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Etomidate and Etomidate Analog Binding and Positive Modulation of γ-Aminobutyric Acid Type A Receptors:

Evidence for a State-dependent Cutoff Effect

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Abstract

Background: Naphthalene-etomidate, an etomidate analog containing a bulky phenyl ring substituent group, possesses very low γ-aminobutyric acid type A ($GABA_A$) receptor efficacy and acts as an anesthetic-selective competitive antagonist. Using etomidate analogs containing phenyl ring substituents groups that range in volume, we tested the hypothesis that this unusual pharmacology is caused by steric hindrance that reduces binding to the receptor's open state.

Methods: The positive modulatory potencies and efficacies of etomidate and phenyl ring–substituted etomidate analogs were electrophysiology defined in oocyte-expressed $\alpha_1\beta_3\gamma_{2L}$ $GABA_A$ receptors. Their binding affinities to the $GABA_A$ receptor's two classes of transmembrane anesthetic binding sites were assessed from their abilities to inhibit receptor labeling by the site-selective photolabels ^{3}[H]azi-etomidate and tritiated R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid.

Results: The positive modulatory activities of etomidate and phenyl ring–substituted etomidate analogs progressively decreased with substituent group volume, reflecting significant decreases in both potency ($P = 0.005$) and efficacy ($P < 0.0001$). Affinity for the $GABA_A$ receptor's two $\beta^+ - \alpha^-$ anesthetic binding sites similarly decreased with substituent group volume ($P = 0.003$), whereas affinity for the receptor's $\alpha^+ - \beta^-/\gamma^+ - \beta^-$ sites did not ($P = 0.804$). Introduction of the N265M mutation, which is located at the $\beta^+ - \alpha^-$ binding sites and renders $GABA_A$ receptors etomidate-insensitive, completely abolished positive modulation by naphthalene-etomidate.

Conclusions: Steric hindrance selectively reduces phenyl ring–substituted etomidate analog binding affinity to the two $\beta^+ - \alpha^-$ anesthetic binding sites on the $GABA_A$ receptor's open state, suggesting that the binding pocket where etomidate's phenyl ring lies becomes smaller as the receptor isomerizes from closed to open.

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Competing Interests

The authors declare no competing interests.

THE γ-aminobutyric acid type A ($GABA_A$) receptor is a member of the Cys-loop superfamily of ligand-gated ion channels that also includes the glycine, nicotinic acetylcholine receptor, and serotonin type 3 receptors.[1] Each individual $GABA_A$ receptor is comprised of five subunits—most commonly a combination of α, β, and γ subunits in a presumed 2, 2, and 1 stoichiometry—arranged pseudosymmetrically around a central ion channel that is selective for chloride ions. Within the receptor's extracellular domain, interfaces between adjacent subunits can form binding sites for ligands that positively modulate receptor function, including the endogenous agonist γ-aminobutyric acid (GABA), which binds at the receptor's two $\beta^+ - \alpha^-$ subunit interfaces, and benzodiazepines, which bind at its $\alpha^+ - \gamma^-$ subunit interface.[2,3]

Two homologous but distinct classes of binding sites for intravenous general anesthetics have also been located between $GABA_A$ receptor subunits using photoaffinity labeling and mutagenesis techniques.[4–7] However, unlike the binding sites for GABA and benzodiazepines, these sites are found within the receptor's hydrophobic transmembrane domain. One class of sites is photolabeled by the photoreactive etomidate analog ^{3}[H]azi-etomidate and located at the receptor's two $\beta^+ - \alpha^-$ subunit interfaces (approximately 50 Å below the sites that bind GABA), whereas the other class of sites is photolabeled by the photoreactive barbiturate tritiated R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid and located at the receptor's $\alpha^+ - \beta^-$ and $\gamma^+ - \beta^-$ subunit interfaces. Photoaffinity protection studies using these two photolabels reveal that while etomidate binds very selectively to the $\beta^+ - \alpha^-$ sites as compared to the $\alpha^+ - \beta^-/\gamma^+ - \beta^-$ sites, pentobarbital exhibits the reverse selectivity, and propofol exhibits essentially no binding site selectivity.[5] It is unclear why some anesthetics bind selectively to particular subunit interfaces. However, such information could provide important clues for the development of novel $GABA_A$ receptor subtype–selective clinical drugs.

Within the context of allosteric models of receptor function, general anesthetics and other $GABA_A$ receptor positive modulators enhance receptor function because they bind with higher affinity when the receptor is in the open state as compared to the closed state, increasing the fraction of open-state receptors.[8–10] Such conformational state-selective binding implies that the physical properties of these binding sites change as the receptor isomerizes from closed to open, leading to an increase in modulator affinity. However, the nature of such changes is unknown.

We recently reported that appending a phenyl ring substituent group onto etomidate's existing phenyl ring (forming naphthalene-etomidate) essentially abolished conformational state selectivity and binding site selectivity, reducing etomidate's intrinsic efficacy and turning the drug into an anesthetic-selective competitive antagonist at the $GABA_A$ receptor.[11] To explain this finding, we hypothesized that the binding pocket where etomidate's phenyl ring lies becomes smaller as the receptor isomerizes from closed to open, sterically hindering naphthalene-etomidate from binding to the open state (fig. 1). If this hypothesis were correct, then the abilities of other phenyl ring–substituted etomidate analogs to bind to the $GABA_A$ receptor's open state and produce positive modulation are predicted to progressively decrease with substituent size as steric hindrance increases. In the current studies, we tested this "state-dependent cutoff" hypothesis by defining the $GABA_A$ receptor

pharmacology of a series of etomidate analogs possessing phenyl ring substituent groups that range in molecular volume.

Materials and Methods

Etomidate, Etomidate Analogs, and Anesthetic Photoaffinity Labels

Figure 2 shows the molecular structures of etomidate and the etomidate analogs containing phenyl ring substituent groups. Etomidate was purchased from Bachem Americas (USA). Etomidate analogs were synthesized by Aberjona Laboratories (USA) and their structures confirmed by nuclear magnetic resonance spectroscopy. For electrophysiologic experiments, drugs (*i.e.*, etomidate and etomidate analogs) were prepared as stock solutions in dimethyl sulfoxide and diluted in buffer to achieve the desired concentrations. The final dimethyl sulfoxide concentration (less than or equal to 0.1% v/v) produces no functional effects on $GABA_A$ receptors.[93][H]Azi-etomidate and tritiated R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid were synthesized previously.[12,13] The molecular volumes of etomidate and etomidate analogs were computationally determined using Discovery Studio (Biovia, USA). The octanol/buffer partition coefficient of each etomidate analog was determined as previously described.[13,14]

$GABA_A$ Receptor Electrophysiology

Oocytes were harvested from *Xenopus* frogs using a protocol approved by and in accordance with rules and regulations of the Institutional Animal Care and Use Committee at the Massachusetts General Hospital, Boston, Massachusetts. They were injected with messenger RNA encoding the α_1, β_3 or β_3 (N265M), and γ_{2L} subunits of the human $GABA_A$ receptor and incubated in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4) containing 0.05 mg/ml of gentamicin for 18 to 48 h at 18°C before electrophysiologic study. Electrophysiologic recordings were performed at room temperature using the whole cell two-electrode voltage-clamp technique as previously described.[15] A GABA concentration–peak current response curve was generated for each oocyte to define the GABA concentration that elicits 5% of the current evoked by 1 mM GABA (*i.e.*, EC_5). The oocyte was then perfused with EC_5 GABA alone for 15 s followed immediately by EC_5 GABA plus drug (etomidate or etomidate analog) at the desired concentration for 60 s, and the peak current response was recorded. Drug concentrations ranged up to those necessary to reach a plateau peak electrophysiologic response or aqueous saturation. To account for variable receptor expression among oocytes, all responses were normalized to the peak current response evoked by 1 mM GABA measured in the same oocyte. To remove GABA and/or drugs and to allow receptors to recover from desensitization, oocytes were perfused with buffer for at least 3 min between electrophysiologic recordings.

