Gating Allosterism at a Single Class of Etomidate Sites on $\alpha_1\beta_2\gamma_2L$ GABA$_A$ Receptors Accounts for Both Direct Activation and Agonist Modulation*

Dirk Rüsch‡§, Huijun Zhong‡, and Stuart A. Forman‡¶

From the ¶Department of Anesthesia and Critical Care, Massachusetts General Hospital, Boston, Massachusetts 02114, the §Department of Anesthesia, Harvard Medical School, Boston, Massachusetts 02115, and the ¶Department of Anesthesia and Critical Care, University Hospital Schleswig-Holstein, 24105 Kiel, Germany

At clinical concentrations, the potent intravenous general anesthetic etomidate enhances $\gamma$-aminobutyric acid, type A (GABA$_A$) receptor activity elicited with low $\gamma$-aminobutyric acid (GABA) concentrations, whereas much higher etomidate concentrations activate receptors in the absence of GABA. Therefore, GABA$_A$ receptors may possess two types of etomidate sites: high affinity GABA-modulating sites and low affinity channel-activating sites. However, GABA modulation and direct activation share stereoselectivity for the $(R)/(+)$-etomidate isomer and display parallel dependence on GABA$_A$ $\beta$ subunit isoforms, suggesting that these two actions may be mediated by a single class of etomidate site(s) that exert one or more molecular effects. In this study, we assessed GABA modulation by etomidate using leftward shifts of electrophysiologically GABA concentration responses in cells expressing human $\alpha_1\beta_2\gamma_2$ receptors. Etomidate at up to 100 $\mu$m reduced GABA EC$_{50}$ values by over 100-fold but without apparent saturation, indicating the absence of high affinity etomidate sites. In experiments using a partial agonist, P4S, etomidate both reduced EC$_{50}$ and increased maximal efficacy, demonstrating that etomidate shifts the GABA$_A$ receptor gating equilibrium toward open states. Results were quantitatively analyzed using equilibrium receptor gating models, wherein a postulated class of equivalent etomidate sites both directly activates receptors and enhances agonist gating. A Monod-Wyman-Changeux co-agonist mechanism with two equivalent etomidate sites that allosterically enhance GABA$_A$ receptor gating independently of agonist binding most simply accounts for direct activation and agonist modulation. This model also correctly predicts the actions of etomidate on GABA$_A$ receptors containing a point mutation that increases constitutive gating activity.

The major mediator of synaptic inhibition in the brain is the GABA$_A$ receptor-chloride channel complex, which is formed from five homologous subunits arranged around a gated membrane pore. Multiple GABA$_A$ receptor subunit classes ($\alpha, \beta, \gamma, \delta, \epsilon, \pi$, and $\rho$) and isoforms have been identified, and most native receptors in brain contain a combination of $\alpha$, $\beta$, and $\gamma$ subunits (1, 2). GABA$_A$ receptors are modulated by sedatives, anxiolytics, anticonvulsants, and many general anesthetics, including neurosteroids (3–5), barbiturates (6), propofol (7, 8), etomidate (9, 10), and halogenated volatile inhalants (12–14).

Clinical concentrations of these anesthetics enhance GABA$_A$ receptor activity (15), which is observed electrophysiologically as increased multichannel responses to low GABA concentrations (e.g. EC$_5$ or EC$_{10}$). Responses to maximally activating GABA concentrations are either not or weakly enhanced by general anesthetics. As a result, general anesthetics shift GABA concentration-response relationships leftward (reduce EC$_{50}$), which could be caused by either enhanced GABA binding to agonist sites or to enhanced gating of ligand-bound receptors (16, 17).

Supra-clinical concentrations of general anesthetics can also directly activate GABA$_A$ receptors (agonism or GABA-mimetic activity) (18–20). The widely different concentrations of general anesthetics that induce GABA modulation versus direct activation suggest that there may be two distinct types of sites on GABA$_A$ receptors as follows: high affinity sites that enhance apparent GABA sensitivity, and low affinity sites that directly activate receptors. Alternative mechanisms, postulating a single class of sites that change affinity for general anesthetic depending on the receptor state, can also account for multiple actions. These unified mechanisms fall into two generic classes: orthosteric partial agonism (where anesthetics act via the GABA sites) and allosteric co-agonism (where anesthetics act via sites distinct from GABA sites). In one electrophysiological study, three effects of n-octanol on $\alpha_1\beta_2\gamma_2$ GABA$_A$ receptors, GABA modulation, direct activation, and inhibition, were explained by partial agonism (21). In a study of the effects of pentobarbital on brain synaptosomal GABA$_A$ channels, Cash and Subbarao (22) considered an allosteric co-agonist mechanism where distinct pentobarbital and GABA-binding sites couple independently to the receptor open-closed equilibrium via Monod-Wyman-Changeux (MWC) allosterism (preferential binding to the open state).

These different mechanisms predict different effects of general anesthetic on experimental parameters that reflect the equilibria between closed and open receptors. If two distinct classes of saturable sites have sufficiently different affinities for an anesthetic, then reliable measures of GABA modulation should display maximal effects at anesthetic concentrations below those that induce maximal direct activation. Partial agonist and co-agonist mechanisms also predict different relationships between GABA EC$_{50}$ and anesthetic concentrations.
Orthosteric partial agonism predicts that as general anesthetic concentration rises, GABA EC\textsubscript{50} will at first decrease (positive modulation) but then increase again as competition for occupation of GABA-binding sites develops. In contrast, allosteric co-agonism predicts that GABA EC\textsubscript{50} will only decrease, approaching a minimum in the same anesthetic concentration range where direct activation reaches a maximum. Furthermore, models that postulate a single class of sites mediating multiple anesthetic actions predict that structurally similar anesthetics (e.g., stereoisomers) will likely display similar relative potencies for their multiple actions.

The intravenous general anesthetic etomidate (ethyl-1-((a-methylbenzyl)imidazole-5-carboxylate) presents several advantages for distinguishing between possible mechanisms of GABA\(_A\) receptor modulation. It is a very potent clinical anesthetic, causing loss of righting responses in tadpoles at about 3 \(\mu\)M (19, 20) and loss of responsiveness in humans at about 2 \(\mu\)M (23). Modulation of apparent GABA affinity for GABA\(_A\) receptors (i.e., \(K_D\) or EC\textsubscript{50}) is seen with etomidate concentrations as low as 0.1 \(\mu\)M (9, 24). Etomidate alone at concentrations above 10 \(\mu\)M directly activates GABA\(_A\) receptors, exhibiting maximal efficacy up to 50% relative to GABA, depending on subunit combination (9, 25). Moreover, etomidate contains a chiral center. Both direct activation of GABA\(_A\) receptors and modulation of GABA responses by \((R)^{(+)}\)-etomidate is seen at about 20 times lower concentrations than with \((S)^{(-)}\)-etomidate, closely paralleling the relative anesthetic potency of these enantiomers in animals (19, 20, 26). Together with recent transgenic animal experiments (27, 28), stereoselectivity tightly links etomidate anesthesia to GABA\(_A\) receptors containing \(\beta_2\) and \(\beta_3\) subunits. Stereoselectivity also constitutes the strongest evidence for etomidate-binding sites within the GABA\(_A\) receptor-ionophore complex (26).

