain. $R$-Etomidate and barbiturates (pentobarbital or $R$- or $S$-mTFD-MPAB) potentiated $[^3\text{H}]\text{muscimol}$ binding to the same extent (Table 1).

To begin characterizing anesthetic-binding sites in the $\alpha_1\beta_3\gamma_2$ GABA$_A$-R, we photolabeled samples with $R-[^3\text{H}]\text{azietomidate}$ or $R-[^3\text{H}]\text{mTFD-MPAB}$ (Fig. 2) at anesthetic concentrations and compared the patterns of subunit photolabeling to those seen for the $\alpha_1\beta_3$ GABA$_A$-R (15, 16). When GABA$_A$-R subunits were resolved by SDS-PAGE after photolabeling, the two preparations appeared essentially the same based upon Coomassie Blue stain, with three bands migrating at $\sim56$, $\sim59$, and $\sim61\text{ kDa}$ (Fig. 2A). For the $\alpha_1\beta_3$ GABA$_A$-R, the N-terminal

| Compound        | Concentration | $R$     | $S$     |
|-----------------|---------------|---------|---------|
| mTFD-MPAB       | 100           | 179 ± 19% | 166 ± 8% |
| (+/−)-Pentobarbital | 1,000       | 170 ± 24% | 24% |
| Etomidate       | 10            | 174 ± 29% | 29% |

**Table 1: The enhancement by general anesthetics of $[^3\text{H}]\text{muscimol}$ binding to purified $\alpha_1\beta_3\gamma_2$ GABA$_A$-R**

Anesthetic modulation was calculated as the ratio (%) of specific $[^3\text{H}]\text{muscimol}$ binding (2 min) in the presence versus the absence of modulator. The results are the means ± S.D. from three independent experiments.
sequence analyses had established that the ~56-kDa band contained the FLAG-tagged α1 subunit, whereas the 59- and 61-kDa bands contained β3 subunits differing in their glycosylation patterns, with β3 subunit in the α1 band and α1 subunit in the β3 bands at ~15% levels (15). N-terminal sequencing of material eluted from each band of the 56- and 61-kDa bands contained primarily the α1 subunit, and the 59- and 61-kDa bands contained primarily the β3 subunit, although the γ2 subunit (beginning at Lys-2) was distributed in all three bands. When the subunit incorporation of R-[3H]azietomidate or R-[3H]mTFD-MPAB was monitored by fluorography (Fig. 2B), for α1β3 and α1β3γ2 GABA_A Rs, R-[3H]azietomidate and R-[3H]mTFD-MPAB were incorporated primarily, but not exclusively, into the 56- and 59-kDa bands, respectively. For R-[3H]azietomidate-photolabeled α1β3γ2 GABA_A R, sequence analysis of subunit fragments isolated from digests enriched in α1 or β3 subunits identified etomidate-inhibitable photolabeling of the same amino acids (α1Met-236 in αM1 and β3Met-286 in βM3) as in the GABA_A R purified from bovine brain (14) or in the α1β3 GABA_A R (data not shown) (15).

R-[3H]mTFD-MPAB Photolabels an Anesthetic-binding Site Distinct from, but Coupled Energetically to, the Etomidate- and Agonist-binding Sites in the α1β3γ2 GABA_A R—R-mTFD-MPAB acts as a potent tadpole general anesthetic, characterized by an EC_50 of 3.7 μM and as a GABA_A R potentiator at anesthetic concentrations, whereas S-mTFD-MPAB was 10-fold less potent as an anesthetic and potentiated weakly GABA_A R responses (16). In the absence of GABA, R- and S-mTFD-MPAB produced a concentration-dependent inhibition of R-[3H]mTFD-MPAB photolabeling, characterized by IC_50 values of 1.4 ± 0.2 and 34 ± 6 μM, respectively, with high concentrations of either enantiomer inhibiting subunit incorporation by >95% (Fig. 3A). In contrast, R-mTFD-MPAB at concentrations up to 10 μM increased R-[3H]azietomidate photolabeling of the GABA_A R by ~25% (EC_50 = 1.4 μM), with inhibition seen only at higher concentrations (IC_50 = 63 ± 8 μM) (Fig. 3B). S-mTFD-MPAB only inhibited R-[3H]azietomidate photolabeling (IC_50 = 50 ± 12 μM).

In the presence of GABA, R-etomidate at 1 mM inhibited R-[3H]mTFD-MPAB photolabeling by <20%, although it completely inhibited R-[3H]azietomidate photolabeling with an IC_50 of 7 ± 1 μM (Fig. 3, C and D). In the absence of GABA, R-etomidate inhibited R-[3H]azietomidate photolabeling with an IC_50 of 21 ± 1 μM, although it increased R-[3H]mTFD-MPAB photolabeling by ~100%, to the level seen in the presence of GABA. The concentration dependence of enhancement (EC_50 = 9 ± 4 μM) was close to the IC_50 of 20 μM for R-etomidate inhibition of R-[3H]azietomidate photolabeling. In the presence of GABA, R-etomidate at 1 mM inhibited
GABAAR General Anesthetic-binding Sites

FIGURE 3. R-[^3]H]mTFD-MPAB binds to site(s) in the α1β3γ2 GABAAR that are distinct from, but coupled energetically to, the etomidate and GABA-binding sites. GABAARs in the absence (○, □, △) or presence (■) of GABA were photolabeled on an analytical scale with R-[^3]H]mTFD-MPAB (A, C, and E) or R-[^3]H]azietomidate (B, D, and F) in the presence of increasing concentrations of nonradioactive R-mTFD-MPAB ( ○ ) or S-mTFD-MPAB ( △ ) ( — GABA; A and B), R-etomidate (C and D), or pentobarbital (E and F), and ^3^H incorporation into GABAAR subunits was determined by SDS-PAGE and liquid scintillation counting. The concentration dependences of inhibition (IC_{50} ) and potentiation (EC_{50} ) were fit as described under "Experimental Procedures," and the values of IC_{50}/EC_{50} of the plotted lines are included under the "Results." The amounts of R-[^3]H]mTFD-MPAB incorporation in the presence of 1 mM pentobarbital or R-[^3]H]azietomidate incorporation in the presence of 1 mM R-etomidate are indicated by long dashed lines.