Photoaffinity Label Competition Experiments

$\alpha_1\beta_3\gamma_{2L}$ $GABA_A$ receptors containing a FLAG epitope on the N terminus of the α_1 subunit were heterologously expressed in a tetracycline-inducible, stably transfected human embryonic kidney 293S cell line and affinity-purified on an anti-FLAG resin.[5,16] Purified receptors in the presence of 1 mM GABA were photolabeled with either ^{3}[H]azi-etomidate

(~2 μM) or tritiated R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid (~1 μM) with addition of nonradioactive competing drugs as previously described.[5,17] Photolabel incorporation into each receptor subunit was then measured by resolving subunits by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, excising the Coomasie blue–stained bands corresponding to each subunit, and measuring tritium incorporation by liquid scintillation counting. The normalized specific radioactivity was then calculated by subtracting the radioactivity measured in the presence of 300 μM etomidate (for ^{3}[H] azi-etomidate experiments) or either 60 μM or 100 μM R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid (for tritiated R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid experiments) and then dividing it by the radioactivity measured in the absence of competing drug. Stock solutions of etomidate analogs were prepared at 200 mM in ethanol, and each photolabeled sample contained ethanol at a 0.5% (v/v) final concentration.

Data Analysis

Individual concentration-response curves for potentiation of EC_5 GABA-evoked currents were fit with Prism 6.0h software (GraphPad, USA) using its built-in three-parameter equation for stimulation (equation 1):

$$\text{Normalized Current Amplitude} = \text{Minimum} + \frac{\text{Maximum} - \text{Minimum}}{1 + 10^{(\text{LogEC}_{50} - [\text{drug}])}} \quad (1)$$

where minimum is the normalized peak current amplitude in the absence of drug, maximum is the normalized peak current amplitude at infinitely high drug concentrations, [drug] is the drug concentration, and EC_{50} is the drug concentration that evokes a peak current amplitude that is halfway between the maximum and minimum values. By definition for EC_5 GABA-evoked currents, the minimum value was constrained to 5%.

Drug concentration-response curves for inhibition of photoaffinity labeling by the two photoaffinity labels were fit with Prism 6.0h software using its built-in equation for inhibition (equation 2):

$$\text{Normalized Specific Counts per Minute} = \frac{100}{1 + 10^{([\text{drug}] - \text{LogIC}_{50})}} \quad (2)$$

where [drug] is the drug concentration and IC_{50} is the drug concentration that produces a normalized specific counts per minute value of 50.

To derive estimates of the microscopic dissociation constants for drug binding to the $GABA_A$ receptor's open state, normalized peak electrophysiologic current amplitudes evoked by EC GABA plus drug were transformed into P_{open} values by assuming that a maximally activating GABA concentration (1 mM) produces a P_{open} of 0.85. The resulting relationship between P_{open} and drug concentration is then defined by the coagonist model by the logistic equation (equation 3):[9]

$$P_{open} = \frac{1}{1 + L_0\left(\frac{1 + [GABA] / Kd_{closed}}{1 + [GABA] / Kd_{open}}\right)^2\left(\frac{1 + [drug] / Kd_{closed}}{1 + [drug] / Kd_{open}}\right)^n} \quad (3)$$

where P_{open} is the fraction of receptors that are open in the presence of EC_5 GABA plus drug, L_0 is the closed/open receptor ratio in the absence of GABA or drug, [drug] is the drug concentration, Kd_{closed} and Kd_{open} are the drug's respective microscopic dissociation constants for the $GABA_A$ receptor closed and open states, and n is the number of drug binding sites. Because all of our electrophysiologic experiments were done using an EC_5 GABA concentration (*i.e.,* a concentration that produces a P_{open} equal to 0.05 × 0.85 = 0.0425) and assuming a median literature value for L_0 of 40,000, equation 3 simplifies to the following logistic equation (equation 4):[8–10,18,19]

$$P_{open} = \frac{1}{1 + 22.5^*\left(\frac{1 + [drug] / Kd_{closed}}{1 + [drug] / Kd_{open}}\right)^n} \quad (4)$$

We thus obtained the value of Kd_{open} for each dataset by fitting the relationship between P_{open} and drug concentration open to equation 4 with Prism 6.0h software.

Statistical Analysis

At each drug concentration, individual electrophysiologic data points were obtained using different oocytes. Sample sizes were defined based on our previous experience. Errors for mean data points are reported as ± SD. The results of linear and nonlinear least squares fitting are reported as the fitted value and its 95% CI. The effects of naphthalene-etomidate on wild-type and mutant $\alpha_1\beta_3\gamma_{2L}$ $GABA_A$ receptors were assessed using a Mann-Whitney test. To avoid output saturation, oocytes producing 1 mM GABA-evoked peak currents greater than 5 μA were discarded. There was no lost or missing data. All fitting was performed with Prism 6.0h or Igor Pro 6.1 (Wavemetrics, USA). Statistical significance was assumed for $P < 0.05$.

Results

Potentiation of EC_5 GABA-evoked Currents by Etomidate and Etomidate Analogs

Figure 3A shows representative individual electrophysiologic traces recorded upon application of EC_5 GABA alone or EC_5 GABA along with 100 μM etomidate or etomidate analog. It reveals that although all of the drugs potentiated EC_5 GABA-evoked currents, the magnitude of potentiation progressively decreased with increasing substituent group volume. To assess whether these substituent size-dependent differences in $GABA_A$ receptor positive modulatory activity were due to differences in drug potencies or efficacies (or both), we quantified the potentiating actions of drugs over a range of drug concentrations. The resulting drug concentration-response curves for potentiation of EC_5 GABA-evoked currents

are shown in figure 3B. Etomidate was the most potent and efficacious drug with an EC_{50} of 1.5 μM (95% CI, 1.1 to 1.9 μM) and a maximal current at (infinitely) high concentrations that was 104% (95% CI, 99 to 109%) of that evoked by 1 mM GABA, a maximally activating GABA concentration. Conversely, *m*-dimethoxy-etomidate was the least potent and efficacious drug with an EC_{50} of 210 μM (95% CI, 51 to 830 μM) and a maximal current at high concentrations that was only 11% (95% CI, 8 to 14%) of that evoked by 1 mM GABA. For all drugs studied, EC_{50} and maximum peak currents recorded at high drug concentrations along with octanol/buffer partition coefficients and molecular volumes are reported in table 1.

Figure 4 plots the relationship between a drug's potency as reflected by the logarithm of its EC_{50} (fig. 4A) or its efficacy as reflected by the maximum peak current elicited at high drug concentrations (fig. 4B) and the volume of its phenyl ring substituent group. A linear fit of the relationship between log EC_{50} (in μM) *versus* substituent group volume (in $Å^3$) had a slope of 0.037 (95% CI, 0.016 to 0.058), which was significantly different from 0 ($P = 0.005$), and a coefficient of variation (r^2) of 0.7557. A linear fit of the relationship between the maximal current (in %) *versus* substituent group volume (in $Å^3$) had a slope of −2.1 (95% CI, −2.6 to −1.6), which was also significantly different from 0 ($P < 0.0001$), and a coefficient of variation of0.9396. Thus, on average, each 10-$Å^3$ increase in substituent group volume approximately doubled the EC_{50} and reduced the maximal current by 20%. We observed no relationship between a drug's hydrophobicity (as reflected by its octanol/buffer partition coefficient) and either its potency or efficacy (data not shown).