In this study, we examined the relationship between etomidate-induced direct activation of GABA\(_A\) receptors and GABA modulation assessed with EC\textsubscript{50} shifts. We also used a partial agonist, P4S, to investigate whether etomidate-induced leftward shift is due to enhancement of agonist-receptor binding or channel gating. Activity was measured electrophysiologically in voltage-clamped Xenopus oocytes or HEK293 cells expressing human \(\alpha_1\beta_2\gamma_2\) GABA\(_A\) receptors. Results obtained over a wide range of etomidate concentrations were comparatively analyzed using equilibrium partial agonist and co-agonist models that account for multiple etomidate effects via a single class of GABA\(_A\) receptor-binding sites. Finally, we tested whether an MWC co-agonist model predicts etomidate effects on mutant GABA\(_A\) receptors characterized by a high degree of spontaneous activity.

**EXPERIMENTAL PROCEDURES**

**Expression of Recombinant \(\alpha_1\beta_2\gamma_2\) GABA\(_A\) Receptors in Xenopus laevis Oocytes and HEK293 Cells—**DNA plasmids encoding human \(\alpha_1\), \(\beta_2\), and \(\gamma_2\) GABA\(_A\) receptors subunits in pcDNA3 vectors (Invitrogen) were gifts from Dr. Paul Whiting (Merck). The \(\alpha_1\)L294T mutation was introduced as described previously (29). Subunit mRNAs were mixed in a w/w ratio of 1:1:1:4:4 and injected into oocytes as described previously (19). Injected oocytes were incubated at 18 °C for 48–144 h before electrophysiological experiments.

**Maintenance of HEK293 cells (ATCC: Manassas, VA) and methods for transient transfection of GABA\(_A\) receptors have been described (29).** Cells were transfected with GABA\(_A\) subunit cDNA mixtures at a ratio of 1:1:1:2:5, along with a marker plasmid encoding green fluorescent protein.

**Chemicals—**All chemicals were from Sigma unless otherwise indicated. GABA was stored at −80 °C as an aqueous 1 M stock solution, and aliquots were thawed and diluted into electrophysiology solution each day. P4S was freshly prepared each day as a 1 M stock. \((R)^{(+)}\)-etomidate (Bedford Laboratories, Bedford, OH) solutions were also prepared daily.

**Two Microelectrode Oocyte Electrophysiology—**Oocyte electrophysiological measurements were carried out at 21–23 °C in ND-96 solution containing NaCl 96, KCl 2, MgCl\(_2\) 0.8, CaCl\(_2\) 1, HEPES 5, pH 7.5, as described previously (19). Oocytes were placed in a custom-built low volume (20 \(\mu\)l) flow chamber, and currents were elicited with ND-96 solutions containing etomidate, GABA (± etomidate), or P4S (± etomidate), delivered by gravity from glass reservoirs via polytetrafluoroethylene (PTFE) tubing and fiber-actuated solenoid valves. Two sets of EC\textsubscript{50} values were analyzed using a program fit the same data set with (i) the Hill function and (ii) a composite Hill-Richardson model

\[
\text{EC}_{50} = \frac{\min \text{GABA}}{1 + \left(\frac{\text{GABA}}{K_{\text{HILL}}^\text{GABA}}\right)^{n_{\text{HILL}}}}
\]

where \(K_{\text{HILL}}^\text{GABA}\) is the Hill constant. The \(n_{\text{HILL}}\) parameter reflects the slope of the Hill equation.

Whole-cell solution exchange times, estimated from currents elicited by 0.1–1 mM GABA, were 2 \(\mu\)s. Whole-cell currents and was continuously present during the agonist concentration-response experiments. Oocyte currents were elicited with etomidate plus alternating with 1 mM GABA plus etomidate for normalization.

**Analysis of GABA Concentration Responses—**Data analysis was performed offline. For responses elicited with GABA alone, peak currents were corrected by subtracting the base-line leak current, which was usually below 0.1 \(\mu\)A in oocytes. Corrected peak currents were normalized to the average of pre- and post-control maximal GABA responses measured at 1–10 mM, \(I_{\text{max}}^\text{GABA}\). Direct activation by etomidate was estimated by subtracting the average leak current from sweeps where there was no etomidate from the average leak when etomidate was present in the same oocyte. Oocyte current responses elicited with etomidate plus GABA were corrected by subtracting only the average leak in buffer (i.e., \(I_{\text{max}}^\text{GABA}\) minus \(I_{\text{max}}^\text{ETOM}\)). Leak-corrected responses were normalized to the average of pre- and post-control responses to etomidate plus maximal activating GABA (1–10 mM), \(I_{\text{max}}^\text{GABA}+\text{ETOM}\). Scaling of responses in the presence and absence of etomidate was achieved by multiplying the ratio of currents elicited with maximal GABA plus etomidate and with maximal GABA alone, \(I_{\text{max}}^\text{GABA}+\text{ETOM}/I_{\text{max}}^\text{GABA}\). At etomidate concentrations below 100 \(\mu\)M, inhibition by etomidate was minimal, and \(I_{\text{max}}^\text{GABA}+\text{ETOM}\) was larger than \(I_{\text{max}}^\text{GABA}\) in most oocytes.

In the absence of etomidate, \(\alpha_1\beta_2\gamma_2\) GABA\(_A\) receptors display negligible spontaneous activity. Therefore, normalized data from individual oocytes were fitted by non-linear least squares with Equation 1 (Hill

\[
I = I_{\text{max}} \left(\frac{[\text{GABA}]^n}{[\text{GABA}]^n + K_{\text{HILL}}^\text{GABA}^n}\right)
\]

where \(I_{\text{max}}\) is the maximum response at saturating [GABA], \([\text{GABA}]\) is the concentration of GABA, and \(K_{\text{HILL}}\) is the Hill constant.
or logistic), where EC_{50}^{GABA} is the half-maximal activating GABA concentration, and \( n \) is the Hill coefficient, \[ \frac{GABA}{P_{\text{max}}} = \frac{1}{1 + (EC_{50}^{GABA} / [GABA])^n} \] (Eq. 1)

The ratios of EC_{50}^{GABA} values measured in the presence and absence of etomidate (left-shift ratios), \( EC_{50}^{GABA}/EC_{50}^{GABA} \), were calculated for data recorded in individual oocytes.