R-[^3]H]mTFD-MPAB photolabeling by <15%, which indicated that the affinity of R-etomidate for those binding sites is >100-fold weaker than for the R-[^3]H]azietomidate sites. The GABA enhancement of R-[^3]H]mTFD-MPAB photolabeling (EC_{50} = 50 μM, not shown) established that there was positive allosteric coupling between the GABA-binding sites in the extracellular domain and the R-[^3]H]mTFD-MPAB-binding sites, as seen previously for R-[^3]H]azietomidate sites in brain GABAARs (14). The etomidate potentiation of R-[^3]H]mTFD-MPAB photolabeling and the reciprocal R-mTFD-MPAB potentiation of R-[^3]H]azietomidate photolabeling (in the absence of GABA) established that there was also positive allosteric coupling between those binding sites.

Pentobarbital was ~8-fold more potent as an inhibitor of R-[^3]H]mTFD-MPAB photolabeling than of R-[^3]H]azietomidate photolabeling (Fig. 3, E and F). Pentobarbital, which anesthetizes tadpoles with EC_{50} = 150 μM (27), inhibited R-[^3]H]mTFD-MPAB photolabeling with IC_{50} values of 75 ± 6 and 106 ± 18 μM in the presence and absence of GABA, respectively. In contrast, it inhibited R-[^3]H]azietomidate photolabeling with IC_{50} values of 600 ± 120 and 1,700 ± 230 μM.

R-[^3]H]mTFD-MPAB Photolabels β3Met-227 in β3M1—Because nonradioactive R-mTFD-MPAB was 60-fold more potent as an inhibitor of R-[^3]H]mTFD-MPAB photolabeling than of R-[^3]H]azietomidate photolabeling, the high affinity R-mTFD-MPAB-binding site must be distinct from the R-[^3]H]azietomidate/etomidate-binding site. To identify the labeled residues, α1β3γ2 GABAAR was photolabeled with R-[^3]H]mTFD-MPAB (0.6 μM) on a preparative scale in the absence or presence of 1 mM pentobarbital, and we used previously developed strategies (14, 15) to identify photolabeling within each of the four transmembrane helices of the β3 subunit, the subunit with the highest ^3^H incorporation. When an EndoLys-C digest of material enriched in β3 subunits (eluted from the 59- and 61-kDa gel bands) was fractionated by reversed-phase HPLC (Fig. 4A), all ^3^H was recovered in hydrophobic fractions, consistent with

FIGURE 4. R-[^3]H]mTFD-MPAB photolabels β3Met-227 in the β3M1 transmembrane helix. GABAARs were photolabeled on a preparative scale in the presence of 1 mM GABA with R-[^3]H]mTFD-MPAB (0.6 μM) in the absence (○, □) or presence (■) of 1 mM pentobarbital, and GABAAR subunits were isolated by SDS-PAGE. A, rpHPLC fractionation of EndoLys-C digests of β3 subunits (59–61 kDa gel bands). Fractions 28–29 containing the peak of ^3^H were pooled for sequencing (B). B and C, ^3^H (○, □) and picomoles of PTH-derivatives (△) released during Edman sequencing of β3 subunit fragments beginning at β3Arg-216 (B) and β3His-191 (C). B, primary sequence began at β3Arg-216 (35 pmol, both conditions), and the peak of ^3^H release in cycle 12 indicated photolabeling of β3Met-227 in β3M1 at 980 cpm/pmol (—pentobarbital) and 160 cpm/pmol (+pentobarbital). C, aliquots of β3 subunit from the same preparative labeling were digested with EndoGlu-C and sequenced without fractionation. The sequencing filters were treated with OPA prior to cycle 16, and thereafter, the only sequence detected originally began at β3His-191 (4–5 pmol). The peak of ^3^H release in cycle 37 confirmed R-[^3]H]mTFD-MPAB photolabeling of β3Met-227 at 920 cpm/pmol in the absence and 170 cpm/pmol in the presence of pentobarbital.
photolabeling restricted to the GABA$_R$ transmembrane domain. N-terminal sequencing of the pool of the fractions containing the peak of $^3$H identified a fragment beginning at $\beta$3Arg-216 near the beginning of the M1 helix as the primary sequence, with a peak of $^3$H release in cycle 12 consistent with photolabeling of $\beta$3Met-227 (Fig. 4B). Based upon the amounts of $^3$H and $\beta$3Met-227 released at that cycle, $\beta$3Met-227 was photolabeled at a calculated efficiency of 980 cpm/pmol ($\sim$3% of $\beta$3 labeled), and photolabeling was inhibited by $>80\%$ in the presence of pentobarbital.

Because the sequenced samples also contained a fragment beginning at $\beta$3Ala-280, before $\beta$M3, at $\sim$15% the level of the primary sequence, we used an alternative sequencing strategy to confirm the pentobarbital-inhibitable photolabeling of $\beta$3Met-227. With $\beta$3Met-227 positioned in the subunit primary structure 37 amino acids after $\beta$3Glu-190 and a proline ($\beta$3Pro-206) in between, we took advantage of the fact that OPA, which reacts with primary amines but not proline, a secondary amine, can be used to prevent further Edman degradation of any peptide not containing a proline at the cycle of treatment (25, 26). When an EndoGlu-C digest of material enriched in photolabeled $\beta$3 subunit was sequenced, after treatment with OPA at cycle 16, the only sequence remaining began originally at $\beta$3His-191. The observed peak of $^3$H release in cycle 37 confirmed that $\beta$3Met-227 was photolabeled at 920 cpm/pmol and that 1 mM pentobarbital reduced its labeling by $\sim$80% to 170 cpm/pmol (Fig. 4C).