Etomidate and Etomidate Analog Inhibition of $GABA_A$ Receptor Photolabeling by ^{3}[H]Azi-etomidate and Tritiated R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) Barbituric Acid

Because ^{3}[H]azi-etomidate and tritiated R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid photoaffinity label each of the two classes of transmembrane anesthetic binding sites in a highly selective manner (*i.e.*, more than 50-fold selectivity for their respective sites), they have been used in competition assays to quantify the affinities of other ligands to each of those sites.[5] We applied this approach in the presence of 1 mM GABA to quantify the binding affinity of etomidate and our seven etomidate analogs to these two classes of binding sites when the receptor is in the open/desensitized state. Figure 5 shows that etomidate and etomidate analogs inhibited specific ^{3}[H]azi-etomidate (fig. 5A) and tritiated R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid (fig. 5B) photolabeling of $\alpha_1\beta_3\gamma_{2L}$ $GABA_A$ receptors in a concentration-dependent manner. A fit of each dataset to equation 2 yielded half-maximal inhibitory concentrations that are given in table 2. Figure 6 plots the relationship between the logarithm of a drug's half-maximal inhibitory concentration (in μM) and its substituent group volume (in $Å^3$). A linear fit of this relationship when using ^{3}[H]azi-etomidate as the photolabel (fig. 6A) had a slope of 0.040 (95% CI, 0.019 to 0.061), which was significantly different from 0 ($P = 0.004$), and a coefficient of variation of 0.7821. Thus, on average, each 10-$Å^3$ increase in substituent group volume increased the half-maximal inhibitory concentration by two- to three-fold. A similar fit when using tritiated R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid as the photolabel (fig. 6B) had a slope

of −0.005 (95% CI, −0.028 to 0.017), which was not significantly different from 0 (P = 0.602), and a coefficient of variation of only 0.04814.

For each drug, the binding site selectivity ratio was defined from the two sets of photoaffinity labeling experiments as the half-maximal inhibitory concentration for inhibiting ^{3}[H] azi-etomidate labeling divided by the half-maximal inhibitory concentration for inhibiting tritiated R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid labeling.[5] In figure 6C, the logarithm of this ratio is plotted as a function of substituent group volume and shows that binding site selectivity for the two $\beta^{+} - \alpha^{-}$ subunit interfacial binding sites progressively decreased (*i.e.,* the selectivity ratio increased) with substituent group volume ($P < 0.001$) with the four analogs possessing the largest substituent groups exhibiting essentially no selectivity for the two $\beta^{+} - \alpha^{-}$ subunit interfacial binding sites (*i.e.,* the sites photolabeled by ^{3}[H]azi-etomidate) *versus* the $\alpha^{+} - \beta^{-}/\gamma^{+} - \beta^{-}$ interfacial binding sites (*i.e.,* the sites photolabeled by tritiated R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid).

Potentiation of EC_5 GABA-evoked Currents by Naphthalene-Etomidate: The Impact of a Mutation That Renders $GABA_A$ Receptors Insensitive to Etomidate

Figure 7A shows representative electrophysiologic traces recorded upon application of EC_5 GABA alone or EC_5 GABA along with 100 μM naphthalene-etomidate to oocytes expressing either wild-type $\alpha_1\beta_3\gamma_{2L}$ $GABA_A$ receptors (left) or etomidate-insensitive mutant $\alpha_1\beta_3$(N265M)γ_{2L} $GABA_A$ receptors (right). Figure 7B summarizes the results of a series of such experiments performed using 6 different oocytes/experimental condition. It shows that naphthalene-etomidate approximately doubled the peak amplitudes of EC_5 GABA-evoked currents (from 4.4 ± 1.2% to 10 ± 2.4% of the current produced by 1 mM GABA; P = 0.002) in oocytes expressing wild-type receptors but had no potentiating effect on those expressing receptors containing the N265M mutation (4.9 ± 0.5% to 4.4 ± 0.6% of the current produced by 1 mM GABA; P = 0.132).

Discussion

The current studies were motivated by our previous observation that naphthalene-etomidate, an etomidate analog containing a bulky substituent group on its phenyl ring, exhibits pharmacologic properties that are quite distinct from those of etomidate.[11] Unlike etomidate, naphthalene-etomidate binds nonselectively to the $GABA_A$ receptor's two classes of transmembrane anesthetic binding sites and produces relatively little positive modulation of $GABA_A$ receptors even at high, near-aqueous saturating concentrations. Consequently, it exhibits the pharmacology of an anesthetic competitive antagonist capable of reversing the $GABA_A$ receptor actions of anesthetics that bind to these sites and represents a potential lead compound for the development of anesthetic reversal agents. As a possible explanation for this unexpected pharmacology, we hypothesized that steric hindrance caused by the presence of the large phenyl ring substituent group selectively reduced naphthalene-etomidate's binding affinity for the $GABA_A$ receptor's open state *versus* its closed state. Such behavior would occur if the binding pocket where etomidate's phenyl ring lies becomes smaller as the receptor isomerizes from closed to open. To test this hypothesis, we

defined the effect of varying phenyl ring substituent group volume on the abilities of etomidate analogs to (1) bind to each of the two classes of transmembrane anesthetic binding sites on the $GABA_A$ receptor when the receptor is in the open/desensitized state and (2) positively modulate receptor function. If our hypothesis were correct, then the abilities of these drugs to bind to the $GABA_A$ receptor's two $\beta^+ - \alpha^-$ transmembrane binding sites and positively modulate receptor function should progressively decrease as the steric hindrance became greater with increasing substituent group volume.

Our electrophysiologic studies indeed showed that $GABA_A$ receptor positive modulatory ability progressively decreases with substituent group volume. Our drug concentration-response curves for EC_5 GABA potentiation indicate that this reflects significant reductions in both drug potency and efficacy. Our photoaffinity protection studies showed that with progressively larger substituent volume, the binding affinities of these drugs for the $\beta^+ - \alpha^-$ sites on the open/desensitized state also decrease whereas those to the $\alpha^+ - \beta^-/\gamma^+ - \beta^-$ sites exhibit no such trend. Consequently, there is a progressive increase in the selectivity ratio, reaching values that approximate 1 (*i.e.*, no selectivity) for the four analogs having the largest substituent groups.

Figure 8 plots the relationship between a drug's potency for positively modulating the $GABA_A$ receptor as reflected by electrophysiologic EC_{50} values *versus* its binding affinity for the $\beta^+ - \alpha^-$ sites (A) or the $\alpha^+ - \beta^-/\gamma^+ - \beta^-$ sites (B) as reflected by half-maximal inhibitory concentration values for inhibiting photolabeling by ^{3}[H]azi-etomidate and tritiated R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid, respectively. It shows a significant correlation between drug binding affinity for the $\beta^+ - \alpha^-$ sites and positive modulatory potency with a slope that is not significantly different from unity (0.91 with a 95% CI of 0.64 to 1.18). Thus, every 50% reduction in binding affinity for this class of sites produced (on average) a 50% reduction in positive modulatory potency. In contrast, we found no correlation between drug binding affinity for the $\alpha^+ - \beta^-/\gamma^+ - \beta^-$ sites, and positive modulatory potency as a fit of the data yielded a slope that was not different from 0 (0.10 with a 95% CI of −1.8 to 2.0; $P = 0.903$). These finding strongly suggest that these drugs modulate $GABA_A$ receptor function primarily—if not exclusively—by binding to the $\beta^+ - \alpha^-$ sites.