**Determination of Model Parameters**—By using equations defining equilibrium \( P_{\text{open}} \) as functions of GABA and etomidate concentrations, parameters for the partial agonist model (Fig. 5) and allosteric co-agonist models (Fig. 6) were independently fitted to pooled concentration-response data from oocytes. Data for these fits were converted to estimated \( P_{\text{open}} \), by re-normalizing to a maximal open probability \( P_{\text{max}} \) of 0.85 in the presence of GABA alone. \( P_{\text{max}}^{GABA} \) was based both on published single channel estimates (31–33) and on the degree of current enhancement observed in the presence of high GABA plus etomidate. Because GABA EC_{50}^{GABA} values varied among individual oocytes, we fitted GABA parameters for the models to a restricted set of GABA concentration responses from 10 oocytes characterized by EC_{50}^{GABA} values between 32 and 48 \( \mu \)M. Etomidate direct activation parameters were fitted to the oocyte data shown in Fig. 1 (right panel).

For the partial agonist model, Equation 4 from Kurata et al. (21) was fitted to estimated \( P_{\text{open}} \) as GABA data to derive \( K_p \) (the microscopic GABA dissociation constant), \( c \) (the binding allosteric factor for GABA), and \( n_p \) (the gating efficacy with both sites occupied by GABA). An analogous equation was fitted to estimated direct activation \( P_{\text{open}} \) versus [etomidate] data to derive \( K_p \) (the microscopic etomidate dissociation constant), \( b \) (the binding allosteric factor for etomidate), and \( n_p \) (the gating efficacy with both sites occupied by etomidate). These six parameters were used in Equation 6 from Kurata et al. (21) to generate \( P_{\text{open}} \) values for combinations of GABA and etomidate. The remaining model parameter, \( \alpha_p \) (gating efficacy with one agonist site occupied by GABA and the other site occupied by etomidate), was varied manually, and EC_{50}^{GABA}/EC_{50}^{GABA} ratios were derived from model-generated \( P_{\text{open}} \) versus [GABA] curves for etomidate concentrations of 0.32, 1.3, 32, 320, and 100 \( \mu \)M (Fig. 4, bottom). Least squares analysis was applied to log-transformed model versus experimental EC_{50}^{GABA}/EC_{50}^{GABA} ratios to derive the optimal \( X \) value.

For the Monod-Wyman-Changeux co-agonist models, parameters for GABA binding (\( K_p \), the microscopic equilibrium dissociation constant) and efficacy (\( c \), the ratio of GABA open state dissociation constant to closed state dissociation constant) were derived from the estimated \( P_{\text{open}} \) versus [GABA] data set by non-linear least squares fitting to Equation 3 (34, 35) where \( L_0 \) (the basal gating equilibrium constant for unliganded receptors; [RU/0]) was constrained to an experimentally determined value of 70,000 (see Fig. 6B), and the number of GABA sites was constrained to two, \[ P_{\text{open}} = \frac{1}{1 + L_0 (1 + [GABA] / K_p) (1 + [GABA] / cK_p)^n} \] (Eq. 3)

Parameters for etomidate binding and efficacy (\( K_p \) and \( n \), defined analogously to \( K_p \) and \( c \) above) in models with different numbers of etomidate sites were derived from the direct activation estimated \( P_{\text{open}} \) versus [etomidate] data set by non-linear least squares fits to a similar Equation 4, with \( L_0 \) constrained to 70,000, and the number of etomidate sites (\( n_p \)) constrained to integers between 1 and 5,

Simulated \( P_{\text{open}} \) values for the MWC co-agonist models containing different numbers of etomidate sites were generated by using Equation 5, using \( L_0 = 70,000, K_p, c \) from the fit to Equation 3, and \( K_p, c \) from the appropriate fit to Equation 4,

\[ P_{\text{open}} = \frac{1}{1 + L_0 (1 + [GABA] / K_p) (1 + [Etom] / K_E)^c} \] (Eq. 5)

Model calculations were performed using spreadsheet software (Excel, Microsoft Corp., Redmond, WA), and the results were transferred to Origin worksheets for display and analysis. Statistical analyses were performed with Excel. Non-linear least squares fits were performed using Origin software (version 6.1, Microcal Software, Inc., Northampton, MA).

**RESULTS**

**Direct Activation and GABA Modulation by (R)-Etomidate in Human \( \alpha_1\beta_2\gamma_2 \) GABA_A Receptors Expressed in Xenopus Oocytes and HEK293 Cells**—We and others have previously reported (19, 20, 25) electrophysiological data from Xenopus oocytes expressing \( \alpha_1\beta_2\gamma_2 \) GABA_A receptors that compared direct activation by etomidate with maximal activation by GABA. Our results showed that etomidate is a moderately efficacious agonist, eliciting about 20% of the maximal GABA-activated current in oocytes, with an EC_{50} of 64 \( \mu \)M and Hill coefficient of 1.5 (19).

A potential limitation in electrophysiological studies of ligand-gated channels using Xenopus oocytes, because of their size, is slow agonist concentration-jump rates. If the current activation rate is slower than receptor desensitization, then peak currents can be significantly underestimated, leading to underestimated EC_{50} values and Hill slopes. In our low volume flow chamber for oocytes (20 \( \mu \)l) using superfusion rates of 5 ml/min, we achieve 4 volume exchanges/s. This is almost 20 times faster than maximal desensitization rates of 0.25 s^{-1} recently reported by Boileau et al. (36) for HEK293-expressed \( \alpha_1\beta_2\gamma_2 \) receptors. Based on the observation that overexpression of \( \gamma_2 \) minimizes fast desensitization (36), we used 4–5-fold excess \( \gamma_2 \) relative to \( \alpha_1 \) and \( \beta_2 \) mRNA or cDNA. We then compared normalized peak responses of the oocyte-expressed \( \alpha_1\beta_2\gamma_2 \) GABA_A receptors with currents from small HEK293 cells and excised membrane patches, elicited using a device that exchanges solutions in less than 1 ms (Figs. 1 and 2). When peak HEK293 cell and patch currents elicited with etomidate were normalized to maximal GABA-activated responses, the apparent potency and efficacy of etomidate direct activation was similar to that observed in oocyte currents (Fig. 1, right). In addition, maximal GABA-induced desensitization rates in the currents we recorded from HEK293 cells and patches (\( n = 20 \)) averaged 0.7 s^{-1}. Simulations using a solution exchange rate of 4 s^{-1} (an underestimate of activation rate at high GABA) and desensitization rates of 0.25 and 0.7 s^{-1} suggest that oocyte peak currents at high GABA may be underestimated by about 20–30%. In concentration-response analyses, this degree of error would cause underestimation of EC_{50} values and Hill slopes by less than 30%.