**FIGURE 5.** $R^-[3H]mTFD-MPAB$ photolabels $\alpha$1Ala-291, $\alpha$1Tyr-294, and $\gamma$2Ser-301 in the $\alpha$1 and $\gamma$2 M3 transmembrane helices. $A, {^3}H(●)$ and picomoles of PTH-derivatives (□) released during Edman sequencing of a GABA$_R$ subunit fragment beginning at $\alpha$1Asp-287 (7 pmol). The peaks of $^3$H release in cycles 5 and 8 indicated photolabeling of $\alpha$1Ala-291 and $\alpha$1Tyr-294. For this sequencing experiment, material isolated by rpHPLC from an EndoGlu-C digest of $\alpha$1 subunit (B, fractions 25–27) was sequenced for four cycles, establishing that the primary sequence began at $\alpha$1Ser-251 before $\alpha$1M2, a fragment predicted to extend to $\alpha$1Glu-313 near the C terminus of $\alpha$1M3 (C). After cycle 4, the sample was treated with OPA to block all free N termini, which was confirmed by five more cycles of Edman degradation, and then treated with cyanogen bromide to cleave at methionines before sequencing for 15 additional cycles. $D, {^3}H(●)$ and picomoles of PTH-derivatives (□) released during Edman sequencing of a GABA$_R$ subunit fragment beginning at $\gamma$2Asp-297 (0.6 pmol). The peak of $^3$H release in cycle 5 indicated labeling of $\gamma$2Ser-301. Material isolated by rpHPLC from an EndoGlu-C digest of 59–61 kDa gel bands (E, fractions 28–29) was sequenced for 10 cycles, establishing the presence of the fragment beginning at $\gamma$2Val-212 (F) as a secondary sequence along with the primary sequence beginning at $\beta$3His-191. The sample was treated with OPA after cycle 10 to block all free N termini, sequenced an additional 5 cycles to confirm block, then treated with cyanogen bromide, and sequenced for an additional 15 cycles. The efficiencies of photolabeling of the residues are tabulated in Table 5.

$R^-[3H]mTFD-MPAB$ Photolabels $\alpha$1Ala-291 and $\alpha$1Tyr-294 in $\alpha$1M3—Although the amino acids photolabeled by $R^-[3H]$azetomidate ($\beta$3Met-286 in $\beta$M3 and $\alpha$1Met-236 in $\alpha$M1) are located in the GABA$_R$ structure at the $\beta^+\alpha^-$ subunit interfaces (15), the amino acid photolabeled by $R^-[3H]mTFD-MPAB$, $\beta$3Met-227, is located within the $\beta$3M1 helix at the $\alpha^+\beta^-$ and $\gamma^+\beta^-$ subunit interfaces in proximity to amino acids from $\alpha$1M2/M3 or $\gamma$2M2/M3. To determine whether there was also photolabeling of amino acids in $\alpha$1M3, we devised a strategy to sequence a fragment beginning at $\alpha$1Asp-287 at the $\alpha$3 N terminus that entailed the rpHPLC fractionation of an EndoGlu-C digest of material enriched in $\alpha$1 subunits, the use of OPA, and digestion with cyanogen bromide to cleave after methionines (Fig. 5, A–C). When the fragment beginning at $\alpha$1Asp-287 was sequenced (Fig. 5A), the peaks of $^3$H release in cycles 5 and 8 indicated $R^-[3H]mTFD-MPAB$ photolabeling of $\alpha$1Ala-291 and $\alpha$1Tyr-294 at photolabeling efficiencies of $\sim$50 cpm/pmol. This identification was confirmed by sequencing for 50 cycles a fragment beginning at $\alpha$1Ser-251 before $\alpha$1M2, produced by digestion with EndoGlu-C, with OPA treatments prior to cycles 3 and 28 corresponding to $\alpha$1Pro-253 and $\alpha$1Pro-278. Peaks of $^3$H release in cycles 41 and 44 confirmed photolabeling of $\alpha$1Ala-291 ($\sim$90 cpm/pmol) and $\alpha$1Tyr-294 ($\sim$60 cpm/pmol), and 1 mM pentobarbital inhibited incorporation into both residues by $>80\%$ (data not shown).
GABA_A R General Anesthetic-binding Sites

R-[3H]mTFD-MPAB Photolabels γ2Ser-301 in γ2M3—To characterize photolabeling in γ2M3, we sequenced the fragment beginning at γ2Asp-297 by use of a protocol similar to that used to sequence the homologous α1Asp-287 fragment (Fig. 5, D–F). Material recovered from an rpHPLC fractionation of an EndoGlu-C digest of labeled subunits was sequenced, N-terminally blocked, treated with cyanogen bromide, and resequenced. To maximize the amount of γ2 and minimize the amount of α1 subunit, material was used from the β subunit gel bands that contain more γ2 than α1 subunit. Rather than use the rpHPLC fractions where the α1Ser-251 fragment had eluted (Fig. 5B), we used fractions eluting at higher organic solvent that contained the peak of 3H (from photolabeled β3Met-227 in the β3His-191 fragment) and the γ2Val-212 fragment that begins before γ2M1 and extends through M3 (Fig. 5, E and F). When that material was sequenced after cyanogen bromide digestion (Fig. 5D), the fragment beginning at γ2Asp-296 was present as a secondary sequence, with the primary sequence beginning at β3Pro-228 and no detectable α1 subunit sequences. There was a peak of 3H release in cycle 5, the cycle that contained β3Ile-232 from the primary sequence and γ2Ser-301 from the secondary sequence. Because there was no evidence of photolabeling of β3Ile-232 (Fig. 4B, cycle 17), the peak of 3H release in cycle 5 indicated R-[3H]mTFD-MPAB photolabeling of γ2Ser-301, the amino acid in γM3 homologous to α1Ala-291. We confirmed this identification by using a protocol that took advantage of the unique distributions of Trp and Pro in the three subunits in the M2–M3 region to chemically isolate γM3 during sequencing. When labeled subunits from gel bands enriched in either α1 or β3 were treated with BNPS-skatole to cleave at tryptophans and sequenced for 50 cycles with OPA treatment at cycle 7, a peak of 3H release was seen in cycle 45 that confirmed R-[3H]mTFD-MPAB photolabeling of γ2Ser-301 at ~100 cpm/pmol (data not shown).

R-[3H]mTFD-MPAB Photolabeling in Other Transmembrane Helices—By sequencing appropriate rpHPLC fractions from EndoLys-C digests of β3 subunits (14), we found that within β3M3 R-[3H]mTFD-MPAB photolabeled β3Met-286, the amino acid photolabeled by R-[3H]azietomidate, and β3Phe-289. However, those residues were photolabeled at ~20 cpm/pmol, i.e. ~2% the efficiency of photolabeling of β3Met-227 from the same photolabeling experiment. Any photolabeling within α1M1, if it occurred, was at <3% the efficiency of β3Met-227.