In silico studies utilizing homology models of the $GABA_A$ receptor and substituted cysteine modification-protection studies indicate that amino acid N265 of the receptor's β subunit is located at the $\beta^+ - \alpha^-$ subunit interface, forms part of the etomidate binding site, and is in close proximity to etomidate's phenyl ring.[20] Mutation of the asparagine to methionine renders $GABA_A$ receptors insensitive to positive modulation by etomidate and other positive modulators that bind to the $\beta^+ - \alpha^-$ site, and significantly reduces the hypnotic and immobilizing potency of etomidate (but not R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid, which binds with very high selectivity to the $\alpha^+ - \beta^-/\gamma^+ - \beta^-$ sites).[19,21–24] We utilized mutant $\alpha_1\beta_3(N265M)\gamma_{2L}$ $GABA_A$ receptors in an attempt to detect any potential positive modulatory action resulting from drug binding to the $\alpha^+ - \beta^-/\gamma^+ - \beta^-$ sites without the confounding impact of positive modulatory actions resulting from binding to the $\beta^+ - \alpha^-$ sites. These studies showed that the N265M mutation completely abolishes positive modulation produced by naphthalene-etomidate, a representative low-

efficacy etomidate analog that binds with similar affinities to both classes of transmembrane anesthetic binding sites. These results suggest that while these drugs can bind to the $\alpha^+ - \beta^-/\gamma^+ - \beta^-$ sites (albeit with relatively low affinity), such binding is unproductive as it does not produce positive modulation.

Can our electrophysiologic results be reasonably explained by the coagonist model simply as a progressive reduction in open-state binding affinity with increasing substituent volume? To test that possibility, we transformed the peak electrophysiologic currents shown in figure 3B into P_{open} values and fit the data as a function of drug concentration to equation 4. For all fits, we constrained the dissociation constant for the closed state (Kd_{closed}) to our previously determined value of 44 μM for etomidate[11] and the number of binding sites (n) to 2 to reflect positive modulation resulting only from drug binding to the two $\beta^+ - \alpha^-$ sites as suggested by our mutation studies. Figure 9A plots the relationship between P_{open} and drug concentration along with the fits to equation 4. Although there was only a single free parameter (Kd_{open}) in each fit and there was a tendency to overestimate the efficacies of the more efficacious compounds, key features of our studies were reproduced by the model as drug potency and efficacy progressively decreased with increasing substituent group volume. Figure 9B plots the relationship between the Kd_{open} calculated from the fits *versus* substituent group volume. A linear fit of this relationship had a slope of 0.038 (95% CI, 0.027 to 0.049), which was significantly different from 0 ($P < 0.001$), and a coefficient of variation of 0.9219. The plot shows that the derived value of Kd_{open} increased progressively with substituent volume as predicted by our hypothesis and indicates that, on average, each 10-$Å^3$ increase in substituent group volume increases Kd_{open} by two- to threefold. This corresponds to a reduction in binding energy of approximately 0.5 kcal/mol for each 10-$Å^3$ increase in substituent group volume.

The importance of anesthetic molecular size in governing anesthetic actions on the $GABA_A$ receptor and other protein targets is well established and has often been attributed to steric hindrance, which limits anesthetic binding to sites having circumscribed dimensions. In one of their seminal works on anesthetic–protein interactions, Franks and Lieb found that although normal alcohol potency for inhibiting firefly luciferase progressively increases with alkyl chain length, this trend stops (and activity is eventually lost) as their lengths exceed those of the binding pocket.[25] In a follow-up study, they reported that the alcohol chain length at which this “cutoff” in activity occurs depends upon the conformational state of the enzyme.[26] Jenkins *et al.* similarly reported a size cutoff in the abilities of volatile anesthetics to positively modulate $GABA_A$ receptors that could be altered by varying the volume of the putative volatile anesthetic binding site using site-directed mutagenesis.[27] We have previously shown a progressive reduction and then cutoff at a molecular volume of 120 $Å^3$ in the abilities of both alcohols and volatile anesthetics to positively modulate the structurally homologous serotonin type 3A receptor.[28] The current data show an analogous cutoff as the binding affinities (to the $\beta^+ - \alpha^-$ sites) and positive modulatory activities of the etomidate analogs progressively decreased with substituent volume with analogs having the largest substituent groups possessing very low affinities and little modulatory activities.

In summary, our results show that the $GABA_A$ receptor positive modulatory activities of phenyl ring–substituted etomidate analogs (1) decrease with substituent group volume,

reflecting decreases in both analog potencies and efficacies; (2) are strongly correlated with their affinities for the $GABA_A$ receptor's two $\beta^+ - \alpha^-$ (but not their $\alpha^+ - \beta^-/\gamma^+ - \beta^-$) transmembrane anesthetic binding sites; and (3) can be generally accounted for by a simple model in which open-state binding affinity progressively decreases with increasing substituent group volume. Although we found that all of these drugs can bind to the $\alpha^+ - \beta^-/\gamma^+ - \beta^-$ transmembrane anesthetic binding sites, and those with the largest substituent groups do so with affinities that are similar to their affinities for the $\beta^+ - \alpha^-$ sites, we found no evidence that such binding causes significant positive receptor modulation. Together, these results are consistent with the hypothesis that the binding pocket within each $\beta^+ - \alpha^-$ anesthetic binding site where etomidate's phenyl ring lies becomes smaller as the receptor isomerizes from closed to open, sterically hindering etomidate analogs with phenyl ring substituent groups from binding to the open state, thus decreasing their open-state affinities and reducing their positive modulatory potencies and efficacies in a manner that directly correlates with their substituent volumes.

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Editor's Perspective

What We Already Know about This Topic

- Positive γ-aminobutyric acid type A ($GABA_A$) receptor modulators bind with higher affinity when the receptor is in the open state, increasing the fraction of open state receptors and, thereby, enhancing receptor function
- Appending a phenyl ring substituent group onto the phenyl ring of etomidate abolished its conformational state selectivity and binding site selectivity, reducing the intrinsic efficacy of etomidate and turning it into an anesthetic-selective competitive $GABA_A$ receptor antagonist

What This Article Tells Us That Is New

- γ-Aminobutyric acid type A ($GABA_A$) receptor positive modulatory activities of phenyl ring–substituted etomidate analogs decreased with increasing substituent group volume, reflecting decreases in both potencies and efficacies of the analogs
- Their $GABA_A$ receptor positive modulatory activities were strongly correlated with their affinities for the two $\beta^+ - \alpha^-$ transmembrane anesthetic binding sites of the $GABA_A$ receptor
- Open-state binding affinity decreased progressively with increasing substituent group volume