We also compared etomidate modulation of GABA-activated currents in oocytes and HEK293 cells and patches (Fig. 2). As we reported previously for oocytes, 3.2 \( \mu \)M etomidate dramatically enhanced current responses elicited with GABA concentrations that activate a small fraction (5%) of maximal currents (maximal activation at 1–10 mM GABA). Etomidate also modestly enhanced (by 11 ± 4.3%) peak currents elicited with 1 mM GABA in both oocytes and HEK293 cells. The magnitude of the GABA concentration-response left shift at 3.2 \( \mu \)M etomidate was also similar in these two expression systems (Fig. 2, bottom). Individual oocytes displayed EC_{50}^{GABA}/EC_{50}^{GABA} ratios ranging from 0.22 to 0.06 (mean ± S.D. = 0.084 ± 0.027; \( n = 5 \)). Combined data from HEK293 patches and cells (\( n = 5 \)) gave EC_{50}^{GABA}/EC_{50}^{GABA} = 0.10 ± 0.018. In all, results obtained using
**Fig. 1. Direct activation of α3β7γ2δ GABA<sub>A</sub> receptor-mediated currents by etomidate.** Left, traces show currents recorded from an HEK cell patch. Three traces were activated using etomidate (concentrations labeled in μM). For normalization, a trace activated with 1 mM GABA is also shown. A notable difference between etomidate-activated currents from oocytes and HEK293 membrane patches is that patch currents displayed larger “surge” currents, evident after discontinuing etomidate above 100 μM. This indicates the presence of an inhibitory etomidate action that rapidly reverses before deactivation is complete. Right, etomidate direct activation responses from HEK293 cells and patches (open circles) and oocytes (solid squares) are plotted. Oocyte data include some previously published data (19) combined with new results. Points represent mean ± S.D. of at least four measurements. Peak responses to etomidate (surge current peak was used, if present) were normalized to maximal GABA currents (1 mM).

**Fig. 2. Enhancement of GABA-activated currents in HEK293 cells and Xenopus oocytes by 3.2 μM etomidate.** Top, traces show four currents recorded from an HEK293 cell expressing α3β7γ2δ GABA<sub>A</sub> receptors. Labels indicate GABA and etomidate concentrations. Cells were exposed to etomidate alone for 500 ms prior to activation with GABA plus etomidate (3.2 μM etomidate alone did not elicit an observable current). Bottom left, an EC50 shift study from a single Xenopus oocyte. Solid boxes represent the control GABA concentration response (EC50 = 36 ± 2.4 μM; nH = 1.4 ± 0.27), and open circles represent the GABA concentration response during continuous exposure to 3.2 μM etomidate (EC50 = 3.4 ± 0.60 μM; nH = 1.2 ± 0.18). EC50<sub>GABA</sub>/EC50<sub>Eto</sub> = 0.09 ± 0.018. All currents were normalized to control responses in 10 mM GABA. Note that etomidate increases maximal response at 10 mM GABA by about 10%. Bottom right, combined results, normalized to 1 mM GABA responses, from HEK293 cells, and patches are plotted as mean ± S.D. Control GABA concentration response (solid squares; n ≳ 5) was fitted with EC50<sub>GABA</sub> = 45 ± 5.2 μM and nH<sub>GABA</sub> = 1.2 ± 0.15. In the presence of 3.2 μM etomidate (open circles; n ≳ 4), EC50<sub>GABA</sub>/EC50<sub>Eto</sub> = 4.7 ± 0.61 μM and nH<sub>GABA</sub> = 1.3 ± 0.16. EC50<sub>GABA</sub>/EC50<sub>Eto</sub> = 0.10 ± 0.018. In the presence of etomidate, maximal GABA responses were enhanced by 7%.

**GABA Modulation Does Not Saturate at Up to 100 μM Etomidate**—GABA modulation assessed with leftward shifts in concentration-response curves demonstrate that GABA EC50 values continuously diminish at etomidate concentrations between 0.32 and 100 μM (Fig. 3). Normalized control concentration responses at 0 etomidate varied among individual oocytes, with GABA EC50 values ranging from about 20 to 60 μM (mean ± S.D. = 36 ± 12 μM; n = 35). To correct for variability among oocytes, EC50 ratios (EC50<sub>GABA</sub>/EC50<sub>Eto</sub>) were calculated based on measurements in individual oocytes, rather than on grouped averages.

In the presence of 0.32 μM etomidate, GABA EC50<sub>Eto</sub> was 2–3-fold lower than EC50<sub>EC50<sub>GABA</sub></sub>/EC50<sub>Eto</sub> (EC50<sub>GABA</sub>/EC50<sub>Eto</sub> ratio = 0.4 ± 0.15) and dropped steeply as etomidate concentration increased to 10 μM. EC50<sub>GABA</sub>/EC50<sub>Eto</sub> ratios determined at 10, 32, and 100 μM etomidate (Fig. 3) averaged 0.019, 0.014, and 0.010, respectively, and pairwise comparisons of shifts at these three etomidate concentrations were all significantly different (p ≤ 0.03 using two-tailed Student’s t tests). Fig. 3, bottom, shows that EC50<sub>GABA</sub>/EC50<sub>Eto</sub> does not reach a minimum at etomidate concentrations up to 100 μM, although the EC50<sub>GABA</sub>/EC50<sub>Eto</sub> ratio versus etomidate relationship appears to flatten somewhat above 32 μM.

Attempts to quantify EC50<sub>GABA</sub> values while exposing oocytes to etomidate concentrations above 100 μM were unsuccessful, because of rapid and irreversible rundown of current responses, which was apparently caused by both desensitization of GABA<sub>A</sub> receptors and very slow washout of etomidate from oocytes. By using voltage-clamped HEK293 cells, where etomidate washout is much faster, experiments comparing responses to submicromolar GABA and 1 mM GABA in the presence of 100 μM etomidate (data not shown) indicated a GABA EC50<sub>Eto</sub> near 0.4 μM, corresponding to an EC50<sub>GABA</sub>/EC50<sub>Eto</sub> ratio of 0.009, which closely matched the value derived from oocyte experiments (Fig. 3, bottom).

**Etomidate Effects on Currents Elicited with P4S**—Reduced GABA EC50 values in the presence of etomidate could be due to either enhanced GABA binding or to enhanced gating efficacy of GABA-bound receptors (16, 17). It is not possible to distinguish unambiguously these possibilities from macrorcurrents...
stimulated with GABA, because high concentrations of GABA alone activate nearly all receptors ($P_{\text{max}}^{\text{open}} \approx 0.85$). To test whether etomidate enhances GABA$_A$ receptor gating, we used a partial agonist, P4S, that acts at the GABA sites (Fig. 4). In whole oocytes, 70 ± 24 μM P4S induced half-maximal activation of GABA$_A$ receptors, and P4S concentrations up to 10 mM (140 × EC$_{50}$) elicited 40 ± 3.2% ($n = 3$) of the maximal GABA-activated current. Thus, P4S is a partial agonist in α$_1$β$_2$γ$_2$L receptors. In the presence of 3.2 μM etomidate, the P4S EC$_{50}$ was reduced to 6.3 ± 0.79 μM (EC$_{50}^{\text{P4S}/\text{EC}_{50}^{\text{GABA}}} = 0.09 \pm 0.03$), and the apparent efficacy of 1–10 μM P4S increased dramatically, eliciting currents that averaged 10% higher than those elicited with 1 mM GABA (Fig. 4).