The sequencing protocols used to characterize photolabeling in α1M3 and γ2M3 (Fig. 5) involved sequencing through α1M2, γ2M2, and β3M2, and any photolabeling within the M2 helices, if it occurred, was at <3% the efficiency of β3Met-227. Sequence analyses of fragments beginning at β3Ile-414 before β3Met and αThr-377 before αM4 that were isolated by rpHPLC from proteolytic digests of β3-enzriched material established that photolabeling, if it occurred, within β3M4 was at <0.3% and within α1M4 at <1% the efficiency of labeling of β3Met-227.

R-[3H]mTFD-MPAB Binds to Sites at the α+-β- and γ+-β- Subunit Interfaces Equivalent to the Etomidate-binding Site at the β-α Interface—The high degree of amino acid sequence conservation between the GABA_A R M1–M4 helices and those of GLIC or GluCl allows simple and consistent alignment of those GABA_A R regions in homology models based upon GLIC or GluCl (15). In an α1βγ2 GABA_A R homology model based upon the structure of GLIC (Fig. 6), the residues photolabeled by R-[3H]mTFD-MPAB are located in two different subunit interfaces (α+-β- and γ+-β-) (Fig. 6C). In the α+-β- interface, β3Met-227 in the M1 helix is opposite both α1Ala-291 and α1Tyr-294 in the M3 helix and located between them on an axis perpendicular to the membrane, whereas in the γ+-β- interface it is opposite γ2Ser-301 in M3 and slightly below it. In both cases there is a pocket between the subunits that is large enough to accommodate R-mTFD-MPAB (volume of 275 Å3). Shown in Fig. 6, D–F, are expanded views of these binding sites with R-mTFD-MPAB docked in the lowest energy orientation predicted by computational docking. R-mTFD-MPAB was predicted to bind with its reactive diazirine positioned in close proximity to the photolabeled amino acids in β3M1 and α1M3 or γ2M3, the NCH3 group of barbituric acid oriented toward α1M2–15’ or γ2M2–15’ (α1Ser-270/γ2Ser-280), and the C5 allyl group oriented toward β3M2–10’ and β3M2–14’ (β3Thr-260/β3Ile-264). β3Pro-228 in β3M1 is predicted to be a major determinant of the shape of this binding pocket, as noted previously for the homologous proline in α1M1 (α1Pro-233) in the etomidate-binding site at the β+-α- interface (15).

The binding sites for R-mTFD-MPAB at the α+-β- and γ+-β- subunit interfaces are homologous to the etomidate-binding site at the β+-α- subunit interface identified by photolabeling with R-[3H]azietomidate and R-[3H]TDBzl-etomidate (15), which is shown in Fig. 6G with R-azietomidate docked in its predicted lowest energy orientation. The alignment of α1, β3, and γ2 M1–M3 transmembrane helices (45% identity, Fig. 6, bottom) illustrates that α1Ala-291 and γ2Ser-301 occupy the same positions in the R-mTFD-MPAB-binding sites as β3Met-286 (labeled by R-azietomidate) in the etomidate-binding sites. Similarly, β3Met-227 occupies the same position in the R-mTFD-MPAB-binding sites as αLeu-232 does in the etomidate sites, 4 amino acids or one helical turn above α1Met-236 (also labeled by R-azietomidate). Thus, the R-mTFD-MPAB and R-etomidate binding sites are at different subunit interfaces, but they are located at the same depth in the transmembrane domain.

In addition to the four intersubunit-binding sites identified by R-[3H]azietomidate and R-[3H]mTFD-MPAB, there is a fifth potential site in the transmembrane domain at the α+-γ- subunit interface, the same interface that in the extracellular domain contains the benzodiazepine site. This site may be photolabeled by R-[3H]mTFD-MPAB, because the residues it photolabeled in αM3 at the α+-β- interface are present in the second α subunit at the α+-γ- interface. We have not yet been able to characterize photolabeling in γM1, which is necessary to determine whether this fifth intersubunit site is also photolabeled.

Selectivities of Etomidates and Barbiturates for Intersubunit-binding Sites—To determine whether the >50-fold selectivity of R-mTFD-MPAB and ~10-fold selectivity of pentobarbital for the anesthetic-binding sites at α+/γ+-β- interfaces and the >100-fold selectivity of R-etomidate for the sites at the β+-α- interfaces were general properties of barbiturates and etomidates, we screened other etomidates (Table 2) and barbi-
to the sites at the midate and anesthetics, because IC50 values were determined from total investigative comparisons can be made of the potencies of different anesthetics (Table 3) as inhibitors of $\alpha^+\beta^-$ and $\gamma^+\beta^-$ interfaces that are homologous to the etomidate-binding sites at the $\beta^+\alpha^-$ interfaces. A, side view of an $\alpha_1\beta_3\gamma_2$ GABA$_A$R homology model built using a GLIC crystal structure (Protein Data Bank code 3P50), with $\alpha$-helices displayed as cylinders, $\beta$-sheets as ribbons, and subunits color-coded as follows: $\alpha_1$, light yellow; $\beta_3$, light blue, and $\gamma_2$, light green. B and C, views down the intracellular channel of the GABA$_A$R extracellular ($B$) and transmembrane ($C$) domains. A–C, locations are indicated of the pockets containing the binding sites for GABA (green), benzodiazepine (blue), etomidate (brown), and $R$-mTDF-MPAB (red). D and E, views of $R$-mTDF-MPAB docked in the pocket at the $\alpha^+\beta^-$ interface, viewed from the lipid ($D$) and from the base of the extracellular domain ($E$). F and G, views of the lipid of $R$-mTDF-MPAB docked at the $\gamma^+\beta^-$ interface ($F$) and $R$-azietomidate docked at the $\beta^+\alpha^-$ interface ($G$). D–G, docked anesthetic is shown in stick format in its lowest energy orientation, color-coded by element (carbon, gray; oxygen, red; nitrogen, blue; and fluorine, light blue) within the Connolly surface representation of the volumes defined by the ensemble of the 100 lowest energy-minimized docking solutions. Residues photolabeled by $R^{{[3H]}}$mTDF-MPAB are shown in stick format and color-coded as follows: $\beta_3$Met-27, red; $\alpha_1$L3-291, magenta; $\alpha_1$Tyr-294, purple; $\gamma_2$Ser-301, orange; $\beta_3$Met-286, cyan, and $\beta_3$Phe-289, yellow-green. Residues photolabeled by $R^{{[3H]}}$azietomidate/$^{{[3H]}}$TDFBzl-etomidate (G only) are color-coded as follows: $\beta_3$Met-286, cyan; $\beta_3$Val-290, dark green; and $\alpha_1$Met-236, lime green. Also color-coded in G are $\beta_3$Asn-265 (brown, M2–15’), the in vivo etomidate/propofol/pentobarbital sensitivity determinant (5, 6) and $\beta_3$Phe-301 (blue), the residue photolabeled by an anesthetic steroid in a homopentameric $\beta_3$ GABA$_A$R (42). The color-coded residues of D–G are also highlighted in the aligned GABA$_A$R subunit sequences spanning the M1–M3 helices (bottom of figure).