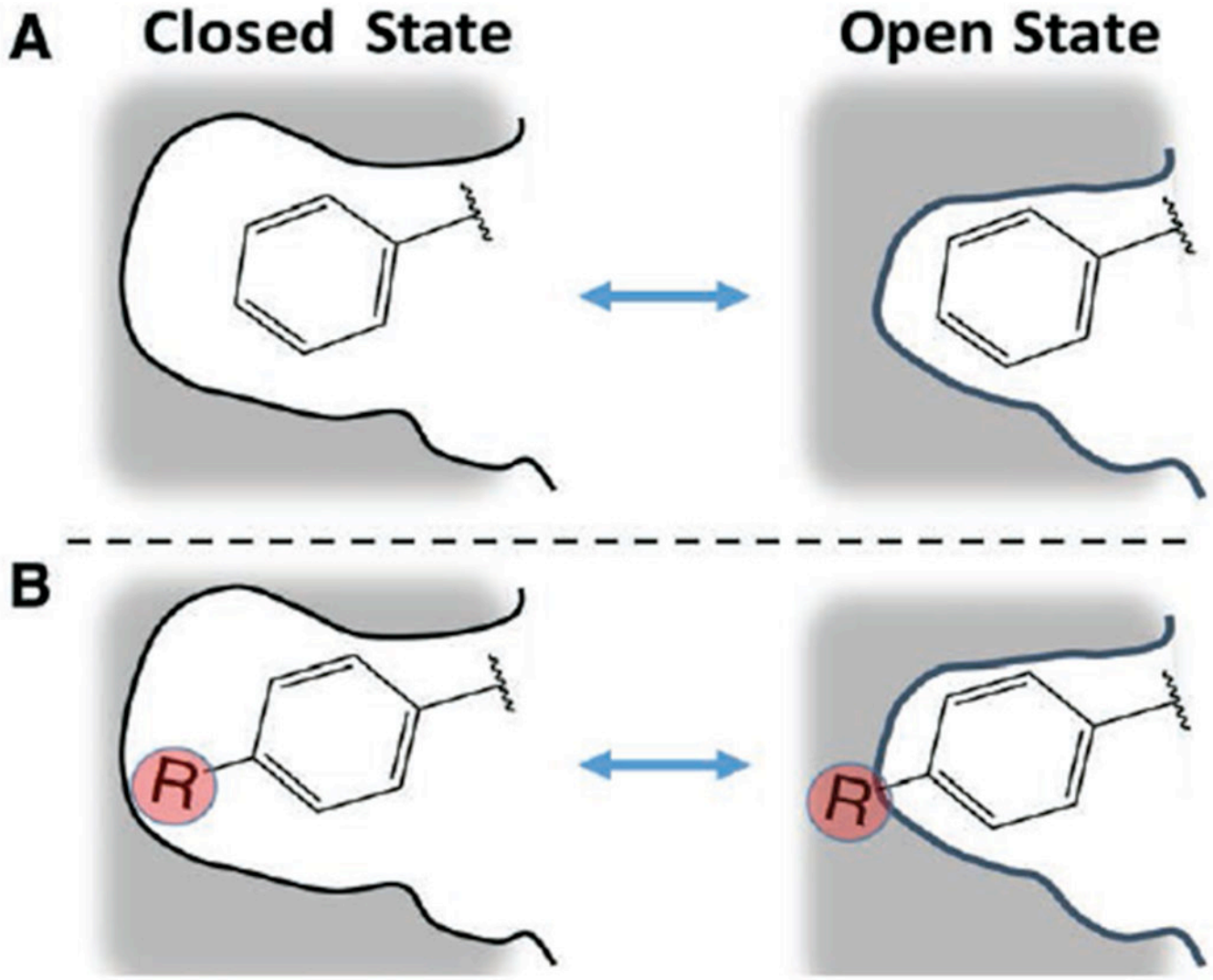

Fig. 1.

Illustration depicting the "state-dependent cutoff" hypothesis. It is hypothesized that the size of the hydrophobic pocket where etomidate's phenyl ring lies becomes smaller when the γ-aminobutyric acid type A receptor isomerizes from the closed to the open state. This reduction in pocket size directly impacts binding affinity. (*A*) For etomidate, Van der Waals interactions strengthen because there is a closer fit between the anesthetic and its binding site. This increases etomidate's binding affinity for the open state (*vs.* the closed state), imparting high intrinsic efficacy. (*B*) For etomidate analogs with bulky phenyl ring substituent groups, the open state's smaller pocket size also introduces steric hindrance. This repulsive force reduces binding affinity to the open state, thus decreasing both potency and intrinsic efficacy relative to etomidate. Only the drug's phenyl ring is shown in this cartoon. *R* is the substituent group, which is located at the para position of the ring in this example.

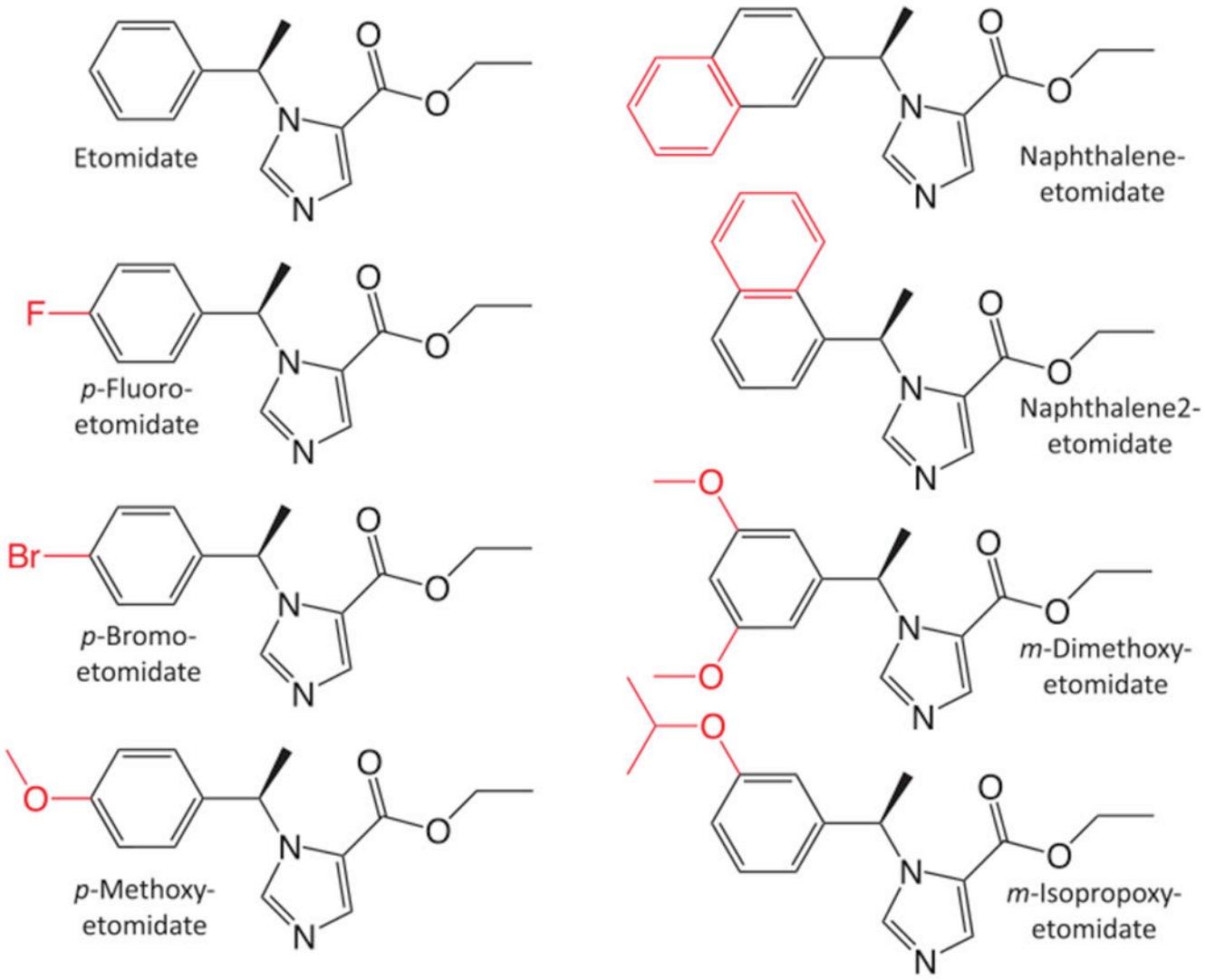

Fig. 2.

Molecular structures of etomidate and etomidate analogs containing phenyl ring substituent groups. N = nitrogen; O = oxygen.