**Analyses of GABA and Etomidate Data Based on Partial Agonist and Co-agonist Models**—We limited our analysis to two quantitative equilibrium models that minimized the number of free parameters by assuming two equivalent GABA sites and equivalent etomidate sites as follows: a partial agonist model (21) and an MWC allosteric co-agonist model (22). After independently fitting portions of the models to estimated $P_{\text{open}}$ versus [GABA] or [etomidate] data, model-generated $P_{\text{open}}$ values in the presence of both GABA and etomidate were compared with experimental results (Figs. 5 and 6).

**Partial Agonism**—The partial agonist model is depicted in Fig. 5A. Six out of its seven free equilibrium parameters ($K_D$, $K_E$, $a$, $b$, $\chi_D$, and $\chi_E$) were directly fitted and are reported in the legend to Fig. 5. The remaining parameter, $\chi_{GB}$, was optimized to a value of 100, to account for the large etomidate-induced leftward shifts in GABA concentration responses (Fig. 5D). Independent of $\chi_{GB}$, the fitted partial agonist model predicts a biphasic relationship between etomidate concentration and GABA EC$_{50}$ with a minimum EC$_{50}^{\text{P4S}}$ at about 30 μM etomidate. At etomidate concentrations near or above the fitted $K_E$ value of 120 μM, EC$_{50}^{\text{P4S}}$ is predicted to rise because of competitive occupation of agonist sites (Fig. 5, B and D).

We tried to model our P4S results (Fig. 4) using the partial agonist mechanism for etomidate. Estimated $P_{\text{open}}$ versus [P4S] data were used to derive $K_p$, $a$, and $\chi_p$ values (reported in the legend to Fig. 5), and $\chi_{P4S}$ was set 20-fold higher than $\chi_p$ (i.e. equal to the optimal $\chi_{GB}/\chi_D$ ratio). When these parameters were combined with fitted $K_E$, $b$, and $\chi_E$ values, the model predicted a large leftward shift but no significant increase in the maximum efficacy of P4S (Fig. 5E; $\chi_{P4S} = 10$). Further increasing $\chi_{P4S}$ produced larger leftward shifts and increased maximal efficacy, but the competitive interaction between P4S and etomidate in the model kept the predicted efficacy of 1–10 μM P4S plus etomidate well below that of maximal GABA.

**MWC Co-agonism**—Unlike the partial agonist model, MWC co-agonism provides no a priori constraint on the number of
etomidate sites. Fig. 6A depicts the model with two equivalent etomidate sites. Each MWC model is defined by the number of equivalent etomidate sites, \( n_E \), and only five additional free parameters, \( K_P \), \( K_G \), \( c \), \( d \), and \( d \). Only \( K_P \) and \( d \) are dependent on the number of etomidate sites in the model. All these models specify two equivalent GABA sites and therefore predict that GABA EC_{50} should show an inverse square root dependence on etomidate direct activation, \( 1^{\text{RtoG}}_{\text{GABA}} \) (16, 29, 35). Fig. 6B demonstrates that for oocytes exposed to 10, 32, or 100 \( \mu M \) etomidate, where both direct activation and EC_{50} were measured, an inverse square root relationship is indeed present (log-log slope = 0.48). Analysis using EC_{50}^{RtoG} instead of EC_{50} resulted in a similar fitted slope of \(-0.51 \pm 0.083 \) but did not improve the correlation coefficient of the line \( (R = -0.82) \). Extrapolation of the fitted line in Fig. 6B to the average GABA EC_{50} value of 36 \( \mu M \) provided an estimate of GABA\(_A\) receptor spontaneous activation probability in the absence of both etomidate and GABA (the R ↔ O transition in Fig. 6A). The basal \( P_{\text{open}} \) estimate \( (P_o) \) was \( 1.4 \times 10^{-5} \), corresponding to an \( L_o \) value of 70,000 that was used in fitting the four additional model parameters (see “Experimental Procedures”).

Fitting Equation 3 to pooled re-normalized GABA concentration-response data resulted in estimates for \( K_G = 57 \mu M \) and \( c = 1.5 \times 10^{-8} \). With \( n_E \) constrained to integers between 1 and 5, a set of \( K_G \) and \( c \) values (Table I) were fitted to etomidate direct activation data (Equation 4). The fit for the single etomidate site model was inferior to the two-site model fit (3-fold reduction in \( \chi^2 \)), but there was no appreciable improvement or degradation in the fits to models with more than two etomidate sites (Fig. 6F, circles). When model predictions for EC_{50} enhancement (Fig. 6D) and EC_{50} ratios (Fig. 6E) were compared with experimental results, it was apparent that the \( n_E = 2 \) model provided a much better fit than models with fewer or more etomidate sites (Fig. 6F, squares and triangles). The \( n_E = 2 \) model fit gave \( K_E = 35 \mu M \) and \( d = 7.7 \times 10^{-3} \).

To model the interaction of etomidate and P4S, Equation 3 was fitted to estimated \( P_{\text{open}} \) versus [P4S] data, deriving \( K_P \) and \( c \) values of 44 \( \mu M \) and \( 5.5 \times 10^{-8} \), respectively. When these values were substituted into the MWC co-agonist model (Equation 5) together with etomidate binding and efficacy parameters for the \( n_E = 2 \) model (Table I), it accurately predicted both the increased apparent potency and efficacy of P4S in the presence of etomidate (Fig. 6G). Thus, the same MWC etomidate binding and efficacy parameters describe the interaction of the drug with both GABA and P4S.

A Test of MWC Co-agonism Using a Receptor Mutation That Alters Gating—An important feature of MWC gating models is their ability to account for the behavior of receptors containing mutations that display "constitutive" or "spontaneous" activation in the absence of agonist, by varying the basal equilibrium between closed and open channels (\( L_o \)). Indeed, Chang and Weiss (35) showed that an MWC allosteric gating mechanism with one fixed set of equilibrium GABA binding and efficacy values \( (K_G \) and \( c \)) could elegantly account for the apparent
GABA sensitivities (EC50 values) of oocyte-expressed wild-type α1β2γ2 GABA_A receptors and multiple mutant receptors containing substitutions at the highly conserved leucines of M2 domains (L9s). This was achieved by noting that as experimental estimates of the spontaneous opening probability (P_o = (1 + L_o o)) of mutant channels increased, their GABA EC50 values decreased. Analogously, the MWC co-agonist model predicts that etomidate will appear to be a more potent and efficacious agonist when mutations that increase spontaneous gating are introduced into GABA_A receptors.

Oocytes expressing α1(L264T)β2γ2 receptors displayed large picrotoxin-sensitive “leak” currents in the absence of GABA, which are associated with spontaneously open GABA_A channels. The portion of the leak current associated with channel activity was estimated using picrotoxin (2 μM), which produced an apparent outward current (actually a reduced leak current) that averaged 10 ± 3.2% of the maximal GABA-activated current (Fig. 7, top). In oocytes expressing α1(L264T)β2γ2 receptors, etomidate concentrations as low as 10 nM directly elicited inward currents, and etomidate concentrations above 10 μM produced current amplitudes comparable with those elicited with high GABA (100 μM).

The n_E = 2 MWC co-agonist model derived for wild-type α1β2γ2 (Fig. 6) was adjusted to reflect the spontaneous activity of α1(L264T)β2γ2 receptors by setting L_o = 10, leaving K_G, c, K_E, and d values unchanged. The model with L_o = 10 accurately predicted both the increased apparent potency and efficacy of etomidate direct activation in the mutant receptors (Fig. 7, bottom). Furthermore, the MWC model predicts that the etomidate-induced leftward shifts (EC50 ratios) in the mutant receptors should be the same as those in wild-type receptors (Fig. 7, bottom left). Indeed, EC50/EC50 ratios in oocytes expressing α1(L264T)β2γ2L receptors were similar to those derived from wild-type studies: 0.5 ± 0.13 (mutant) versus 0.4 ± 0.15 (wild-type) at 0.32 μM etomidate, and 0.3 ± 0.10 (mutant) versus 0.20 ± 0.059 (wild type) at 1.0 μM etomidate.

**DISCUSSION**

The major goals of this study were to examine both the concentration dependence and the mechanism of etomidate-induced modulation of GABA_A receptor activity. Voltage clamp electrophysiology data were mostly obtained from oocytes, providing rough estimates of receptor open probabilities. These were analyzed using binding-gating models that are characterized only by microscopic equilibrium constants between adjacent states. Based on comparison to parallel experiments in rapidly superfused HEK293 cells and patches (Figs. 1–3), our oocyte studies appear quite adequate for comparative evaluation of equilibrium mechanistic models that do not incorporate transition rates or receptor desensitization.

**GABA Modulation Is Not Mediated by a High Affinity Site for Etomidate**—Our first aim was to determine whether high affinity (K_E ≤ 10 μM) etomidate sites on GABA receptors mediate enhancement of GABA responses. The presence of these sites model predictions for EC50 ratios (lines representing models with different n_E values are as in D) are overdrawn on oocyte data from Fig. 3, bottom. F, error analysis comparing the predictions of models with different n_E values to oocyte results. Sum-of-squares deviations are plotted for EC50 enhancement (squares), direct activation (circles), and EC50 ratios (triangles). G, modeling the effects of etomidate on P4S-activated currents. Data from Fig. 4, bottom, were re-normalized assuming GABA EC50A = 0.85 (dashed line). The line overlaying control data (squares) represents a fit to Equation 3 (substituting K_G for K_E), with L_o = 70,000 and n_E = 2. Fitted K_E = 44 ± 10 μM and c = 0.0055 ± 0.0017. The line overlaying the data with 3.2 μM etomidate (circles) was calculated using Equation 5 with L_o = 70,000, K_E = 44 μM, c = 0.0055, K_G = 35 μM, d = 0.0077, and n_E = 2.
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In contrast, GABA EC$_{50}$ ratios (Fig. 3) do not reach a minimum at etomidate concentrations below 100 $\mu$M) elicited an apparent outward current, due to inactivation of constitutively active mutant GABA$_A$ receptors. GABA currents can be observed in the presence of 3.2 $\mu$M etomidate concentrations. The model predicts that very little enhancement of activation reaches its maximum (Fig. 1). Points were suggested by previous studies of GABA modulation, which typically show half-maximal enhancement at 2–5 $\mu$M etomidate and maximal enhancement at 10–20 $\mu$M (e.g. Fig. 6D, squares) (19, 20, 25). In contrast, GABA EC$_{50}$ ratios (Fig. 3) do not reach a minimum at etomidate concentrations below 100 $\mu$M. These data suggest that GABA modulation saturates in the same etomidate concentration range (near 300 $\mu$M) where direct activation reaches its maximum (Fig. 1).

Many studies of GABA modulation by general anesthetics have used a single low GABA concentration, for instance, one that activates 5% of maximal responses (EC$_{5}$). Based on single channel estimates of $P_{\text{open}}$ at high [GABA] near 85% (31–33), the greatest observable anesthetic enhancement at GABA EC$_{5}$ would be 24-fold (100/0.05/0.85), and this measurement will underestimate modulation by compounds that enhance receptor activity more than 24-fold. Etomidate is clearly an example where this is the case. EC$_{50}$ ratios also reduce errors in measuring positive modulation caused by combined inhibiting and enhancing drug effects (37).

**Etomidate Enhances GABA$_A$ Receptor Gating**—The observation that etomidate increases maximal P4S agonist efficacy (Fig. 4) indicates that etomidate shifts the equilibrium between agonist-bound closed and open GABA$_A$ receptors toward the open state. The partial agonist P4S has been used previously to demonstrate enhanced gating of GABA$_A$ receptors by propofol and volatile anesthetics (17, 38). This result is also consistent with single channel evidence for stabilization of GABA$_A$ receptor open states in the presence of etomidate (24). Based on P4S modulation alone, we cannot rule out the possibility that etomidate also enhances P4S binding to agonist sites, but quantitative modeling suggests that a gating effect alone can account for reduced GABA and P4S EC$_{50}$ values (see below).

**Single Site Equilibrium Models for Etomidate Actions on GABA$_A$ Receptors**—Our evidence that both GABA modulation and direct activation are maximized at similar etomidate concentrations adds quantitative support to the hypothesis that identical sites mediate both actions. Previous studies showed that both GABA modulation and direct activation display similar stereoselectivity for (R)(+) versus (S)(−) etomidate (19, 20, 26) and similar dependence on $\beta$ subunit isoform (9, 25). One amino acid at the 15′ position of $\beta$ subunit M2 domains (Ser in $\beta_{1}$ and Asn in $\beta_{2,3}$) was shown to influence both direct activation and GABA modulation in parallel (10, 27, 39, 40). The remarkable correspondence of affinity, stereoselectivity, and subunit dependence makes it highly unlikely that distinct etomidate sites mediate direct activation and GABA modulation. Previously, there has been both speculation about (40–42) and modeling of (21, 22) single site mechanisms to explain multiple general anesthetic effects on GABA$_A$ receptors. For etomidate, the data presented above represent the first critical test of this hypothesis and comparison of the proposed mechanisms.

The partial agonist mechanism of Kurata et al. (21) suggests that two equivalent GABA activation sites (Fig. 5) display binding allostery and also bind general anesthetics, resulting in direct activation, enhanced activation at low GABA concentrations, and inhibition at high GABA concentrations. The model enabled fitting of most free parameters to estimated $P_{\text{open}}$ versus [GABA] or $P_{\text{open}}$ versus [etomidate] data. To account for the large GABA EC$_{50}$ decreases induced by etomidate, this model required that the RGE state (Fig. 5A; receptors with one GABA and one etomidate bound) gate 20 times more efficaciously ($\chi_{GKE} = 100$) than the RG$_2$ state ($\chi_{G2} = 5$). In essence, this model explains multiple actions using a single type of etomidate site with a single binding parameter, $K_{d}$, but each action is mediated through distinct underlying mecha-
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...nisms that are associated with different model parameters. Thus, direct activation is described by the gating efficacy parameter, χ<sub>E</sub>. GABA enhancement under selected conditions is partially due to positive binding allosterism between agonist sites in the model (b < 1), whereas most of the GABA enhancement in the model is driven by positive gating allosterism (χ<sub>GE</sub> > χ<sub>E</sub>). The optimized partial agonist model does a reasonable job predicting estimated P<sub>open</sub> when both etomidate and GABA are present (Fig. 5, C and D).

However, there are significant problems with this and other partial agonist models. Our fitted model incorrectly predicts that GABA EC<sub>50</sub> reaches a minimum near 30 μM etomidate and rises at higher concentrations (Fig. 5D). The fitted etomidate parameters that adequately describe interactions with GABA clearly cannot simulate the interaction between etomidate and P4S, even when we postulate 100-fold gating enhancement when both P4S and etomidate are bound (Fig. 5E). Partial agonist models predict that all the actions of etomidate should display similar stereoselectivity, but the apparent inhibitory potencies of (R)1- vs. (S)1-etomidate do not display the same stereoselectivity seen for direct activation and GABA modulation (19). Also, structure-function studies using subunit chimeras and point mutations suggest that etomidate (40) and other intravenous general anesthetics (41–43) activate GABA<sub>A</sub> receptors via sites that are distinct from the GABA agonist sites. Thus, there are more reasons to reject than to accept partial agonist models for etomidate.

The MWC co-agonist mechanism (Fig. 6) is a simple model that incorporates symmetric allosteric channel gating by both GABA and etomidate at distinct and independent sets of sites. It is highly constrained and defined by only five equilibrium parameters, yet it predicts with remarkable accuracy the combined effects of GABA and etomidate on GABA<sub>A</sub> receptors. Moreover, a single underlying mechanism (positive gating modulation) mediated by a single class of equivalent etomidate-binding sites accounts for both GABA modulation and direct activation in the model. Most important, the same etomidate binding and efficacy parameters that describe GABA modulation also correctly predict both the leftward shift and the increase in apparent efficacy of P4S in the presence of etomidate (Fig. 6C). Because agonist modulation (EC<sub>50</sub> ratio) is similar with either GABA or P4S, our results support an implicit feature of the MWC model, that the impact of etomidate on channel gating is independent of agonist site occupancy and agonist efficacy. Furthermore, the co-agonist model is consistent with both etomidate stereoselectivity studies and structure-function studies of GABA versus etomidate agonism.

In contrast to sequential binding-gating models of receptor activation, MWC models (16, 44) incorporate the assumption that unliganded receptors can spontaneously activate with some low probability P<sub>0</sub> = (1 + L<sub>0</sub>)<sup>-1</sup>. The L<sub>0</sub> value derived from the model-predicted relationship between etomidate direct activation and GABA EC<sub>50</sub> (Fig. 6B) has nearly 10-fold uncertainty. Nonetheless, it is within 2-fold of previous L<sub>0</sub> estimates for α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> receptors in oocytes (35) and HEK293 cells (29). Binding and efficacy parameters were derived by fitting independent estimated P<sub>open</sub> versus [GABA] or [etomidate] data sets. Based on direct activation, EC<sub>50</sub> enhancement and EC<sub>50</sub> ratios, the n<sub>E</sub> = 2 model best described experimental results (Fig. 6E). This outcome fits nicely with subunit substitution and site mutation studies that have located determinants of etomidate actions on GABA<sub>A</sub> receptor β subunits (9, 25) and others indicating the presence of two β<sub>2</sub> subunits per α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> channel (45, 46).

Fitted efficacy parameters for the n<sub>E</sub> = 2 model (Table I) suggest that etomidate binds about 130-fold (d<sup>-1</sup>) more tightly to open versus closed receptors and that occupation of both sites therefore shifts the open-closed equilibrium 130<sup>2</sup> = 16,900-fold toward the open state, corresponding to a stabilization energy of ~5.8 kcal/mol. The model also implies that GABA binds 670-fold (d<sup>-1</sup>) more tightly to open versus closed receptor sites. Two GABAs shift the equilibrium 450,000-fold toward open states (~7.7 kcal/mol). The P<sub>open</sub> value for the model for GABA is 0.86, and adding etomidate increases P<sub>open</sub> to 1.0, because the open state stabilization energies of etomidate and GABA are additive. Experimentally, we observed that etomidate enhances maximal GABA activated currents by about 10%, consistent with a n<sub>E</sub> value near 0.9 in the absence of etomidate.

One effect of etomidate that the co-agonist model predicts, but which was not consistently observed, is increased Hill slopes of GABA activation. Specifically, Hill slopes are predicted to approach 2.0 and then fall again in the presence of high etomidate concentrations (Fig. 6C). However, in the presence of 1–32 μM etomidate, GABA activation Hill slopes from both oocytes and from HEK293 cell and patch experiments were in the 1.0–1.7 range (Figs. 2 and 3). These low experimental Hill slopes prevented us from utilizing a global approach to simultaneously fitting all the free parameters in the model. Reasons why experimental Hill slopes may not behave as the model predicts include inhomogeneities of either GABA sensitivity or desensitization rate within cellular receptor populations, which in turn could be caused by variable subunit assembly (36, 37) or sub-domains of clustered versus unclustered receptors (47). Also, the two GABA sites may not be equivalent (48), or they may be negatively and allosterically coupled to each other or to etomidate sites (49).

A variety of other mechanisms of general anesthetic modulation can account for our experimental results but not without introducing additional states and free parameters. Partial agonist models were discussed above. Allosteric modulation of GABA affinity (i.e. reduced K<sub>G</sub>) has been proposed to explain leftward shift by halothane (50). Although agonist affinity enhancement by etomidate would produce leftward shifts in GABA and P4S concentration responses, it cannot explain direct activation, the enhanced maximal efficacy of P4S, and the impact of the α<sub>1</sub>L264T mutation on apparent etomidate sensitivity. All of these phenomena are explained by etomidate enhancement of the receptor open-closed equilibrium (gating). Furthermore, if we postulate an MWC mechanism where etomidate alters both gating and GABA binding, our results imply that the binding effect will be small, because the gating effect alone (determined from direct activation measurements) quantitatively accounts for the observed leftward shifts. Allosteric gating modulation has also been proposed previously to explain leftward shifts (discussed above). MWC co-agonism is in essence just this mechanism but formally incorporates opening transitions from states that are not fully agonist-bound (i.e. R and RG in Fig. 6).