GABA$_A$R General Anesthetic-binding Sites

Similar to pentobarbital, phenobarbital bound with $\sim$10-fold selectivity to the binding sites at $\alpha^+\gamma^+\beta^-$ interfaces, but addition of bulk to the ring, as in thiopental, reduced the selectivity to only $\sim$1.6-fold, and brallobarbital bound with $\sim$3-fold higher affinity to the “etomidate”-binding site. In contrast to the $\sim$60-fold binding selectivity of $R$-mTDF-MPAB, $S$-mTDF-MPAB bound with lower affinity and nonselectively to both classes of sites. We also examined the effects of stereoisomers of MPPB, as $R$-MPPB acts as an anesthetic and GABA$_A$R potentiator, although $S$-MPPB acts as a convulsant and GABA$_A$R inhibitor (28, 29). The anesthetic isomer bound with 9-fold higher affinity to the sites at the $\alpha^+\gamma^+\beta^-$ interfaces than at the $\beta^+\alpha^-$ interfaces, and similarly to $R$- and $S$-mTDF-MPAB, the difference between $R$- and $S$-MPPB was the decreased affinity of $S$-MPPB for the sites at the $\alpha^+\gamma^+\beta^-$ interfaces.

For the barbiturates studied, $R$-mTDF-MPAB possessed the highest affinity and selectivity for the $\alpha^+\gamma^-\beta^-$ sites. Comparison with $R$-MPAB indicates that the $m$-TDF substituent of $R$-mTDF-MPAB is important for both site selectivity and bind-
**TABLE 2**
Affinities of etomidates for GABA<sub>A</sub>R anesthetic-binding sites at the β<sup>+</sup>–α<sup>−</sup> (R<sup>2</sup>Hzetomidate) and α<sup>+</sup>/γ<sup>−</sup>–β<sup>−</sup> (R<sup>3</sup>HmTFD-MPAB) subunit interfaces<sup>a</sup>

| Drug          | GABA | R-[3H]Azetomidate | Ratio IC<sub>50</sub>| EC<sub>50</sub> Anesthesia | Partition Coefficient |
|---------------|------|-------------------|---------------------|--------------------------|----------------------|
|               | IC<sub>50</sub> (μM) | IC<sub>50</sub> (μM) | IC<sub>50</sub>(R<sup>2</sup>TFD-MPAB) | (μM) |                     |
| R-etiometate   | +    | 11 ± 3            | >1,000              | <0.01                    | 300<sup>b</sup>       |
| S-etiometate   | +    | 135 ± 9           | >1,000              | <0.14                    | 300<sup>b</sup>       |
| R-azetomidate  | +    | 3 ± 0.3           | 200 ± 50            | 0.50                     | 2.2<sup>b</sup>       |
| R-TBzI-etiometate | +  | 1.8 ± 0.2         | 50 ± 20             | 0.04                     | 0.7<sup>b</sup>       |
| S-PeTFD-etiometate | + | 125 ± 20          | 46 ± 7              | 3.0                      | 4.9<sup>d</sup>       |
| R-P<sub>2</sub>-etiometate | + | 105 ± 20          | 53 ± 9              | 2.0                      | 17<sup>e</sup>        |

<sup>a</sup> IC<sub>50</sub> values, the total anesthetic concentrations resulting in 50% inhibition of GABA<sub>A</sub>R photolabeling, were determined as described under "Experimental Procedures"; EC<sub>50</sub> for anesthesia indicates tadpole loss of righting reflex.

<sup>b</sup> See Ref. 17.

<sup>c</sup> See Ref. 18.

<sup>d</sup> See Ref. 19.

**TABLE 3**
Affinities of barbiturates for GABA<sub>A</sub>R anesthetic-binding sites at the β<sup>+</sup>–α<sup>−</sup> (R<sup>2</sup>Hzetomidate) and α<sup>+</sup>/γ<sup>−</sup>–β<sup>−</sup> (R<sup>3</sup>HmTFD-MPAB) subunit interfaces<sup>a</sup>

| Drug          | GABA | R-[3H]Azetomidate | Ratio IC<sub>50</sub>| EC<sub>50</sub> Anesthesia | Partition Coefficient |
|---------------|------|-------------------|---------------------|--------------------------|----------------------|
|               | IC<sub>50</sub> (μM) | IC<sub>50</sub> (μM) | IC<sub>50</sub>(R<sup>2</sup>TFD-MPAB) | (μM) |                     |
| R-mTFD-MPAB   | –    | 63 ± 8            | 1.4 ± 0.2           | 62                       | 3.7<sup>d</sup>       |
| R-mTFD-MPAB   | +    | 76 ± 14           | 1.3 ± 0.1           | 58                       | 3.7<sup>d</sup>       |
| S-mTFD-MPAB   | –    | 50 ± 12           | 34 ± 6              | 1.5                      | 38<sup>b</sup>        |
| pentobarbital | +    | 600 ± 120         | 75 ± 6              | 8                        | 160<sup>c</sup>       |
| thiopental    | +    | 169 ± 16          | 106 ± 18            | 1.6                      | 30<sup>c</sup>        |
| phenobarbital | +    | 5,060 ± 650       | 390 ± 70            | 13                       | 330<sup>d</sup>       |
| thiobarbital  | +    | 220 ± 30          | 790 ± 60            | 0.3                      | ND<sup>c</sup>        |
| R-MPAB        | +    | 310 ± 30          | 28 ± 3              | 10                       | ND<sup>c</sup>        |
| R-mBr-MPAB    | +    | 64 ± 4            | 5 ± 0.5             | 13                       | 330<sup>d</sup>       |
| MPPB          | +    | 570 ± 80          | 61 ± 4              | 9.3                      | anesthetic<sup>c</sup>|
| S-MPPB        | +    | 610 ± 50          | 360 ± 31            | 1.7                      | convulsant<sup>c</sup>|

<sup>a</sup> IC<sub>50</sub> values, the total anesthetic concentrations resulting in 50% inhibition of GABA<sub>A</sub>R photolabeling, were determined as described under "Experimental Procedures"; EC<sub>50</sub> for anesthesia indicates tadpole loss of righting reflex.

<sup>b</sup> See Ref. 16.

<sup>c</sup> See Ref. 27.

<sup>d</sup> Data were calculated with ALOPS 2.1 program, VCCLAB, Virtual Computational Chemistry.

The binding affinity of R-mTFD-MPAB had 30-fold higher affinity than R-MPAB at the α<sup>+</sup>/γ<sup>−</sup>–β<sup>−</sup> sites and only 5-fold higher affinity at the β<sup>−</sup>–α<sup>−</sup> sites. Substitution of mBr (R<sub>2</sub>-mBr-MPAB) increased binding affinity at all interfaces by 5-fold compared with R-MPAB.

**Binding of Propofol and Propofol Analogs to Intersubunit-binding Sites**—Propofol at 300 μM inhibited both R-[3H]mTFD-MPAB (Fig. 7A) and R-[3H]azetomidate (Fig. 7B) photolabeling by >90%, consistent with competitive inhibition at both sites. In the absence or presence of GABA, propofol was ~2–3-
fold more potent as an inhibitor of R-[3H]azietomidate photolabeling. In three photolabeling experiments in the presence of 1 mM pentobarbital or 1 mM TFD-MPAB were 25 ± 13 and 92 ± 46 μM, respectively, with the ratio of IC_{50} [azietomidate]/IC_{50} [TFD-MPAB] for paired experiments equal to 0.6 ± 0.1. In the absence of GABA, the IC_{50} values for R-[3H]azietomidate and R-[3H]mTFD-MPAB were 25 ± 13 and 92 ± 46 μM, respectively, with the ratio of IC_{50} values for paired experiments equal to 0.28 ± 0.03. In the absence of GABA, propofol at low concentrations produced a small enhancement (∼50%) of R-[3H]mTFD-MPAB photolabeling with the concentration dependences of enhancement and inhibition consistent with EC_{50} = 12 μM, the IC_{50} for propofol inhibition of R-[3H]azietomidate photolabeling in the absence of GABA, and IC_{50} = 40 μM, the IC_{50} for R-[3H]mTFD-MPAB inhibition (+GABA) (Fig. 7A, dashed line).

We also determined the inhibition of R-[3H]mTFD-MPAB and R-[3H]azietomidate photolabeling in the presence of GABA by 2,6-di-sec-butylphenol, a propofol analog that is similar in potency to propofol as a GABA_{A}R potentiator and anesthetic (EC_{50} = 2 μM), and 2,6-di-tert-butylphenol, which at 300 μM was inactive as a GABA_{A}R modulator or anesthetic and did not alter responses to propofol (30). 2,6-di-sec-Butylphenol was equipotent as an inhibitor of photolabeling by both photoprobes (IC_{50} = 90 μM), although the inactive isomer, 2,6-di-tert-butylphenol, at 300 μM inhibited photolabeling by <10% (Fig. 7, C and D). Because our experimental IC_{50} values are determined from total, rather than free, drug concentrations and the partition coefficient of 2,6-di-sec-butylphenol (or 2,6-di-tert-butylphenol) is 6-fold greater than that of propofol (Table 4), it is not possible to determine from our data whether 2,6-di-sec-butylphenol is actually more or less potent than propofol. However, differences in hydrophobicity (partition coefficient) cannot account for the capacity of 2,6-di-sec-butylphenol to act as an anesthetic and bind to the GABA_{A}R intersubunit-binding sites, although 2,6-di-tert-butyl phenol neither acts as an anesthetic nor binds to the intersubunit anesthetic-binding sites.

**Interactions of Alphaxalone and Octanol with Intersubunit Anesthetic-binding Sites**—In the presence of GABA, alphaxalone, a synthetic anesthetic steroid, at concentrations up to 30 μM had little or no effect on photolabeling by R-[3H]mTFD-
MPAB (Fig. 7E) or R-[3H]azietomidate (Fig. 7F). In the absence of GABA, alphaxalone increased photolabeling at both sites with EC_{50} values of ~500 nM, similar to the potentiation of R-[3H]azietomidate photolabeling of brain GABA_{A}R by alphaxalone or neurosteroids (31). Alphaxalone binds neither to the R-[3H]azietomidate nor R-[3H]mTFD-MPAB-binding site, which is consistent with early studies demonstrating additive effects of alphaxalone and pentobarbital (32).

Octanol acts as an anesthetic and GABA_{A}R potentiator with EC_{50} values of ~60 μM (33). In the presence of GABA, octanol at 1 mM inhibited R-[3H]mTFD-MPAB (Fig. 7G) photolabeling by 70% and R-[3H]azietomidate (Fig. 7H) photolabeling by 50%. If we assume this inhibition is competitive, analysis yields IC_{50} values of 450 ± 70 and 1,600 ± 400 μM for R-[3H]mTFD-MPAB and R-[3H]azietomidate, respectively. However, in the absence of GABA, octanol at concentrations up to 1 mM had no effect on photolabeling.

Effects of GABA and Etomidate on R-[3H]mTFD-MPAB Photoincorporation at the Amino Acid Level—For R-[3H]azi
tomidate-photolabeled GABA_{A}R purified from bovine brain, the enhancement of photolabeling seen at the subunit level in the presence of GABA or a neurosteroid, as well as the inhibition of photolabeling in the presence of propofol, was also seen at the level of the photolabeled amino acids (14, 31, 34). To determine whether this was also true for the R-[3H]mTFD-MPAB site or whether novel amino acids were photolabeled when subunit photolabeling was enhanced, we characterized photolabeling in β3M1, α1M3, γ2M3, and β3M3 for α1β3γ2 GABA_{A}Rs photolabeled in three conditions as follows: control (no additional drug), +1 mM GABA, or +100 μM etomidate (Table 5). The incorporation at β3Met-277 within β3M1, the residue that accounts for >80% of GABA_{A}R photolabeling, closely paralleled the labeling seen at the subunit level. GABA and etomidate increased photolabeling efficiency by ~50%, and no novel residues were photolabeled in β3M1. The complex sequencing protocols required to identify photolabeling in α1M3 or γ2M3 made quantification more difficult. Qualitatively, GABA increased photolabeling of α1Ala-291, α1Tyr-294, and γ2Ser-301, and no other amino acids were photolabeled. Additional labeling experiments would be necessary to assess the smaller effects of etomidate on those residues.

We also quantified R-[3H]mTFD-MPAB photolabeling of amino acids in the etomidate-binding site (β3Met-286 and β3Phe-289 in the β3M3 helix), which were labeled at ~2% the efficiency of β3Met-277. Etomidate inhibited photolabeling of β3Met-286 and β3Phe-289 by >90%, as expected for the presence of those amino acids in the etomidate-binding site, although it enhanced photolabeling of β3Met-227 in the R-mTFD-MPAB site.

**DISCUSSION**

In this report we provide the first demonstration that there are two structurally related, but pharmacologically distinct, classes of intersubunit general anesthetic-binding sites in the transmembrane domain of human α1β3γ2 GABA_{A}Rs. The binding sites for R-[3H]mTFD-MPAB, a photoreactive barbiturate that acts as a potent, stereoselective GABA_{A}R potentiator and general anesthetic, are located at the α_{-}β^{-} and γ_{+}β^{-} subunit interfaces, centered three helical turns down from the extracellular end of β3M3 (Fig. 6). At anesthetic concentrations, R-mTFD-MPAB does not bind at the previously characterized etomidate-binding sites (14, 15), which are located at the two β_{-}ζ subunit interfaces and are also centered three turns down from the extracellular end of α1M3. Conversely, R-etomidate does not bind at the R-mTFD-MPAB-binding sites. Thus, R-mTFD-MPAB binds to homologous but distinct sites from etomidate and its photoreactive derivatives.

**Pharmacology of the Two Classes of General Anesthetic-binding Sites—R-mTFD-MPAB and R-etomidate**

Each with >50-fold selectivity to their preferred sites, with IC_{50} values similar to the EC_{50} values for GABA_{A}R potentiation in vitro or anesthesia in vivo. Displacing these ligands with nonradioactive anesthetics (see IC_{50} values in Tables 2–4) lead to the conclusion that the two classes of sites are not simply etomidate or "barbiturate" sites. For example, pentobarbital and phenobarbital bound to the α_{-}β^{-} and γ_{+}β^{-} subunit sites with ~10-fold selectivity, whereas thiopental and S-mTFD-MPAB bound with similar affinity to both sites. Furthermore, the barbiturate brallobarbital had an ~3-fold higher preference for the etomidate (β_{-}α^{-}) site, and pTFD-etomidate had 2-fold preference for the barbiturate (α_{+}γ^{-}β^{-}) site. Thus, we refer to these sites by their subunit interface designations. There is precedent for a pharmacological class of anesthetics not binding to isosteric sites in the Cys loop ligand-gated ion channel superfamily. Although some barbiturates that inhibited currents in muscle type nACHRs fully displaced [14C]amobarbital binding, others bound to an unidentified site (35).

Propofol bound with little selectivity at both classes of sites, suggesting it has at least four binding sites. Although the IC_{50} values for R-azietomidate or R-mTFD-MPAB binding are close to anesthetic concentrations, the IC_{50} values for propofol binding to either class of sites (~40 μM) are ~20-fold higher than GABA modulatory or anesthetic concentrations (36). This discrepancy might result if propofol binds with higher affinity to as

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**Table 5**

| Amino acid | Control | +GABA (1 mM) | +Etomidate (100 μM) |
|------------|---------|-------------|---------------------|
| β3M1 Met-227 | 950 ± 30 | 168 ± 5 | 420 ± 60 ± 50 | 660 |
| α1M3 Ala-291 | 92 | <5 | 28 | 41 |
| α1M3 Tyr-294 | 56 | <5 | 36 | 46 |
| γ2M3 Ser-301 | 105 ± 5 | ND | 83 | 130 |
| β3M3 Met-286 | 25 | ND | 7 | 13 |
| β3M3 Phe-289 | 18 | ND | 12 | 10 |

The efficiency of photolabeling of a residue (in cpm/pmol) was calculated using Equation 5 (see under “Experimental Procedures”). The data are presented as mean (± range) when two samples were sequenced. Other values were determined from the sequencing of single samples. ND indicates not determined.
yet unidentified sites in the GABA$_A$R. However, 2,6-di-sec-butyl phenol, which is equipotent with propofol as an anesthetic and GABA$_A$R modulator (30), binds with potency similar to propofol to the two classes of intersubunit anesthetic-binding sites, although 2,6-di-tert-butylphenol, which is inactive as an anesthetic and GABA$_A$R modulator, did not bind to either class of sites (Table 4). These results make it likely that the four intersubunit sites identified by $R$-[$^3$H]azietomidate and $R$-[$^3$H]mTfD-MPAB are the binding sites important for propofol’s anesthetic effects. Interestingly, the potentiation and direct activation by propofol, which has little or no subunit sensitivity determinant positions (41), more at the lipid interface, because an anesthetic steroid photolabeled $\beta_3$Phe-301 (Fig. 6G) in $\beta$M3 in homomeric $\beta$3 GABA$_A$Rs (42).

Anesthetic-binding Sites at $\alpha^+/%y^-/3^- Subunit Interfaces—In the $\alpha$3$\beta$2$\gamma$ homology model (Fig. 6), the amino acids photolabeled by $R$-[$^3$H]mTfD-MPAB are in a pocket formed by residues from $\alpha$1M2/M3 (or $\gamma$2M2/M3) and $\beta$3M2/M1. $R$-mTfD-MPAB is predicted by computational docking to bind with its reactive dizainre in close proximity to the photolabeled residues, the NCH$_2$ of barbituric acid oriented toward $\alpha$M2–15’ or $\gamma$M2–15’, and the C5-allyl oriented toward $\beta$M2–10’/$\beta$M2–14’.

Previous mutational analyses provided evidence that pentobarbital sensitivity determinants were contained within $\beta$M1/$\beta$M2 (43), including $\beta$3Pro–228 (44) that is adjacent to the photolabeled $\beta$3Met–227 in $\beta$M1 and predicted to be a key determinant of the anesthetic binding pocket’s shape (Fig. 6F). Comparison of the amino acids contributing to the $\alpha^+/%y^-/3^-$ and $\beta^+-%/3^-$ binding pockets identifies nonconserved positions likely to contribute to the strong site selectivities of $R$-mTfD-MPAB and $R$-etomidate. Most notable is the difference at position M2–15’, with $\alpha$1Ser–270/$\gamma$2Ser–280 in the $R$-mTfD-MPAB-binding sites and $\beta$3Asn–265 in the $R$-etomidate-binding sites, because the $\beta$3N265S substitution reduces etomidate sensitivity by 10-fold (4). Additional differences in M3 positions can be found in the sequence alignments of Fig. 6.

Because $\beta$M2–15’ is predicted to be an important determinant of the shape of the etomidate-binding site at the $\beta^+-%$ interface (Fig. 6G) (15) and pentobarbital binds with ~8-fold higher selectivity to the $\alpha^+/3^-/3^-$ sites, it is surprising that the anesthetic responses of pentobarbital are reduced in the $\beta$3N265M knock-in mouse (6). This may indicate that the $\beta^+-%$ sites make a greater energetic contribution to the stabilization of GABA$_A$R in the open state. Characterization of the anesthetic effects of $R$-mTfD-MPAB on the $\beta$3N265M GABA$_A$R in vitro and in vivo will clarify whether the substitution prevents transduction of changes initiated by binding to the $\alpha^+/3^-/3^-$ subunit interfaces.

Intrasubunit Sites?—Propofol inhibits the nAChR and the prokaryotic homolog GLIC, and in those proteins it binds to intrasubunit-binding sites within the pocket formed by the transmembrane helix bundle (45, 46). Our studies with $R$-$[^3]$H]mTfD-MPAB (this work) and $R$-[$^3$H]azietomidate and ($[^3]$H)TDF2bl-etomidate (15) provided no evidence of GABA$_A$R intrasubunit-binding sites for those anesthetics, even though we sequenced through each of the $\alpha$ and $\beta$ subunit transmembrane helices. In these peptides, we observed minor labeling of $\beta$3Met–286 and Phe–289 in the $\beta^+-%$ anesthetic-binding site at ~2% the efficiency of $\beta$1Met–227. Thus, we can state that, if any intrasubunit labeling occurred, it must be at levels below this.

Anesthetics and GABA$_A$R Conformational Equilibria—At lower concentrations, most general anesthetics potentiate GABA responses, and at higher concentrations, they directly activate GABA$_A$Rs in the absence of GABA. Direct activation and potentiation of nAChRs and GABA$_A$Rs can be well accounted for by allosteric models that assume that receptors exist in multiple, interconvertible conformational states (47–50). Activators and potentiators shift the conformational equilibria toward the open channel state because they bind with higher affinity to open states than to resting, closed channel states. In purified GABA$_A$R in detergent/lipid micelles, positive energetic coupling between the extracellular and transmembrane domains is preserved as evidenced by anesthetic enhancement of [H]muscimol binding and GABA enhancement of $R$-[H]azietomidate/$R$-[$^3$H]mTfD-MPAB photolabeling. Furthermore, in the absence of GABA, $R$-etomidate enhances $R$-[$^3$H]mTfD-MPAB photolabeling, and reciprocally, $R$-mTfD-MPAB enhances $R$-[H]azietomidate photolabeling. Our studies provide no information about the state-dependent differences in affinity for anesthetics binding at either class of sites. However, smaller differences in binding affinity between open ($K_o$) and closed states ($K_c$) are required for anesthetics binding to four rather than two sites, because the shift in conformational equilibria will be proportional to ($K_o/K_c)^n$, where $n$ is the number of sites.

Because $R$-etomidate does not bind to the $\alpha^+/\%y^-/\%$ sites even at 1 mM, our results provide further evidence that $R$-etomidate directly activates GABA$_A$Rs (17) by binding solely to the $\beta^+-%$ interfaces that also contain the agonist-binding sites in the extracellular domain (14). The selective binding of $R$-mTfD-MPAB to the $\alpha^+/\%y^-/\%-\beta$ subunit interfaces provides the first evidence that potentiation and direct activation (16) can result from anesthetic binding at interfaces not containing the transmitter-binding site.

Conclusions—Our novel finding is that it is possible to synthesize general anesthetics that are selective for sites between
specific subunits in the transmembrane domain of pentameric GABA<sub>δ</sub>Rs. A wide range of general anesthetic structures target these four sites but with variable selectivity, which offers an explanation of the puzzling lack of well defined structure activity relationships among general anesthetics (51–53). These observations suggest that it may be possible to develop agents with novel intersubunit specificity that can be used to target specific nerve pathways and behaviors in a subunit-dependent manner (7). A similar strategy has recently been proposed for the extracellular domain where a potentiator site has been identified at the β<sup>2</sup>–α<sup>2</sup> interface in a pocket equivalent to the transmitter and benzodiazepine sites at the β<sup>2</sup>–α<sup>2</sup>–γ<sup>2</sup> subunit interfaces (13, 54).

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