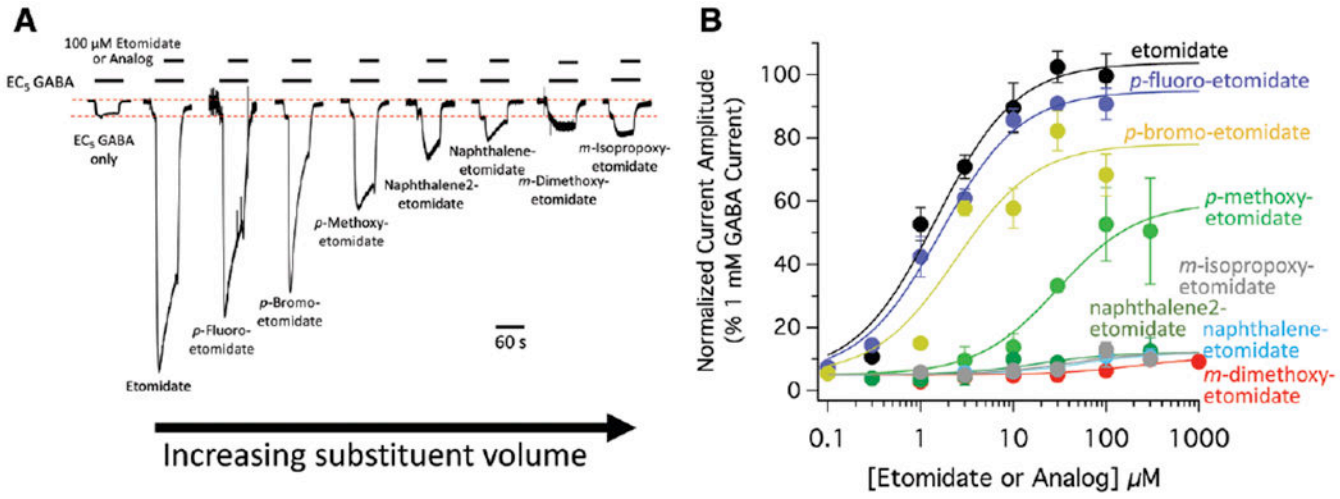

Fig. 3.

Potentiation of $\alpha_1,\beta_3\gamma_{2L}$ γ-aminobutyric acid type A receptor currents by etomidate and etomidate analogs containing phenyl ring substituent groups. (*A*) Electrophysiologic traces showing the potentiating effect of these drugs (all at 100 μM) on currents evoked by a γ-aminobutyric acid (GABA) concentration that elicits 5% of the current evoked by 1 mM GABA (EC_5 GABA). The amplitude of each trace is normalized to that evoked by EC_5 GABA alone, which is indicated by the *dashed red line*. (*B*) Etomidate and etomidate analog concentration-response curves for potentiation of EC_5 GABA-evoked currents. Each *symbol* is the mean ± SD derived from four different oocytes. The *curves* are fits of the datasets to equation 1 with the fitted results given in table 1.

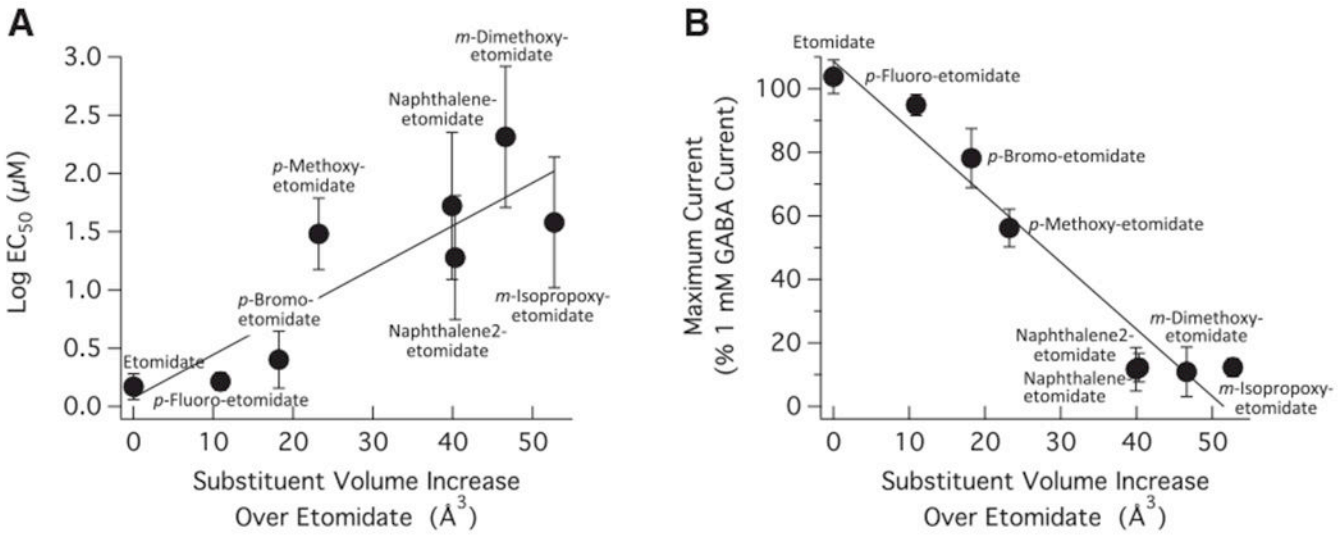

Fig. 4.

The relationship between the logarithm of a drug's EC_{50} (*A*) or the maximal peak current that a drug elicits at high concentrations (*B*) and the size of its phenyl ring substituent group. Substituent group volume is expressed as the increase over that of etomidate. Each data point and its 95% CI were derived by fitting the drug concentration-response curves for potentiation of 5% of the current evoked by 1 mM γ-aminobutyric acid (GABA) shown in fig. 3 to equation 1. The *line* in each is a linear least squares fit of the dataset.

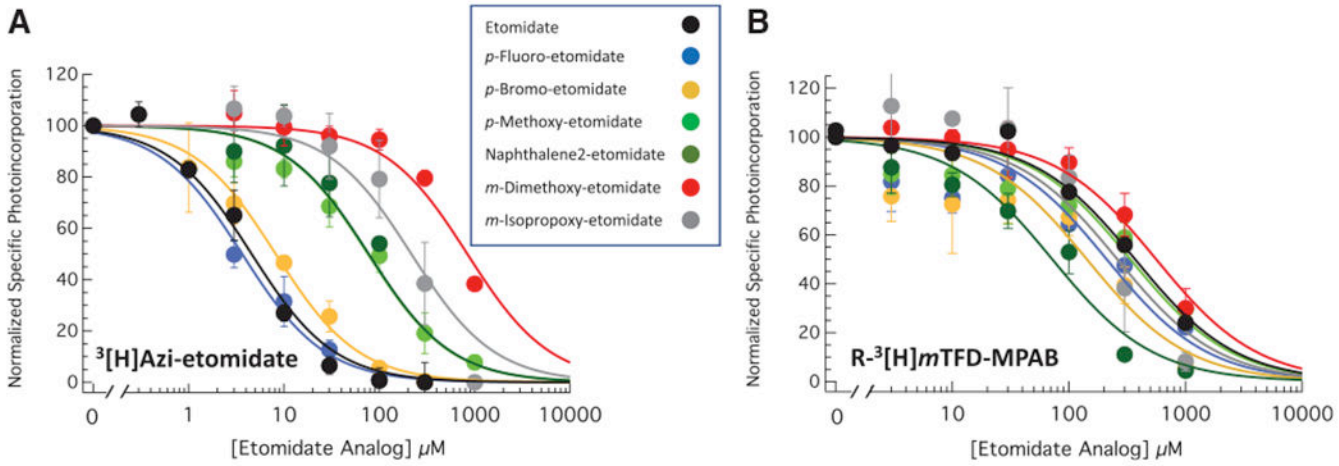

Fig. 5.

Etomidate and etomidate analog concentration-response curves for inhibition of specific ^{3}[H]azi-etomidate (*A*) and tritiated R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid (R-^{3}[H]*m*TFD-MPAB; *B*) photolabeling of $\alpha_1,\beta_3\gamma_{2L}$ γ-aminobutyric acid (GABA) type A receptors. Each *curved line* is a fit of the dataset to equation 2. Each *symbol* is the mean ± SD derived from three or four separate experiments. Data were normalized to counts per minute measured in the absence of competing ligand. Nonspecific photolabeling was defined in the presence of 300 μM etomidate (for ^{3}[H]azi-etomidate photolabeling experiments) and either 60 μM or 100 μM R-5-allyM-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid (for R-^{3}[H]*m*TFD-MPAB photolabeling experiments). All photolabeling was done in the presence of 300 μM GABA to stabilize receptors in the open/desensitized state.