Given its simplicity and the fact that GABA and etomidate parameters were fitted independently, the equilibrium MWC co-agonist model provides surprisingly good estimates of GABA<sub>A</sub> receptor P<sub>open</sub> when both GABA and etomidate are present. Nonetheless, this model represents a low resolution view of receptor modulation by etomidate and GABA. It does not yet incorporate features to account for inhibition of GABA<sub>A</sub> receptor-mediated currents by etomidate, for rates of activation, desensitization, and deactivation in macrocurrents, nor for the existence of multiple open and closed states that are apparent in single channel studies. Additional studies defining the mechanism of etomidate inhibition and others providing kinetic constraints from rapid patch superfusion and single
channel experiments will be required to elaborate the model further.

Etomidate Effects on GABA<sub>A</sub> Receptor Gating Mutants—The value of a model derives both from the insight it provides into real molecular events, such as binding and gating in ligand-gated ion channels, as well as its ability to predict behavior under different conditions, such as with full versus partial agonists. With a simple experimentally based adjustment of one wild-type model parameter, the MWC co-agonist model also accurately predicted the effects of etomidate on a mutant GABA<sub>A</sub> receptor characterized by constitutive activation (Fig. 7). Constitutive activation is modeled by reducing <i>K<sub>d</sub></i> in the wild-type MWC co-agonist model, which then predicts increased apparent sensitivity to etomidate direct activation (and to agonists). Maintaining <i>K<sub>a</sub></i> and <i>d</i> at wild-type values preserves the impact of etomidate on GABA EC<sub>50</sub> ratio, which was also observed experimentally.

Two other GABA<sub>A</sub> receptor single site mutations have been described that cause changes similar to those seen with α<sub>L264T</sub>. GABA<sub>A</sub> receptors containing α<sub>A,291W</sub> (51) or β<sub>L259S</sub> (52) mutations display significant spontaneous activation, increased sensitivity to etomidate direct activation, and apparently reduced etomidate enhancement in the presence of low GABA (left shifts were not measured). In fact, the MWC model predicts that etomidate enhancement of currents elicited with low GABA will appear to decrease when significant constitutive activity is present, because of the reduced <i>P<sub>open</sub></i> range accessible to these receptors. This illustrates how some measures of GABA modulation may be highly dependent on the gating behavior of mutant GABA<sub>A</sub> receptors, even when etomidate binding and efficacy are unchanged. Leftward shifts revealed that etomidate binding and efficacy were in fact altered by the α<sub>L264T</sub> mutation, and the same is likely true for β<sub>L259S</sub> and α<sub>A,291W</sub>. There are some published data that appear to contradict the MWC co-agonist model for etomidate. One report suggested that a mutation at the β subunit 15’ Asn/Ser site differentially affects GABA modulation and direct activation by etomidate (53), but this contrasts with the other studies of this locus (10, 27, 29, 40). Chang et al. (54) reported that rat α<sub>β<sub>L264T</sub></sub> receptors containing a single mutation (β<sub>G219F</sub>) are characterized by low constitutive activity, reduced GABA modulation by etomidate, and slightly enhanced sensitivity to direct activation. Other results reported by Chang et al. (54) and another study of the same site (55) are consistent with the conclusion that mutations at this residue enhance channel gating, which tightly correlates with the degree of altered GABA modulation by anesthetics. However, these findings cannot be easily reconciled with an MWC co-agonist mechanism, and studies of mutations at the homologous site in human α<sub>β<sub>L259S</sub></sub> GABA<sub>A</sub> receptors should help clarify this issue.

Other Anesthetics and Other Ligand-gated Ion Channels—Like etomidate, other general anesthetics both directly activate GABA<sub>A</sub> receptors and modulate GABA responses. In addition to the studies using P45 noted above, other partial GABA agonists have demonstrated enhanced gating in the presence of neurosteroids (56). Single channel and rapid patch superfusion studies have also provided evidence for stabilization of GABA<sub>A</sub> receptor open states in the presence of neurosteroids (4), barbiturates (6, 33), propofol (7), and ethanol (57). Nonetheless, the relationship between direct activation and GABA enhancement by other anesthetic agents remains largely unexplored. Direct activation by general anesthetics is infrequently studied, because this action is not observed in wild-type GABA<sub>A</sub> receptors at concentrations associated with anesthesia in animals and humans.

Given the limited scope of many studies of GABA<sub>A</sub> receptor mutations and anesthetics, in most cases we cannot determine whether they are consistent with MWC co-agonism. Structure-function studies of chimeric subunits and single site mutations suggest that different anesthetics interact with different GABA<sub>A</sub> receptor sites. Like etomidate, barbiturates and propofol apparently interact strongly with β subunits, whereas volatiles and alcohols may interact with sites on α, β, and γ subunits (12, 37, 58). A variety of mutations that apparently alter general anesthetic modulation in GABA<sub>A</sub> receptors seems likely to be gating mutants that behave like α<sub>L264T</sub> (52, 55, 59–62). One study of pentobarbital agonism (41) concluded, like our model, that two equivalent β subunit sites were involved. However, some electrophysiology data for propofol suggest distinct sites for GABA modulation and direct activation (51). Cysteine mutant accessibility studies also suggest that distinct GABA<sub>A</sub> receptor open states are activated by propofol alone versus propofol plus GABA (63).

Both the size dependence of anesthetic modulation (cut-off effect) (58, 64) and the data on mutations are consistent with the idea that small general anesthetics, like short chain alcohols and volatile alkanes and ethers, probably act at more than two GABA<sub>A</sub> receptor sites. Although anesthetic binding to some of these sites must couple to GABA<sub>A</sub> receptor channel gating in order to produce direct activation, other sites could be linked to the GABA-binding site or to desensitization. Each general anesthetic will need to be considered on a case-by-case basis to determine whether MWC co-agonism accounts for its actions on GABA<sub>A</sub> receptors.

Some general anesthetics and alcohols also enhance agonist activation in glycine receptors (65, 66), peripheral and central nicotinic acetylcholine receptors (67, 68), and serotonin type 3 receptors (69). Direct activation of acetylcholine and serotonin receptors has not been reported, but ethanol has been shown to enhance gating efficacy in these channels (68, 69). Thus, allosteric modulation of gating efficacy appears to be a common effect of small general anesthetics on all members of the ligand-gated ion channel superfamily, perhaps mediated by homologous sites.

Acknowledgments—We thank Carol Gelb for technical assistance. Keith Miller, Richard Olsen, and Douglas Raines provided useful comments on the modeling and the manuscript. The Department of Anesthesia and Critical Care, Massachusetts General Hospital, generously donated etomidate.

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Gating Allosterism at a Single Class of Etomidate Sites on \( \alpha_1^{16}\beta_2^{16}\gamma_2^L \) GABA\(_A\) Receptors Accounts for Both Direct Activation and Agonist Modulation

Dirk Rüschn, Huijun Zhong and Stuart A. Forman

J. Biol. Chem. 2004, 279:20982-20992. doi: 10.1074/jbc.M400472200 originally published online March 11, 2004

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