General Anesthetic Binding Sites in Human α4β3δ γ-Aminobutyric Acid Type A Receptors (GABAₐRs)

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Extrasynaptic γ-aminobutyric acid type A receptors (GABAₐRs), which contribute generalized inhibitory tone to the mammalian brain, are major targets for general anesthetics. To identify anesthetic binding sites in an extrasynaptic GABAₐR, we photolabeled human α3β3δ GABAₐR purified in detergent with [3H]azietomidate and a barbiturate, [3H]R-mTFD-MPAB, photoreactive anesthetics that bind with high selectivity to distinct but homologous intersubunit binding sites in the transmembrane domain of synaptic α1β3γ2 GABAₐRs. Based upon 3H incorporation into receptor subunits resolved by SDS-PAGE, there was etomidate-inhibitable labeling by [3H]azietomidate in the α3 and β3 subunits and barbiturate-inhibitable labeling by [3H]R-mTFD-MPAB in the β3 subunit. These sites did not bind the anesthetic steroid alphalone, which enhanced photolabeling, or DS-2, a δ subunit-selective positive allosteric modulator, which neither enhanced nor inhibited photolabeling. The amino acids labeled by [3H]azietomidate or [3H]R-mTFD-MPAB were identified by N-terminal sequencing of fragments isolated by HPLC fractionation of enzymatically digested subunits. No evidence was found for a δ subunit contribution to an anesthetic binding site. [3H]azietomidate photolabeling of β3Met-286 in βM3 and α3Met-269 in αM1 that was inhibited by etomidate but not by R-mTFD-MPAB established that etomidate binds to a site at the β3⁺-α3⁻ interface equivalent to its site in α1β3γ2 GABAₐRs. [3H]azietomidate and [3H]R-mTFD-MPAB photolabeling of β3Met-227 in βM1 established that these anesthetics also bind to a homologous site, most likely at the β3⁺-β3⁻ interface, which suggests a subunit arrangement of β3α4β3δβ3.

γ-Aminobutyric acid type A receptors (GABAₐRs) are the major inhibitory neurotransmitter receptors in the mammalian brain. They are members of the pentameric ligand-gated ion channel superfamily that consists of five homologous subunits, each of which has a large extracellular domain, a transmembrane domain of four transmembrane helices (M1–M4), and an intracellular domain connecting the third and fourth transmembrane helices. GABAₐRs, which are the target of many drugs, among them benzodiazepines and general anesthetics, are heteropentamers, and drug action often depends on the subunit composition. For example, at synaptic receptors, which commonly have a subunit composition of (α)2(β)2γ, arranged βαβγαγ counterclockwise when viewed from the synaptic or extrasynaptic side of the receptor, benzodiazepines act in the extracellular domain between α⁺-γ⁻ subunits at a site homologous to the GABA binding sites at the two β⁺-α⁻ subunit interfaces (Fig. 1) (1–3).

General anesthetics have long been known to bind to sites in the transmembrane domains of pentameric ligand-gated ion channels (reviewed in Refs. 4–7). Photolabeling of endogenous and heterologous GABAₐRs by [3H]azietomidate located the etomidate binding site in the two β⁺-α⁻ subunit interfaces (8, 9), 50 Å from the GABA site and at a position later shown to overlap with the five ivermectin sites in the crystal structure of the homopentameric glutamate-gated chloride channel (GluCl) (10). More recently, a photoreactive, anesthetic barbiturate, R-mTFD-MPAB, has been shown to bind to sites in the γ⁺-β⁻ and α⁺-β⁻ subunit interfaces homologous to the etomidate binding sites, introducing the concept of subtype-dependent action of general anesthetics (11). Whereas etomidate and R-mTFD-MPAB bind with high selectivity to their sites, propofol, pentobarbital, and other barbiturates bind with much less selectivity to these two classes of sites.

The in vivo mechanism of action of etomidate has been firmly linked to the GABAₐR. Heterologously expressed GABAₐRs that have an N256M mutation on the M2 helix of the β3 subunit (β⁻ surface of the interface) are relatively insensitive to etomidate (12), and sleep times in knock-in mice bearing the same mutation are much shorter than in wild-type mice (13).

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Aziotomidate causes normal anesthesia in wild-type mice with the same potency as etomidate, and its action is similarly attenuated in the knock-in mouse (14). R-mTFD-MPAB also causes general anesthesia in mice and is equally potent in wild-type and N256M knock-in mice (15), consistent with the location of its binding sites at the \( \beta^- \) subunit interfaces.

The contrasting subunit-selective actions of these two agents raise questions about the mechanism of general anesthesia itself, because there are 19 known GABA subunits, and which of the possible combinations occur \textit{in vivo} is not yet fully defined. The state of anesthesia involves many behavioral components (16), so subunit-selective general anesthetics might be associated with specific subsets of the behavioral impairments experienced during anesthesia (17). Of particular interest are the relative contributions of phasic (synaptic) and tonic (extrasynaptic) inhibition actions (18, 19). Expression studies in fibroblasts and oocytes establish that multiple combinations of \( \alpha, \beta, \text{ and } \delta \) subunits can combine to form functional receptors, which results in alternative subunit interfaces (25–31).

In this work, we photolabeled detergent-solubilized, purified heterologous \( \alpha3\beta3\delta \) GABA\(_A\)Rs with \([3H]\)aziotomidate and \([3H]\)R-mTFD-MPAB. Two distinct high affinity anesthetic sites were identified: 1) \([3H]\)aziotomidate photolabeling established that aziotomidate and etomidate bind to a \( \beta^+ - \alpha4^- \) interface site that does not bind R-mTFD-MPAB with high affinity; and 2) \([3H]\)aziotomidate and \([3H]\)R-mTFD-MPAB share a common binding site with etomidate at a \( \beta^- \) subunit interface. DS2, a positive allosteric modulator selective for GABA\(_A\)Rs containing a \( \delta \) subunit (32), did not bind to these sites.

### Results

#### Biochemical Characterization of the \( \alpha3\beta3\delta \) GABA\(_A\)R—Comparison of \([3H]\)muscimol binding to \( \alpha3\beta3\delta \) GABA\(_A\)R in membranes and after purification in asolectin/CHAPS established that positive allosteric modulation was retained by etomidate and by DS2, a positive allosteric modulator selective for GABA\(_A\)Rs containing the \( \delta \) subunit (32) (Table 1). In contrast to \( \alpha1\beta3\gamma2 \) GABA\(_A\)Rs, which bound \([3H]\)muscimol with similar affinity in membrane-bound \( (K_{eq} = 50 \text{ nM}) \) and purified \( (K_{eq} = 80 \text{ nm}) \) states (33), \([3H]\)muscimol bound to \( \alpha3\beta3\delta \) GABA\(_A\)Rs in membranes \( (K_{eq} = 13 \text{ nM}) \) with higher affinity than after purification in CHAPS/asolectin \( (K_{eq} = 90 \text{ nm}) \). After purification, etomidate \( (10 \mu M) \) and DS2 \( (30 \mu M) \) increased the specific binding of 2 nM \([3H]\)muscimol by \( \sim 30\% \).

When samples of purified human \( \alpha3\beta3\delta \) GABA\(_A\)R were fractionated by SDS-PAGE and visualized by Coomassie Blue stain, bands were readily visualized at 78 and 58 kDa, along with

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Membrane-bound</th>
<th>Purified</th>
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<tbody>
<tr>
<td>Etomidate</td>
<td>10 ( \mu M )</td>
<td>147 ± 28</td>
<td>129 ± 8</td>
</tr>
<tr>
<td>DS2</td>
<td>30</td>
<td>139 ± 8</td>
<td>129 ± 14</td>
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</tbody>
</table>

Anesthetic modulation was calculated as the ratio (%) of specific binding (2 nM) states (33), \([3H]\)muscimol bound to \( \alpha3\beta3\delta \) GABA\(_A\)Rs with \([3H]\)aziotomidate and \([3H]\)R-mTFD-MPAB. Aliquots of \( \alpha3\beta3\delta \) GABA\(_A\)Rs (8 pmol of \([3H]\)muscimol sites/aliquot) were photolabeled with 3 \( \mu M \) \([3H]\)aziotomidate (lanes 2–4) or 1 \( \mu M \) \([3H]\)R-mTFD-MPAB (lanes 5–7) in the absence (lanes 2 and 5) or presence of 1 mM etomidate (lanes 3 and 6) or 60 \( \mu M \) R-mTFD-MPAB (lanes 4 and 7), and subunits were resolved by SDS-PAGE. After Coomassie Blue staining (CB, representative image in lane 1), the gel was prepared for fluorography (lanes 2–7). Migration positions of molecular mass standards (in kDa) are denoted to the left of lane 1.
fainter bands at 72, 62, and 54 kDa (Fig. 2, lane 1). When extracted materials from in-gel tryptic digests of these bands were characterized by LC/MS/MS (Table 2), fragments of the GABA<sub>R</sub> α4 subunit were most enriched in the 72 kDa band, consistent with the expected mobility of the mature subunit (58 kDa + 3 N-linked glycosylations). Fragments from the β3 subunit were distributed in the 62 and 58 kDa bands, as found for β3 subunit from expressed α1β3γ2 GABA<sub>R</sub>s (11). Fragments from the δ subunit were broadly distributed in the 62, 58, and 54 kDa bands, with α4 subunit fragments also recovered from the 54 kDa band. However, in contrast to the recovery of α4 subunit fragments from the 72 kDa gel band, for the 54 kDa band, no fragments were recovered from the α4 cytoplasmic domain beginning about 30 amino acids after the end of the M3 helix (data not shown). This result suggests that the 54 kDa band contains an N-terminal fragment of the α4 subunit containing the M1–M3 helices that was probably produced by proteolytic cleavage during receptor purification. The major component in the 78 kDa band was identified as the chaperone heat shock 70-kDa protein 1A (HSP70-1).

When material eluted from the 72 kDa band was characterized by Edman degradation, the primary sequence identified (XXLNXPQQNQXXXXXL . . .) matched a region near the predicted N terminus of the human α4 GABA<sub>R</sub> subunit (VCLNESPGQNNKEKL . . .). Multiple amino acids were detected at similar levels at each cycle of Edman degradation of the 62- and 58-kDa samples, which precluded de novo identification of the subunits present. Sequence analysis of material from the 54 kDa band identified a primary sequence (XNDLXXXKCD . . .) matching the N terminus region of the FLAG-tagged human δ GABA<sub>R</sub> subunit sequence (MDIDGDYKDDD . . ., with the underline denoting the FLAG peptide sequence). The N termini of the α4 and δ subunits identified by Edman degradation are those predicted to be the N termini of the mature subunits by the signal sequence cleavage site prediction program P-signal (34). No N-terminal sequence was detected from the 78-kDa material, consistent with the fact that the N-terminal alanine of 70-kDa heat shock protein is acetylated (35), preventing Edman degradation.

**TABLE 2**

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<tr>
<th>Gel band</th>
<th>Protein (gene name)</th>
<th>Peptides detected</th>
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<th>Coverage</th>
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When photoreactive anesthetics—α4β3δ GABA<sub>R</sub>s were photolabeled at anesthetic concentrations with [3H]azietomidate or [3H]R-mTFD-MPAB in the absence or presence of etomidate at 1 mM or non-radioactive R-mTFD-MPAB at 60 μM, concentrations at which they each bind selectively to the β<sup>+</sup> or β<sup>−</sup> intersubunit sites in α1β3γ2 GABA<sub>R</sub>s (11). When 3H incorporation was determined by fluorography after SDS-PAGE (Fig. 2, lanes 2–7), 3H incorporation was highest in the 58/62 kDa gel region for both photoreactive anesthetics. The 72 kDa band (α4) was labeled prominently only by [3H]azietomidate, and that photolabeling was inhibitable by etomidate but not by R-mTFD-MPAB.

[3H]Azietomidate photolabeling in the 54/58/62 kDa gel bands was inhibited to a greater extent by etomidate than by R-mTFD-MPAB, and, conversely, [3H]R-mTFD-MPAB photolabeling of 58/62 kDa gel bands was inhibited to a greater extent by R-mTFD-MPAB than by etomidate. These findings suggested that 1) there is an etomidate/azietomidate binding site associated with the α4 subunit that does not bind R-mTFD-MPAB with high affinity; 2) azietomidate, etomidate, and R-mTFD-MPAB share a common binding site associated with the 58/62 kDa gel band; and 3) there may be an R-mTFD-MPAB binding site associated with the 58/62 kDa gel band that does not bind etomidate.

To further characterize the pharmacological specificity of [3H]azietomidate and [3H]R-mTFD-MPAB incorporation at the subunit level, photolabelings were performed on an analytical scale in the presence of various concentrations of etomidate, R-mTFD-MPAB, the neuroactive steroid alphaxalone, or DS2, with 3H incorporation into the gel bands quantified by liquid scintillation counting (Fig. 3). Etomidate inhibited [3H]azietomidate photoincorporation into the 72 kDa (α4) and 58/62 kDa bands with IC<sub>50</sub> values of ~15 μM, with high con-
centrations producing maximal inhibition of subunit photolabeling by 80 and 60%, respectively. Etomidate also inhibited $[^3H]R\text{-}m\text{-TFD-MPAB}$ photolabeling in the 58/62 kDa bands with similar potency. $R\text{-}m\text{-TFD-MPAB}$ inhibited $[^3H]R\text{-}m\text{-TFD-MPAB}$ photolabeling in the 58/62 kDa bands with an $IC_{50}$ of 2 $\mu M$ and a maximal inhibition of 75%. $R\text{-}m\text{-TFD-MPAB}$ also inhibited $[^3H]\text{azietomidate}$ photolabeling in the 58/62 kDa bands with similar potency, but it inhibited $[^3H]\text{azietomidate}$ labeling in the 72 kDa ($\alpha_4$) band only at the highest concentration tested (60 $\mu M$, $\sim$30% inhibition).

As seen for $[^3H]\text{azietomidate}$ and $[^3H]R\text{-}m\text{-TFD-MPAB}$ photolabeling of $\alpha_1\beta_3\gamma_2$ $GABA_A$-Rs (11), in the absence of $GABA$, the neuroactive steroid alfaxalone at concentrations up to 30 $\mu M$ potentiated photoincorporation into the $GABA_A$-R subunit bands, maximally by $\sim$50%. This result establishes that alfaxalone does not bind to the sites in the purified $\alpha_4\beta_3\delta GABA_A$-R photolabeled by $[^3H]\text{azietomidate}$ or $[^3H]R\text{-}m\text{-TFD-MPAB}$ but that there is positive allosteric linkage between alfaxalone and $\text{azietomidate}/R\text{-}m\text{-TFD-MPAB}$ binding. At concentrations up to 30 $\mu M$, DS2 had little or no effect on photolabeling by $[^3H]\text{azietomidate}$ or $[^3H]R\text{-}m\text{-TFD-MPAB}$ in the presence of $GABA$.

**Localization of $\alpha_4\beta_3\delta GABA_A$-R Residues Photolabeled by $[^3H]\text{azietomidate}$**—To provide an initial characterization of the locations of photolabeled amino acids, we fractionated by reversed phase HPLC (rpHPLC) EndoLys-C digests of subunit bands isolated from $\alpha_4\beta_3\delta GABA_A$-Rs photolabeled with $[^3H]\text{azietomidate}$ or $[^3H]R\text{-}m\text{-TFD-MPAB}$ (Fig. 4). For both photoreactive anesthetics, the digests of the 58/62 kDa band ($\beta_3$ and $\delta$ subunits) contained peaks of $^3H$ in hydrophobic fractions ($\sim$55 and 70% organic solvent) where fragments beginning at the N termini of the $\beta_3$ and $\delta$1 helices are known to elute (36). For the 72 kDa band ($\alpha_4$) labeled by $[^3H]\text{azietomidate}$, the $^3H$ eluted in two peaks, a broad hydrophobic peak (55% organic solvent), which for digests of $\alpha_1$ subunits contains fragments beginning at the N termini of the M1 and M3 helices, and a peak at 40% organic solvent, where fragments from the $\alpha_1$ subunit extracellular domain elute (36). For the 54 kDa band, for each drug, there were peaks of $^3H$ at 40, 55, and 70% organic solvent, corresponding to the peaks seen in either of the higher molecular weight gel bands.

**Etomidate Inhibits $[^3H]\text{Azietomidate}$ Photolabeling of $\alpha_4\text{Met}-269$ ($\alpha_1$M1), $\beta_3\text{Met}-227$ ($\beta_3$M1), and $\beta_3\text{Met}-286$ ($\beta_3$M3)**—Aliquots were sequenced of unfractionated EndoLys-C digests from the 72 and 58/62 kDa gel bands from $GABA_A$-Rs photolabeled with $[^3H]\text{azietomidate}$ in the absence and presence of non-radioactive etomidate (Fig. 5). For the 72 kDa band, there was a major peak of etomidate-inhibitable $^3H$ release in cycle 14 (Fig. 5A). For the digest from the 58/62 kDa band (Fig. 5B), there were peaks of etomidate-inhibitable $^3H$ release in cycles 7 and 12 (pharmacologically specific photolabeling) and peaks of $^3H$ release in cycles 3 and 19 that were not inhibited by etomidate (nonspecific labeling). The 72 kDa gel band digest will contain all possible $\alpha_4$ subunit proteolytic fragments, including...
fragments beginning near the N termini of the M1–M4 helices. For the 58/62 kDa band, digests will include fragments beginning near the N termini of the M1, M3, and M4 helices of the β3 and δ subunits (Fig. 5C). However, the etomidate-inhibitable peak of 3H release in cycle 14 for the 72 kDa band digest occurs in the cycle predicted to contain α1Met-269, the residue homologous to α1Met-236 in the α1 subunit M1 helix that was photolabeled by [3H]azetomidate in the absence (O) or presence (□) of 1 μM etomidate were loaded directly onto sequencing filter without prior purification by rpHPLC. In this experiment, 7,620 (□) and 3,620 (○) cpm were loaded for the 72 kDa (A) band, 22,020 cpm (□) and 17,210 (○) cpm for the 58/62 kDa band (B), and five-sixths of the material from each cycle of Edman degradation was collected for determination of released 3H. Included in C are the subunit fragment sequences containing transmembrane helices that can be sequenced after EndoLys-C digestion.

R-mTFD-MPAB Inhibits [3H]Azetomidate Photolabeling of β3Met-227 but Not α4Met-269—To confirm that [3H]azetomidate photolabeled α4Met-269 and β3Met-227, samples were sequenced after rpHPLC fractionation of EndoLys-C digests of material from the 72, 58/62, and 54 kDa gel bands. To determine whether R-mTFD-MPAB also inhibited photolabeling of these residues, receptors were photolabeled in the absence or the presence of 20 μM R-mTFD-MPAB, a concentration sufficient to occupy ~90% of its high affinity binding sites based upon the inhibition of photolabeling at the subunit level (Fig. 3). When fractions from the 58/62 kDa band were sequenced that contained the fragment beginning at β3Arg-216 to ~15 pmol (Fig. 6A), the peak of 3H release in cycle 12 confirmed labeling of β3Met-227 (220 cpm/pmol) and R-mTFD-MPAB inhibited that labeling by ~80%. When fractions from the 72 kDa (α4) gel band were sequenced (Fig. 6B) that contained the fragment beginning at α4Met-256 (1 pmol), there was a single peak of 3H release in cycle 14, consistent with photolabeling of α4Met-269 in α4M1 at 230 cpm/pmol in the absence or presence of 20 μM R-mTFD-MPAB.

**FIGURE 6.** R-mTFD-MPAB inhibits [3H]azetomidate photolabeling of β3Met-227 (βM1) but not α4Met-269 (αM1). A and B, □, ○ and pmol of PTH-derivatives (□) released during sequencing of subunit fragments beginning at β3Arg-216 and at α4Met-256 isolated by rpHPLC from EndoLys-C digests of subunits in the 58/62 kDa (A, β3/δ) or 72 kDa (B, α4) gel bands isolated by SDS-PAGE from α4β3δ GABAAR Rs (110 pmol of muscimol sites per condition) photolabeled with 3.5 μM [3H]azetomidate in the absence (○) or presence (□) of 20 μM R-mTFD-MPAB. rpHPLC fractions 28 and 29 (A) and 25–28 (B) were sequenced. A, the primary sequence began at β3Arg-216 (labeled 14 pmol, both conditions), and the peak of 3H release in cycle 12 indicated labeling of β3Met-227 at 220 cpm/pmol in the absence and at 50 cpm/pmol in the presence of R-mTFD-MPAB. A secondary sequence was present beginning at β3Ala-280 (βM3, ~1 pmol). B, the fragment beginning at α4Met-256 (labeled 1 pmol) was present, along with fragments beginning at α4Val-313 (2.6 pmol) and α4Ser-238 (~1 pmol). The peak of 3H release in cycle 14 was consistent with labeling of α4Met-269 (230 cpm/pmol) unaffected by R-mTFD-MPAB.

**FIGURE 5.** 3H release during N-terminal sequencing of EndoLys-C digests of 72 kDa (α4) and 62 kDa (β3/δ) subunit bands. Subunit digests from purified human α4β3δ GABAAR, R photolabeled with 3 μM [3H]azetomidate in the absence (○) or presence (□) of 1 μM etomidate were loaded directly onto sequencing filter without prior purification by rpHPLC. In this experiment, 7,620 (□) and 3,620 (○) cpm were loaded for the 72 kDa (A) band, 22,020 cpm (□) and 17,210 (○) cpm for the 58/62 kDa band (B), and five-sixths of the material from each cycle of Edman degradation was collected for determination of released 3H. Included in C are the subunit fragment sequences containing transmembrane helices that can be sequenced after EndoLys-C digestion.
were sequenced. \( \text{[H]} \)azetomidate, and pmol of PTH-derivatives (\( \text{[H]} \)Ala-309 fragment (A, 6 pmol; B, 1 pmol) but not with those of the \( \alpha \text{Ala}-309 \) fragment (A, 4 pmol; B, 2 pmol)).

Releases in cycle 7 correlated well with the amount of the \( \beta 3\text{Ala}-280 \) fragment but not with the amount of the \( \delta 3 \) fragment, the peak of \( \text{[H]} \) release in cycle 7 indicated photolabeling of \( \beta 3\text{Met}-286 \) (130 cpm/pmol), whereas for the 54 kDa band, peaks of \( \text{[H]} \) release were <7 cpm (Fig. 8C). For labeling by \( \text{[H]} \)azetomidate, the peaks of \( \text{[H]} \) release correlated well with the amounts of the \( \beta 3\text{Ala}-280 \) fragment (Fig. 8, B (8 pmol) and C (1 pmol)) but not with those of the \( \delta 3\text{Ala}-309 \) fragment (B, 8 pmol; C, 1 pmol).
In this report, we provide a first characterization of the locations of anesthetic binding sites in a \( \alpha 4 \beta 3 \delta \) \( \gamma 2 \) GABA\( \alpha \)Rs, expressed extrasynaptically in the CNS. We photolabeled purified human \( \alpha 4 \beta 3 \delta \) \( \gamma 2 \) GABA\( \alpha \)Rs with [\( ^{3}H \)]azietomidate and [\( ^{3}H \)]\( R-m \)TFD-MPAB, photoreactive anesthetics that have been used previously to identify two homologous but pharmacologically distinct classes of anesthetic binding sites in \( \alpha 1 \beta 3 \gamma 2 \) GABA\( \alpha \)Rs (11). Based upon the identification of photolabeled amino acids and the results of competition photolabeling assays carried out at the level of intact subunits, we demonstrate that etomidate, but not \( R-m \)TFD-MPAB, binds with high affinity to a site at the \( \beta^+ - \alpha^- \) subunit interface in \( \alpha 4 \beta 3 \delta \) GABA\( \alpha \)Rs that is equivalent to its binding site in \( \alpha 1 \beta 3 \gamma 2 \) GABA\( \alpha \)Rs. In contrast to \( \alpha 1 \beta 3 \gamma 2 \) GABA\( \alpha \)Rs, which bind \( R-m \)TFD-MPAB, but not etomidate, with high affinity to sites at the \( \alpha^- / \gamma^+ - \beta^- \) interfaces in proximity to \( \beta 3 \)Met-227 in \( \beta 1 \)M1, we find that etomidate as well as \( R-m \)TFD-MPAB bind with high affinity to a site in \( \alpha 4 \beta 3 \delta \) GABA\( \alpha \)Rs containing \( \beta 3 \)Met-227. As discussed below, this site is most likely to be at a \( \beta^- - \beta^- \) subunit interface. The sites identified by photolabeling with [\( ^{3}H \)]azietomidate and [\( ^{3}H \)]\( R-m \)TFD-MPAB are distinct from the binding sites for alphaxalone, an anesthetic steroid, or DS-2, a \( \delta \) subunit-selective positive allosteric modulator (32), because neither drug inhibited photolabeling.

\( \alpha 4 \beta 3 \delta \) GABA\( \alpha \)R Composition—Based upon mass spectrometry and Edman degradation, the affinity-purified \( \alpha 4 \beta 3 \delta \) GABA\( \alpha \)Rs used in this work contain \( \alpha 4 \) and \( \beta 3 \) subunits as well as the \( \delta \) subunit, whose presence is assured because the FLAG epitope used for purification is attached near the \( \delta \) subunit N terminus. However, we do not know whether the preparation is characterized by a single dominant subunit composition. Whereas receptors having a \( \beta 3 \alpha \alpha 4 \beta 3 \alpha 4 \delta \) subunit arrangement (counterclockwise when viewed from the extracellular side) with two \( \beta^+ - \alpha^- \) interfaces containing the agonist sites and a \( \delta \) subunit replacing the \( \gamma \) subunit have been reported to be strongly favored in transiently transfected HEK cells (27, 28, 37), other studies indicate that subunit stoichiometry can be variable and dependent upon the subunit cDNA transfection ratios (26). Also, studies using concatenated subunits provide evidence that the \( \delta \) subunit can assume multiple positions in a receptor pentamer and can contribute to a \( \beta^+ - \delta^- \) agonist binding site (25, 27, 30).

In the absence of independent definition of the subunit composition and arrangement in our purified \( \alpha 4 \beta 3 \delta \) GABA\( \alpha \)Rs, consideration of our photolabeling results suggests a \( \beta 3 \alpha 4 \beta 3 \beta 3 \delta \) or \( \beta 3 \delta \beta 3 \alpha 4 \beta 3 \) organization for the stably transfected cell line used in our studies. We favor these stoichiometries because 1) they have a \( \beta 3 - \beta 3 \) interface required for the shared azietomidate/etomidate/\( R-m \)TFD-MPAB binding site, and 2) they have three \( \beta 3 \) subunits to every one \( \alpha 4 \) subunit, consistent with the similar levels of [\( ^{3}H \)]azietomidate incorporation (cpm/pmol) at the amino acid level in the \( \beta 3 \) and \( \alpha 4 \) subunits (Fig. 6) in the presence of a higher level of [\( ^{3}H \)] incorporation in the \( \beta 3 \) gel band than in the \( \alpha 4 \) band (Fig. 2). However, a \( \beta 2 \alpha 4 \delta 3 \beta 2 \) pentameric concatamer, containing a \( \beta^- - \beta^- \) interface, also forms a functional receptor (30).

Discussion

An Etomidate Binding Site at the \( \beta 3^- - \delta^- \) Interface—[\( ^{3}H \)]Azietomidate photolabeled \( \beta 3\)Met-286 in \( \beta 3 M3 \) (\( \beta 3^- \) side of an interface) and \( \alpha 4 \)Met-269 in \( \alpha 4 M1 \) (\( \alpha^- \) side), with etomidate inhibiting labeling by > 90% and \( R-m \)TFD-MPAB by < 15%. Because [\( ^{3}H \)]azietomidate also photolabeled \( \beta 3\)Met-286 in \( \alpha 1 \beta 3 \gamma 2 \) GABA\( \alpha \)Rs and \( \alpha 4 \)Met-269 is homologous to \( \alpha 1 \)Met-236 that was also photolabeled (11), the simplest interpretation of these results is that there is an etomidate/azietomidate binding site at a \( \beta^+ - \alpha^- \) interface homologous to the etomidate site at the \( \beta 3^- - \alpha 1^- \) interfaces in \( \alpha 1 \beta 3 \gamma 2 \) GABA\( \alpha \)Rs. This conservation of etomidate binding sites between \( \alpha 4 \beta 3 \delta \) and \( \alpha 1 \beta 3 \gamma 2 \) GABA\( \alpha \)Rs is not unexpected, in view of the strong conservation of amino acids in the regions of the \( \alpha 1 \) and \( \alpha 4 \) subunit M1 and M2 helices that contribute to the \( \alpha^- \) surface of the etomidate binding sites (Fig. 9) and the fact that etomidate produces similar allosteric modulation in \( \alpha 1 \beta 3 \delta \) and \( \alpha 1 \beta 3 \gamma 2 \) GABA\( \alpha \)Rs (38).

An Etomidate/\( R-m \)TFD-MPAB Binding Site at a \( \beta 3^- \) Interface—The most prominently labeled residue in the \( \beta 3 \) subunit for both [\( ^{3}H \)]azietomidate and [\( ^{3}H \)]\( R-m \)TFD-MPAB was \( \beta 3\)Met-227. Whereas in \( \alpha 1 \beta 3 \gamma 2 \) GABA\( \alpha \)Rs, etomidate enhanced [\( ^{3}H \)]\( R-m \)TFD-MPAB photolabeling of this residue in \( \alpha^- - \beta^- \) and/or \( \gamma^- - \beta^- \) intersubunit sites (11), etomidate inhibited this photolabeling by > 90% in the \( \alpha 4 \beta 3 \delta \) GABA\( \alpha \)R, where \( \beta 3\)Met-227 can potentially contribute to anesthetic binding sites at the \( \alpha 4^- - \beta^- , \delta^- - \beta^- , \) or \( \beta^+ - \beta^- \) intersubunit interfaces.

Several lines of evidence indicate that the \( \beta 3^- - \delta^- \) interface is the most likely interface for the site binding etomidate, azietomidate, and \( R-m \)TFD-MPAB with high affinity. 1) Photolabeling studies with expressed \( \alpha 1 \beta 3 \gamma 2 \) GABA\( \alpha \)Rs establish that etomidate, [\( ^{3}H \)]azietomidate, and [\( ^{3}H \)]\( R-m \)TFD-MPAB all bind with high affinity to the \( \beta 3^- - \delta^- \) interface pocket that is present in \( \alpha 1 \beta 3 \) but not in \( \alpha 1 \beta 3 \gamma 2 \) GABA\( \alpha \)Rs (9, 39). 2) Examination of the amino acid residues that would contribute to the three alternative binding pockets (Fig. 9) identifies non-conservative substitutions contributing to the (+)-surface of the binding pocket that are expected to prevent the high affinity binding of etomidate in an \( \alpha 4^- - \beta^- \) or \( \delta^- - \beta^- \) intersubunit pocket. In \( \alpha 1 \beta 2^3 \gamma 2 \) GABA\( \alpha \)Rs, \( \beta 2^3 \alpha 4 \)M2-265 (\( \beta M2^-15^- \)) is known to be a major determinant of etomidate binding affinity, and in vitro and in vivo mutational analyses establish that replacement by Ser (\( \alpha 4 \)M2-15^-) or Met (\( \delta M2^-15^- \)) reduces etomidate potency by > 10-fold (12, 13, 40—42). Similarly, substitution of \( \beta 3\)Met-286 by Trp (the \( \delta \) residue in the \( \beta 3\)Met-286 position) also inhibits the effects of etomidate (40, 43). Therefore, it is unlikely that etomidate can bind with high affinity at either the \( \alpha 4^- - \beta^- \) or \( \delta^- - \beta^- \) interface.

Contributions of \( \delta \) Subunit Residues to Etomidate/Barbiturate Binding Sites—In our study, we did not identify any \( \delta \) subunit amino acids photolabeled in an anesthetic-inhibitable manner by [\( ^{3}H \)]azietomidate or [\( ^{3}H \)]\( R-m \)TFD-MPAB. Based upon sequence analyses of samples containing variable amounts of \( \beta M3 \) and \( \delta M3 \), any pharmacologically specific photolabeling in \( \delta M3 \) is at < 15% the level of \( \beta M3 \). It is possible that [\( ^{3}H \)]azietomidate does bind in a pocket containing \( \delta M3 \) residues that is homologous to the \( \beta^- - \alpha^- \) site without photolabeling any residue in \( \delta M3 \), because the pocket would lack the methionine side chains favored by azietomidate’s photoreac-
sequence analysis. In fact, δM1 may contribute to a barbiturate binding site, because studies with receptors containing α1, β3, and chimeric γ/δ subunits indicated that pentobarbital sensitivity determinants were contained within a fragment containing the amino terminus and the first 3 amino acids of δM1 (44). Further studies will be necessary to clarify whether general anesthetics also bind with high affinity in the pocket at the β^+−δ^− interface in the cell line used in this study or at the α^+−δ^− interface in ββδδ GABA_\text{ARs}.

**Functional Significance of the Identified Binding Sites**—Photolabeling studies provided a first definition of two classes of pharmacologically distinct binding sites for intravenous general anesthetics at subunit interfaces in the αβδ2 GABA_\text{AR} transmembrane domain (8, 11, 36) that overlap with the binding sites for ivemectin (45). Mutational analyses of the residues identified by photoaffinity labeling as well as neighboring residues in the shared subunit interface pockets have demonstrated their contributions to GABA_\text{AR} gating and as determinants of anesthetic efficacy (12, 40, 43, 46, 47). In addition, the capacity of anesthetics to protect against modification of substituted cysteines has expanded the definition of residues contributing to anesthetic binding sites (48, 49). Mutational analyses will be necessary to determine, for example, whether the β^−−α^− site and β^−−β^− sites identified by photoaffinity labeling are equally important for etomidate enhancement of GABA responses in an αβδδ GABA_\text{AR}.

However, in view of the difficulty of expressing αββδδ GABA_\text{ARs} with defined subunit stoichiometry and subunit arrangement, these studies should be carried out using pentameric concatenated receptors.

**Experimental Procedures**

**Materials**—[^H]Muscimol (36 Ci/mmol) was from Perkin-Elmer Life Sciences. The detergents n-dodecyl β-D-maltopyranoside and CHAPS were from Anatrace-Affymetrix (anagrade quality). R-mTFD-MPAB and [3H]R-mTFD-MPAB (38 Ci/mmol) were prepared previously (50), as was [3H]azetomidate (19.3 Ci/mmol) (39). Soy bean aseloin, R-etomidate, and GABA were from Sigma. DS2 and alaphaxalone were from Tocris. EndoLys-C was from Roche Applied Sciences.

**Purification of αββδδ GABA_\text{AR}**—A detailed description of the expression and affinity purification of αββδδ GABA_\text{ARs} will be presented elsewhere. As described previously for αβδ2 GABA_\text{ARs} (33), a stably transected, tetracycline-inducible HEK293-TetR cell line expressing human GABA_\text{AR} subunits α4, β3 (splice variant 2), and δ containing a FLAG tag near its N terminus (between ΔGly-29 and ΔAsp-30) was induced and grown for 2–3 days, and then membranes were harvested, flash-frozen in liquid N2, and stored at −80 °C until use. GABA_\text{ARs} were solubilized with 30 mM n-dodecyl β-D-maltopyranoside and affinity-purified as described (11), using a FLAG M2 antibody column. Columns were washed with purification buffer supplemented with 200 μM aseloin and 5 mM CHAPS and then eluted with 1.5 mM FLAG peptide in the wash buffer. Aliquots of the eluate fractions were assayed for [^H]muscimol binding, and eluate fractions were flash-frozen in liquid N2 and stored at −80 °C until use. Membranes harvested from 60 15-cm plates contained 5–10 nmol of[^H]muscimol binding sites (15–20 pmol of sites/mg of membrane protein), and the
elute fractions from the purifications used for photolabeling contained 50–70 nm [3H]muscimol sites. Based upon [3H]muscimol binding, the receptor was purified at 10–25% yield from the starting membranes. Because the receptor was eluted in the presence of 1.5 mM FLAG peptide, it was not possible to estimate purity in terms of pmol of muscimol binding/mg of protein. Based upon analyses by SDS-PAGE and LC/MS/MS (see “Results”), GABA<sub>R</sub> subunits were the dominant polypeptides in the preparation.

**Radioligand Binding Assays**—[3H]Muscimol binding to purified GABA<sub>R</sub> was measured by filtration after precipitation with polyethylene glycol (8). The total concentration of sites in eluate fractions was determined at 250 nm [3H]muscimol with 1 mM GABA to determine nonspecific binding. Allosteric modulation of 2 nm [3H]muscimol binding was determined as described (9, 11).

**Sequence Numbering**—For α4, residue 1 is the predicted signal sequence Met; for β3, residue 1 is the predicted N terminus of the mature protein (splice variant 1, QSNVVD . . .), with β3Met-286 at the 15th position in the M2 helix (M2–15’); and for δ, the numbering begins with the signal sequence Met and excludes the inserted FLAG sequence (DYKDDDDK). The primary structure locations of transmembrane helices M1–M4 in the figures correspond to the extent of the individual α-helices in the β3 monomeric GABA<sub>R</sub> crystal structure (Protein Data Bank code 4COF).

**Analysis of the α4β3δ GABA<sub>R</sub> Preparation by LC/MS and N-terminal Sequencing**—Three aliquots (24 pmol of [3H]muscimol sites each) of α4β3δ GABA<sub>R</sub> were separated by SDS-PAGE. Based upon Coomassie Blue staining, bands migrating at 78, 72, 62, 58, and 54 kDa were excised. The bands from one lane were submitted to the Harvard Medical School Taplin Mass Spectrometry Facility for reduction and alklylation, in-gel trypsin digestion, and peptide extraction for microcapillary LC/MS/MS analysis. The material from the equivalent gel bands from the other two lanes was eluted and subjected to N-terminal sequence analysis.

**GABA<sub>R</sub> Photolabeling**—Aliquots of purified α4β3δ GABA<sub>R</sub> in elution buffer were photolabeled at analytical or preparative scale (150–200 μl or 1–2 ml of GABA<sub>R</sub> per condition, respectively) to characterize photoincorporation at the subunit level or to identify individual photolabeled amino acids by protein microsequencing. Aliquots of [3H]azietomidate or [3H]R-mTFD-MPAB were dried under a gentle argon stream and resuspended with GABA<sub>R</sub> solutions for 30 min on ice with gentle vortexing. For preparative photolabeling, non-radioactive drugs were added directly to this resuspension, whereas for analytical photolabeling, drug aliquots were added by the use of a 1-μl syringe (Hamilton 86200) to 10 μl of purified GABA<sub>R</sub>, which was then combined with 90–150 μl of GABA<sub>R</sub> equilibrated with radioglutid. With the exception of studies with alphaxalone, all photolabeling was carried out in the presence of 300 μM GABA. Samples were transferred to 96-well plastic plates or 3.5-cm diameter Petri dishes (Corning catalogue numbers 2797 and 3001) for analytical or preparative scale labeling and irradiated on ice with a 365-nm UV lamp (Spectroline EN–280L) for 30 min at a distance of <1 cm. Samples were then denatured by mixing 2 parts sample with 1 part SDS-PAGE sample buffer, incubated for ~30 min, and fractionated by modified Laemmli SDS-PAGE (11).

**Proteolysis, Reversed-phase HPLC, and N-terminal Sequence Analysis**—Aliquots of labeled subunits isolated from gel bands were digested (2 weeks, 20 °C, 0.3–1 units/sample) with EndoLys-C (Roche Applied Science). Digests were fractionated by RP-HPLC as described (51), except that the gradient began at 95% aqueous solvent (0.08% TFA) and 5% organic solvent (60% acetonitrile, 40% isopropyl alcohol, 0.05% TFA) and progressed
to 100% organic in 75 min by approximating (in 5-min intervals) the quadratic growth curve, \( f(x) = 5 + 0.017 \times x^2 \), where \( x \) is time in minutes and \( f(x) \) is percentage of organic solvent. The flow rate was 200 \( \mu \text{l} / \text{min} \), and fractions were collected every 2.5 min, with 10% assayed for \( ^{3}\text{H} \). Fractions of interest were pooled and drop-loaded onto glass fiber filters for N-terminal sequence analysis on an Applied Biosystems Procise 492 protein sequencer modified so that two-thirds of each cycle were injected for PTH-derivative detection and quantification, whereas one-third was collected for scintillation counting. Some samples were sequenced without rpHPLC separation by loading them onto Applied Biosystems ProSorb\textsuperscript{TM} PVDF filters by diluting the samples 10-fold into 0.1% TFA. The pmol of PTH-derivatives detected were calculated by using rpHPLC peak heights at 269 nm compared with a standard injection.

Photolabeling in \( \alpha4M1 \) or \( \alpha4M3 \) was determined by sequencing appropriate rpHPLC fractions from digests of the 72 kDa gel band. Labeling in \( \beta3M1 \) and \( \beta3M3 \) was identified by sequencing fractions from the 58/62 kDa gel bands. In preliminary studies, we established that that fragments containing \( \deltaM1 \) and \( \deltaM3 \) were present at the highest level in the fractions containing \( \beta3M3 \) from the 58/62 kDa gel band, where they were present at \( \sim 50\% \) the level of the \( \betaM3 \) fragment. The \( \deltaM3 \) fragment was present in the equivalent fractions from the 54 kDa gel band at \( \sim 200\% \) the level of the \( \betaM3 \) fragment. The N termini of \( \betaM3 \) (\( \beta3\text{Ala-280} \)) and \( \deltaM3 \) (\( \delta3\text{Ala-309} \)) were each at the first cycle of Edman degradation. However, comparison of \( ^{3}\text{H} \) release profiles and relative amounts of \( \betaM3 \) and \( \deltaM3 \) during sequence analyses of fractions from the 54 and 58/62 kDa gel bands established that the anesthetic-inhibitable peaks of \( ^{3}\text{H} \) release originated from residues in \( \betaM3 \) rather than \( \deltaM3 \). Sequencing through \( \deltaM1 \) began only after 19 cycles of Edman degradation, at which point PTH-derivative and \( ^{3}\text{H} \) releases were too low to allow characterization of photolabeling in \( \deltaM1 \).

The detected sequences were quantitated by fitting the background-subtracted pmol of the detected peptide to the equation,

\[
M(x) = I_0 \times R^x \quad \text{(Eq. 2)}
\]

where \( M(x) \) represents the pmol in cycle \( x \), \( I_0 \) is the initial amount of the peptide, and \( R \) is the repetitive yield. Cys, Trp, Ser, and His were omitted from the fits due to known problems with their quantitations. The \( ^{3}\text{H} \) incorporation \( E(x) \), the efficiency of photolabeling (in cpm/pmol) of the amino acid in cycle \( x \), was calculated by the following equation.

\[
E(x) = \frac{2 \times (\text{cpm}_x - \text{cpm}_{x-1})}{I_0 \times R^x} \quad \text{(Eq. 3)}
\]

Author Contributions—J. B. C. and K. W. M. conceived and coordinated the study. D. C. C. and J. B. C. designed and analyzed the experiments illustrated in Figs. 2–8 that were performed by D. C. C. Y. J. created the cell line expressing \( \alpha4\beta3\delta \) GABA\(_A\)R, and X. Z. expressed, purified, and assayed the GABA\(_A\)R under the guidance of K. W. M. P. Y. S. and K. S. B. synthesized the photoreactive anesthetics used in the study. J. B. C., D. C. C., and K. W. M. wrote the paper with input from all authors. All authors approved the final version of the manuscript.

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α4β3δ GABA_A R General Anesthetic Binding Sites