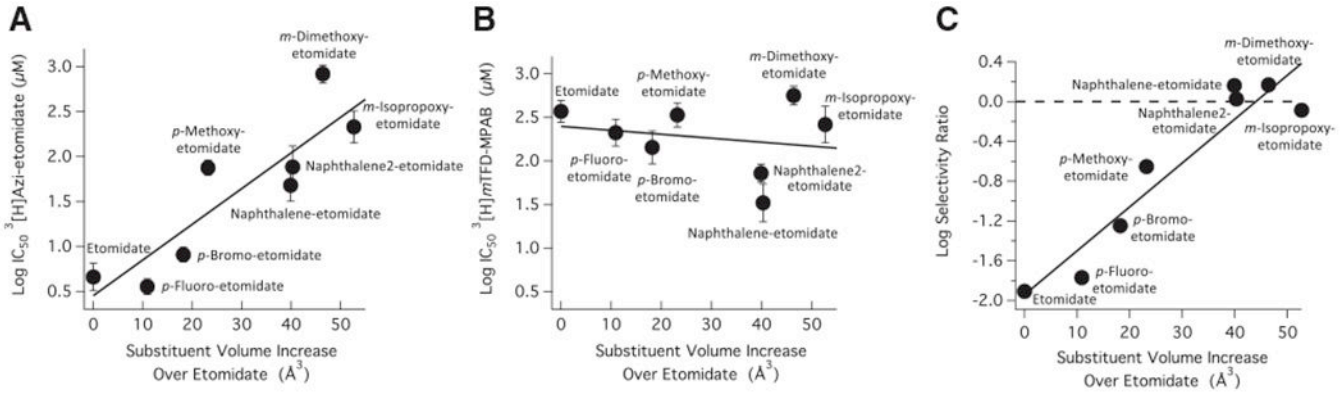

Fig. 6.

The relationship between a drug's half-maximal inhibitory concentration (IC_{50}) for inhibiting ^{3}[H]azi-etomidate photolabeling (*A*) or tritiated R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid photolabeling (*B*) and the size of its phenyl ring substituent group. The relationship between a drug's binding site selectivity ratio (defined as the IC_{50} for inhibiting ^{3}[H]azi-etomidate photolabeling ÷ the IC_{50} for inhibiting tritiated R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid photolabeling) and the volume of its phenyl ring substituent group (*C*). Substituent group volume is expressed as the increase over that of etomidate. *Error bars* on each point indicate the 95% CI. The *line* in each panel is a linear least squares fit of the dataset. Naphthalene-etomidate photoaffinity labeling data are from Ma *et al.*[11]

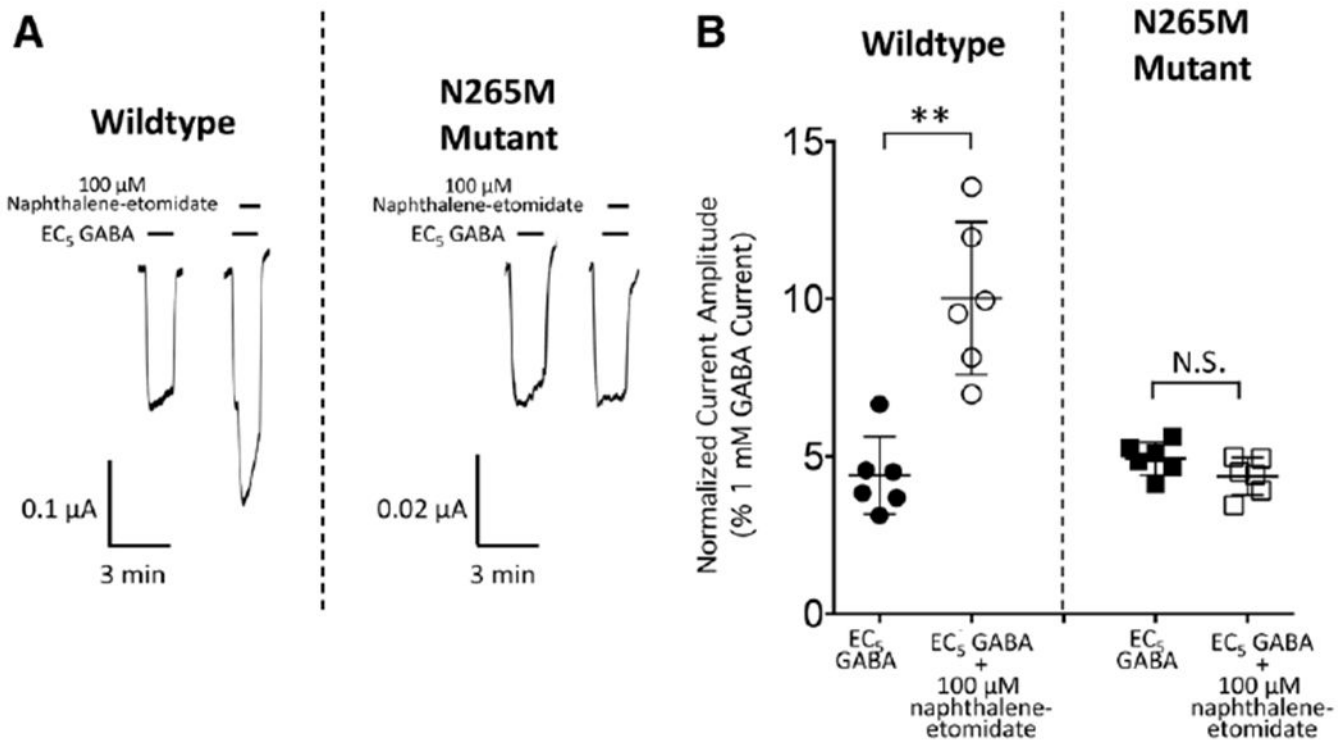

Fig. 7.

Potentiation of wild-type $\alpha_1\beta_3\gamma_{2L}$ γ-aminobutyric acid type A ($GABA_A$) receptor currents or mutant $\alpha_1\beta_3$(N265M)γ_{2L} $GABA_A$ receptor currents by 100 μM naphthalene-etomidate. (*A*) Electrophysiologic traces showing the effect of naphthalene-etomidate on currents evoked by a γ-aminobutyric acid (GABA) concentration that elicits 5% of the current evoked by 1 mM GABA (EC_5 GABA) and mediated by either wild-type (*left*) or mutant $\alpha_1\beta_3$(N265M)γ_{2L} (*right*) $GABA_A$ receptors. (*B*) Summary data (n = 6 oocytes/experimental condition) showing the impact of 100 μM naphthalene-etomidate on currents evoked by EC_5 GABA and mediated by either wild-type (*left*) or mutant $\alpha_1\beta_3$(N265M)γ_{2L} (*right*) $GABA_A$ receptors. In this panel, each *symbol* represents data obtained from a single oocyte, and the *lines* indicate the mean ± SD of each dataset. N.S. = not significant. ** P = 0.002.

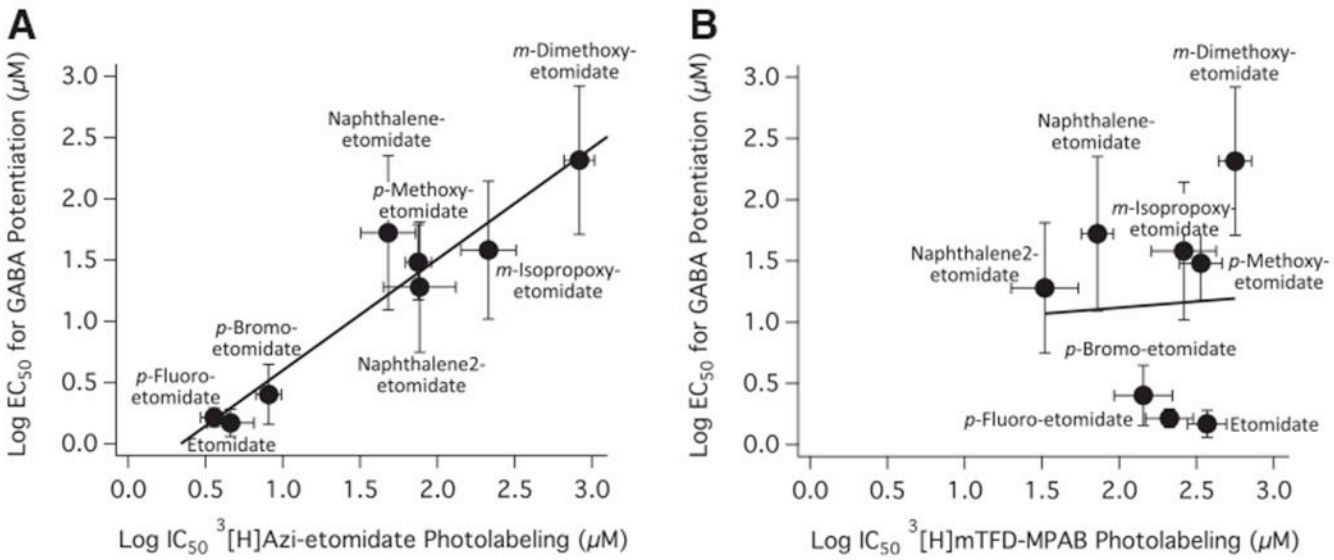

Fig. 8.

The relationship between the logarithm of a drug's EC_{50} and the logarithm of its half-maximal inhibitory concentration (IC_{50}) for inhibiting ^{3}[H]azi-etomidate photolabeling (*A*) or R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid photolabeling (*B*). *Error bars* on each point indicate the 95% CI. The *line* in each panel is a linear least squares fit of the dataset. GABA = γ-aminobutyric acid.

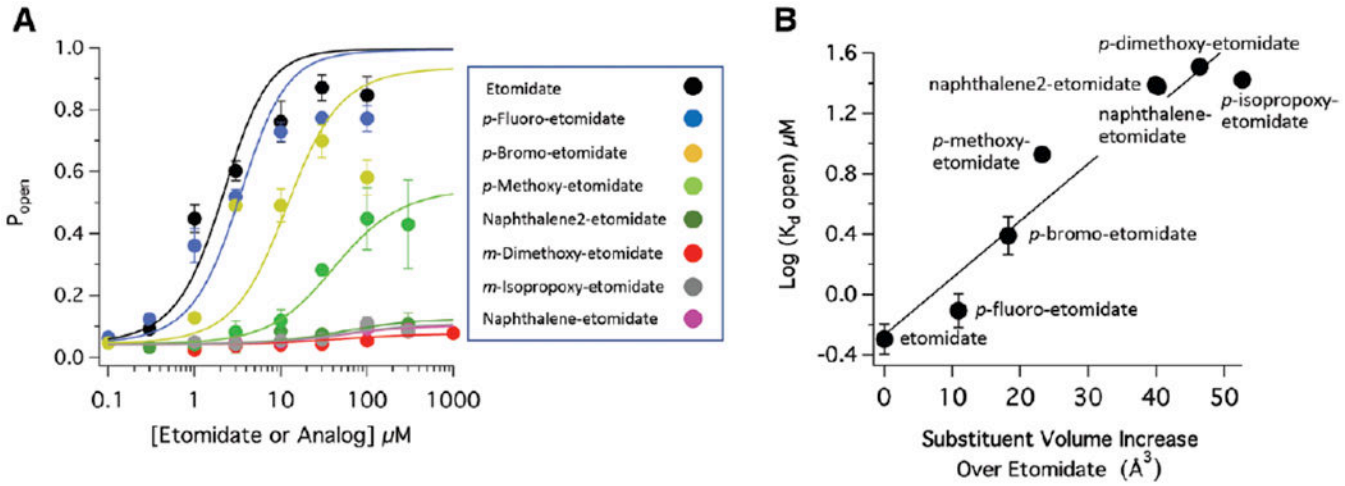

Fig. 9.

Allosteric coagonist model analysis of $\alpha_1\beta_3\gamma_{2L}$ γ-aminobutyric acid type A ($GABA_A$) receptor positive modulation by etomidate and etomidate analogs. (*A*) $GABA_A$ receptor open state probability (P_{open}) as a function of etomidate or etomidate analog concentration. The *curves* are fits of the datasets to equation 4 to define Kd_{open} with Kd_{closed} constrained to 44 μM and the number of binding sites (*n*) constrained to 2. (*B*) The model-dependent relationship between the logarithm of Kd_{closed} and the size of its phenyl ring substituent group over etomidate. *Error bars* on each point indicate the 95% CI. The *line* in each panel is a linear least square fit of the dataset.

Table 1.

Molecular Properties and γ-Aminobutyric Acid Type A ($GABA_A$) Receptor Electrophysiologic Pharmacology of Etomidate and Etomidate Analogs

Compound	Molecular Volume Increase Over Etomidate Å^3	Octanol/Buffer Partition Coefficient (SD)	$GABA_A$ Receptor EC_{50} μM (95% CI)	$GABA_A$ Receptor Maximal Current Amplitude %1 mM GABA (95% CI)
Etomidate	0	731 * (72)	1.5 (1.1–1.9)	104 (99–109)
p-Fluoro-etomidate	10.9	420 (280)	1.6 (1.4–2.0)	95 (92–98)
p-Bromo-etomidate	18.2	870 (370)	2.5 (1.4–4.4)	78 (69–88)
p-Methoxy-etomidate	23.2	1,140 (64)	30 (15–61)	60 (48–71)
Naphthalene2-etomidate	39.9	7,810 (2150)	19 (6–65)	13 (10–15)
Naphthalene-etomidate	40.3	13,510 (250)	53 (12–230)	12 (9–16)
m-Dimethoxy-etomidate	46.4	1,070 (44)	210 (51–830)	11 (8–14)
m-Isopropoxy-etomidate	52.7	1,490 (640)	38 (10–140)	12 (9–15)

* Data from Pejo *et al.*[14]

GABA = γ-aminobutyric acid.

Table 2.

Etomidate and Etomidate Analog Potencies for Inhibiting Photoaffinity Labeling of γ-Aminobutyric Acid Type A ($GABA_A$) Receptors by ^{3}[H]Azi-etomidate and R-^{3}[H]*m*TFD-MPAB

Compound	IC_{50} for Inhibiting Photoaffinity Labeling by ^{3}[H]Azi-etomidate μM (95% CI)	IC_{50}* for Inhibiting Photoaffinity Labeling toy R-^{3}[H]*m*TFD-MPAB μM (95% CI)	Selectivity Ratio*
Etomidate†	4.6 (3.2–6.5)	370 (280–490)	0.012
p-Fluoro-etomidate	3.6 (2.9–4.4)	210 (150–300)	0.017
p-Bromo-etomidate	8.1 (6.7–9.8)	140 (93–220)	0.058
p-Methoxy-etomidate	75.0 (62–91)	340 (250–460)	0.22
Naphthalene2-etomidate	77 (51–115)	72 (57–91)	1.1
Naphthalene-etomidate	48† (28–81 μM)	33† (20–54 μM)	1.5
m-Dimethoxy-etomidate	830 (66–1,040)	560 (440–720)	1.5
m-Isopropoxy-etomidate	210 (140–320)	260 (160–420)	0.81

*(IC_{50} forinhibiting ^{3}[H]azi-etomidate photolabeling) + (IC_{50} for inhibiting R-^{3}[H]*m*TFD-MPAB photolabeling).

†Data from Ma *et al.*[11]

IC_{50} = half-maximal inhibitory concentration; R-^{3}[H]*m*TFD-MPAB = tritiated R-5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid.