An Allosteric Coagonist Model for Propofol Effects on $\alpha1\beta2\gamma2L$ γ-Aminobutyric Acid Type A Receptors

Dirk Ruesch, M.D.,* Elena Neumann, M.D.,† Hinnerk Wulf, M.D.,‡ Stuart A. Forman, M.D., Ph.D.§

ABSTRACT

Background: Propofol produces its major actions *via* γ-aminobutyric acid type A ($GABA_A$) receptors. At low concentrations, propofol enhances agonist-stimulated $GABA_A$ receptor activity, and high propofol concentrations directly activate receptors. Etomidate produces similar effects, and there is convincing evidence that a single class of etomidate sites mediate both agonist modulation and direct $GABA_A$ receptor activation. It is unknown if the propofol binding site(s) on $GABA_A$ receptors that modulate agonist-induced activity also mediate direct activation.

Methods: $GABA_A$ $\alpha1\beta2\gamma2L$ receptors were heterologously expressed in *Xenopus* oocytes and activity was quantified using voltage clamp electrophysiology. We tested whether propofol and etomidate display the same linkage between agonist modulation and direct activation of $GABA_A$ receptors by identifying equiefficacious drug solutions for direct activation. We then determined whether these drug solutions produce equal modulation of GABA-induced receptor activity. We also measured propofol-dependent direct activation and modulation of low GABA responses. Allosteric coagonist models similar to that established for etomidate, but with variable numbers of propofol sites, were fitted to combined data.

Results: Solutions of 19 μM propofol and 10 μM etomidate were found to equally activate $GABA_A$ receptors. These two drug solutions also produced indistinguishable modulation of GABA-induced receptor activity. Combined electrophysiological data behaved in a manner consistent with allosteric coagonist models with more than one propofol site. The best fit was observed when the model assumed three equivalent propofol sites.

Conclusions: Our results support the hypothesis that propofol, like etomidate, acts at $GABA_A$ receptor sites mediating both GABA modulation and direct activation.

What We Already Know about This Topic

- Propofol acts *via* γ-aminobutyric acid type A ($GABA_A$) receptors. Low concentrations of propofol enhance agonist-stimulated $GABA_A$ receptor activity, and high concentrations directly activate receptors.
- Whether these two effects are mediated by different types of propofol sites is unknown.

What This Article Tells Us That Is New

- Quantitative $GABA_AR$ electrophysiology indicates that the propofol-binding sites causing GABA enhancement are the same as those mediating direct activation.
- Allosteric coagonist models fitted to the data suggest that there may be three such propofol sites per receptor.

PROPOFOL and other potent intravenous sedative-hypnotic drugs such as alphaxalone and etomidate act *via* γ-aminobutyric acid type A ($GABA_A$) receptors, a major class of inhibitory ligand-gated ion channels in mammalian brain.[1,2] In both neuronal synaptic receptors and heterologously expressed $GABA_A$ receptors with subunit composition $2\alpha:2\beta:1\gamma$, clinical concentrations of these anesthetics potentiate ion channel activation by GABA, shifting concentration-responses leftward.[3–7] These drugs also slow the deactivation of $GABA_A$ receptor-mediated currents, prolonging decay of GABA-stimulated inhibitory postsynaptic currents.[8–11] At higher concentrations, the potent intravenous anesthetics also directly activate $GABA_A$ receptor channels (*i.e.*, in the absence of GABA).[6,7,10,12–14]

Two contrasting mechanistic models for anesthetic effects in $GABA_A$ receptors have been proposed to account for these observations. One type of model postulates that modulation of GABA responses is mediated by high-affinity anesthetic

* Staff Anesthesiologist, Department of Anesthesia and Intensive Care, University Hospital Giessen-Marburg, Marburg, Germany. † Postdoctoral Fellow, Department of Anesthesiology, Section of Experimental Anesthesiology, University of Tuebingen, Tuebingen, Germany. ‡ Professor, Department of Anesthesia and Intensive Care, University Hospital Giessen-Marburg. § Associate Professo of Anesthesia, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts.

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Address correspondence to Dr. Forman: Department of Anesthesia, Critical Care and Pain Medicine, Jackson 4, Massachusetts General Hospital, 55 Fruit Street, Boston, Massachusetts 02114. saforman@partners.org. Anesthesiology 2012; 116:47–55

sites, whereas direct receptor agonism is mediated by distinct low-affinity anesthetics sites. This type of model with two classes of anesthetic sites has been used to interpret the actions of neuroactive steroids, which include alphaxalone.[14] A second type of model supposes that a single class of drug-binding sites mediate both GABA modulation and direct receptor activation. Quantitative electrophysiological analysis of etomidate effects on $\alpha1\beta2\gamma2L$ $GABA_A$ receptors were modeled using a two-state (inactive and active) Monod-Wyman-Changeux (MWC) allosteric coagonist mechanism.[6] This MWC model fits functional data best with two equivalent etomidate sites per receptor, a stoichiometry that is supported by [^{3}H]-azi-etomidate photolabeling of purified bovine $GABA_A$ receptors.[15]

Despite the strong similarities between propofol actions and those of neuroactive steroids and etomidate, it remains uncertain whether propofol modulation and direct activation are mediated by the same *versus* distinct $GABA_A$ receptor sites. Our first aim was to test the hypothesis that propofol actions, like those of etomidate, behave in accordance with MWC coagonist models. Because the same drug-binding sites mediate both direct-channel activation and modulation of GABA responses in these models, the quantitative relationship between these two drug effects is predicted to be independent of both the specific drug and the number of binding sites. Thus, our hypothesis implies that etomidate and propofol concentrations that elicit equal direct-receptor activation will also produce equal shifts in apparent agonist sensitivity (GABA EC_{50}). To test this idea, we used electrophysiology in *Xenopus* oocytes and identified a pair of equieffacacious direct activating solutions of etomidate and propofol in $\alpha1\beta2\gamma2L$ $GABA_A$ receptors. We then compared how these two drug solutions alter GABA modulation of receptors by quantifying shifts in GABA EC_{50}. Our second goal, contingent on the outcome of the first aim, was to develop a quantitative two-state coagonist model for propofol, including an estimate of the number of propofol sites per $GABA_A$ receptor. We performed quantitative electrophysiological studies of both propofol direct activation and propofol-dependent enhancement of currents elicited with low GABA. Data from all the above experiments were combined and globally fitted to MWC models with a variable number of propofol sites.

Materials and Methods

Animal Care

Xenopus laevis maintenance and oocyte harvest procedures were performed in accordance with National Institutes of Health guidelines and approved by the local committees for animal care of Marburg University Hospital (Marburg, Germany) or Massachusetts General Hospital (Boston, Massachusetts).

Molecular Biology

Plasmids containing DNA encoding wild-type bovine $\alpha1$and human $\beta2$ and $\gamma2L$ $GABA_A$ receptor subunits were provided by Paul Whiting (Merck Sharp & Dohme Research Labs, Essex, United Kingdom). DNAs were linearized and used as templates for messenger RNA synthesis with the mMessage mMachine High Yield Capped RNA Transcription Kit (Ambion Inc., Austin, TX).

Oocyte Procedures and Receptor Expression

Oocytes were collected from human chorionic gonadotropin-injected adult female *Xenopus laevis* anesthetized with 0.2% tricaine and hypothermia. Stage V and VI oocytes were injected with 10–17 ng (30–50 nl) of a messenger RNA mixture encoding $\alpha1$, $\beta2$, and $\gamma2L$ $GABA_A$ receptor subunits. At least three-fold excess $\gamma2L$ RNA was used to ensure uniform incorporation into receptors. Oocytes were cultured at 18°C in ND96 solution (96 mM NaCl, 2 mM KCl, 10 mM HEPES, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, pH 7.5) supplemented with 2.5 mM pyruvate and 20 μg/ml gentamicin.

Drugs, Chemicals, and Preparation of Solutions

Ethyl 3-aminobenzoate methanesulfonate salt (tricaine), collagenase IA, dimethyl sulfoxide, propylene glycol, GABA, 2,6-di-isopropylphenol (propofol), and all salts and buffers were obtained from Sigma-Aldrich (St Louis, MO). R-etomidate was from Bedford Laboratories (Bedford, OH) or Janssen-Cilag GmbH (Neuss, Germany) as the commercially available solution for clinical use containing 2 mg/ml (8.2 mM) drug in 35% propylene glycol.

Etomidate was diluted in ND96 recording solution (without antibiotics) to make a 1 mM stock solution containing 4.3% propylene glycol. This etomidate stock was kept refrigerated and diluted to a final concentration of 10 μM etomidate on each experimental day. Propylene glycol at 0.5% (more than 10-fold higher than the concentration in 10 μM etomidate solutions) did not have any effect on resting leak currents or on GABA-activated currents. Propofol was dissolved in dimethyl sulfoxide to make a 100 mM stock solution, which was diluted into recording solution to the desired propofol concentration. Dimethyl sulfoxide at 1% (about 50-fold higher than the concentration in experiments) did not have any effect on resting leak currents or on GABA-activated currents.

Electrophysiology

Two-microelectrode voltage clamp experiments were performed at room temperature (21°C) 2–7 days after the injection of the messenger RNA mix into oocytes. The electrophysiology equipment and techniques have been previously described.[6,16]

Experimental Protocols

Identification of Equiefficacious Concentrations of Etomidate and Propofol for Direct Activation

Currents elicited by propofol were compared to currents evoked by 10 μM etomidate using the following procedure. Etomidate at 10 μM was applied to an oocyte expressing $GABA_A$ receptors until the peak current reached a plateau (I_{eto} prepropofol). After a 10 min period of oocyte wash in recording solution, the oocyte was exposed to propofol (at 10 μM initially) until the peak current reached a plateau (I_{pro}). Following another 10 min wash period, the oocyte was again exposed to 10 μM etomidate until the peak current reached a plateau (I_{eto} postpropofol). The propofol-induced current was compared with the average of the two etomidate-induced currents. Depending on the ratio of I_{pro}/I_{eto} averaged from three oocytes, the propofol concentration was increased or decreased in steps of 1–3 μM for the next set of three cells. When the equiefficacious propofol concentration (19 μM) was identified (I_{pro}/I_{eto} range 0.95–1.05), the equivalence of direct-activating efficacies of the two drug solutions were confirmed in another seven cells.

GABA Enhancement in the Absence and Presence of Propofol versus Etomidate

GABA solutions ranging from 0.03 μM to 10 mM were prepared in recording solution or recording solution supplemented with either 10 μM etomidate or 19 μM propofol. Oocyte exposure time to GABA (15–60 s) depended on GABA concentration and the time to plateau current. Every test sweep (GABA, GABA + 10 μM etomidate, GABA + 19 μM propofol) was preceded and followed by a sweep elicited by a maximally activating concentration of GABA (10 mM) for normalization. To ensure complete washout of agonist and anesthetic as well as return of the receptors to the resting state, cells were washed with recording buffer for 5 min following every 10 mM GABA control exposure, for 3–5 min following every GABA test exposure, and for 10–15 min after every exposure to GABA + anesthetic.

In one set of experiments (two-drug protocol), we compared the GABA-enhancing effects of 10 μM etomidate and 19 μM propofol in the same cell at one GABA concentration. Because propofol displayed slower washout than etomidate, we exposed oocytes to etomidate before propofol. Current from an oocyte was initially activated by a chosen GABA concentration (I_{GABA}). After wash, the oocyte was exposed to 10 μM etomidate until the elicited-inward current reached a plateau. At this point, a solution with GABA at the same test dose, plus 10 μM etomidate, was applied ($I_{GABA+ETO}$). After wash, the same oocyte was then exposed to 19 μM propofol until the elicited-inward current reached a plateau, then to GABA at the same test dose, plus 19 μM propofol ($I_{GABA+PRO}$). For each concentration of GABA, a minimum of 5 cells was studied.

To control for possible interactions between etomidate and propofol, we also measured GABA enhancement with 10 μM etomidate and 19 μM propofol in separate sets of cells (single-drug protocol). For each concentration of GABA, a minimum of 5 cells was studied.

Propofol Concentration-responses in the Absence and Presence of GABA

Concentration-dependent direct activation by propofol was measured over a range of concentrations (5 μM–1 mM) in oocytes. Each cell was first activated with 10 mM GABA as a control, followed by 5 min wash before activation with propofol. After activation with propofol, oocytes were washed for 10–15 min and the 10 mM GABA response was repeated. If the postpropofol control differed from the prepropofol control by more than 15%, the data were not included for analysis. Propofol responses were normalized to the average of pre- and postpropofol GABA controls. Three to five oocytes were tested at each propofol concentration.

The GABA concentration eliciting 2–3% of maximal response (EC2.5) was established in each oocyte by testing various solutions against 10 mM GABA, with 3–5 min washes between. GABA EC2.5 ranged from 3 to 8 μM (mean ± SD = 5 ± 1.5 μM). After the EC2.5 response was stable for two sequential sweeps, the same oocytes was exposed to the test propofol concentration for 1 min, followed by propofol plus GABA at EC2.5. Oocytes were then washed for 10–15 min and retested against GABA at EC2.5 alone. Each propofol concentration was tested in three to five oocytes.

Data Analysis

Unless otherwise indicated, results are reported as mean ± SD. The criterion for statistical significance was $P < 0.05$ unless otherwise stated.

Comparison of GABA Enhancement by Propofol versus Etomidate

At each GABA concentration studied, normalized leak corrected peak currents elicited by GABA, GABA + 10 μM etomidate, and GABA + 19 μM propofol were compared using two-way ANOVA with Bonferroni correction posttest in GraphPad Prism 5.02 (GraphPad Software, San Diego, CA).

Concentration-response Analyses

Leak-corrected and normalized agonist concentration response curves, both in the absence and presence of anesthetic drugs, were constructed with the data obtained using the single drug protocol and fitted by nonlinear least squares to the logistic (Hill) equation:

$$I = \frac{I_{max} - I_{min}}{1 + 10\exp((\log EC_{50} - \log[\text{Agonist}]) \times nH)} + I_{min}$$

where I is the current evoked by agonist; I_{max} and I_{min} are the maximal and minimal currents, respectively; EC_{50} is the agonist concentration that evokes a current halfway between

I_{max} and I_{min}; agonist is either GABA or propofol; and nH is the Hill coefficient. When direct activation by etomidate or propofol was present during a GABA concentration-response study, I_{min} reflects this basal activity. Nonlinear least-squares method fits to Equation 1 were performed using GraphPad Prism 5.02. Results of these fits are reported as best fit (95% CI). Statistical comparison of fitted parameters for multiple data sets was performed in GraphPad Prism using the "compare" function in the nonlinear least-squares method fitting module. The EC_{50} shifts for each anesthetic were calculated from the fitted EC_{50}s in the presence *versus* absence of drug. The logEC_{50} difference (anesthetic − control) errors were calculated from the fitting errors and used to determine 95% CIs for EC_{50} shifts.

Fitting MWC Allosteric Model Parameters

Average peak current data from GABA concentration-responses in the presence and absence of propofol, propofol direct activation responses, and GABA EC2.5 enhancement were pooled and renormalized to provide estimated P_{open} values:(P^{est}_{open}) This was achieved by assuming that maximum peak current elicited by high GABA concentrations in the presence of etomidate (125% of maximal GABA response) represented 100% channel activation. Nonlinear least-squares regression was use to fit the pooled data to the following equation:

$$P_{open} = \frac{1}{1 + L_0\left(\frac{1 + [GABA]/K_G}{1 + [GABA]/cK_G}\right)^2\left(\frac{1 + [propofol]/K_p}{1 + [propofol]/dK_p}\right)^n}.$$

This equation describes a two-state equilibrium allosteric mechanism with two classes of agonist sites (one for GABA and one for propofol). The model assumes two equivalent GABA sites, while the number of propofol sites is variable (n). L_0 is a dimensionless basal equilibrium gating variable, inversely related to the open probability of unliganded receptors. Based on previous estimates in our lab[6,17] and others,[18] we used a L_0 value of 50,000 in fitting Equation 2 to data. K_G and K_P are equilibrium dissociation constants for GABA and propofol binding to inactive states, and c and d are dimensionless parameters representing the respective ratios of binding constants in active *versus* inactive states. The agonist efficacies of GABA and propofol are inversely related to c and d, respectively. Nonlinear least-squares method fits to Equation 2 were performed using Origin 6.1 software (OriginLab, Northampton, MA). Fitted parameters for MWC models are reported as best fit ± SE.

Results

Our first set of experiments was aimed at identifying a pair of etomidate and propofol concentrations that were equiefficacious for directly activating currents mediated by $\alpha1\beta2\gamma2L$ $GABA_A$ receptors. We chose 10 μM etomidate as our benchmark, because previous studies have shown that 10 μM etomidate predictably activates $GABA_A$ receptors, producing approximately 3–5% of maximal GABA-activated current.[6] In addition, $GABA_A$ receptor currents elicited with 10 μM etomidate do not display desensitization, and this low concentration of etomidate washes out of oocytes within several minutes. Sets of oocytes expressing $\alpha1\beta2\gamma2L$ receptors were activated with 10 μM etomidate and varying propofol concentrations, starting with 10 μM. For each set of oocytes, the average ratio I_{pro}/I_{10eto} was calculated, and in subsequent sets of oocytes, propofol was adjusted upward or downward in steps of 1–3 μM until the average ratio of I_{pro}/I_{10eto} was 0.95 to 1.05. This process identified 19 μM propofol as equiefficacious with 10 μM etomidate (fig. 1). This match was confirmed in a total of 10 oocytes. The ratio of I_{19pro}/I_{10eto} from these oocytes was 0.97 ± 0.11 (mean ± SD). We also observed that current activation by propofol was significantly slower in onset than that by etomidate (fig. 1).

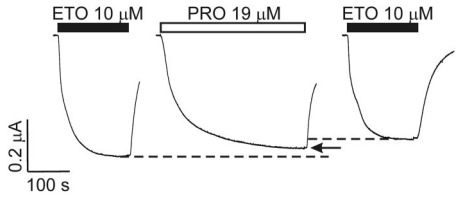

Fig. 1. Direct activation of $GABA_A$ receptors by 10 μM etomidate and 19 μM propofol is equal. Three current traces recorded from a single oocyte expressing $\alpha1\beta2\gamma2L$ γ-aminobutyric acid type A ($GABA_A$) receptors are displayed, illustrating how equiefficacious solutions of etomidate and propofol were identified. Currents were sequentially activated by superfusion with 10 μM etomidate (ETO) or 19 μM propofol (PRO) as illustrated by the *bars* above each trace (*solid* = ETO; *open* = PRO), and the oocyte was washed for 15 min between traces. The current amplitudes change slightly over time, necessitating control ETO applications both before and after the experimental PRO. The maximum current elicited with 19 μM PRO (*arrow*) is approximately the average of the ETO-elicited control currents before and after (*dashed lines*). Current activation with PRO was significantly slower than with ETO. ETO = etomidate; PRO = propofol.

We next compared the effects of 10 μM etomidate *versus* 19 μM propofol on electrophysiological responses to GABA. Oocytes (n ≥ 5) were tested for responses to a single concentration of GABA alone (1 μM, 3 μM, 10 μM, 30 μM, 100 μM, 300 μM, or 1 mM), and then sequentially to GABA supplemented with each anesthetic. Results are summarized in figure 2. Both anesthetics significantly enhanced responses at all seven GABA concentrations tested (two-way ANOVA; $P < 0.001$ at all concentrations), including concentrations eliciting maximal current responses (1 mM GABA; $P < 0.001$ for both etomidate and propofol). In contrast, pairwise comparison of current responses in the presence of 10 μM etomidate *versus* 19 μM propofol showed no significant differences at any GABA concentration ($P > 0.05$). Thus, two different anesthetic solutions with identical direct activating effects on $GABA_A$ receptors also produce the same degree of enhancement in GABA-elicited channel activity.

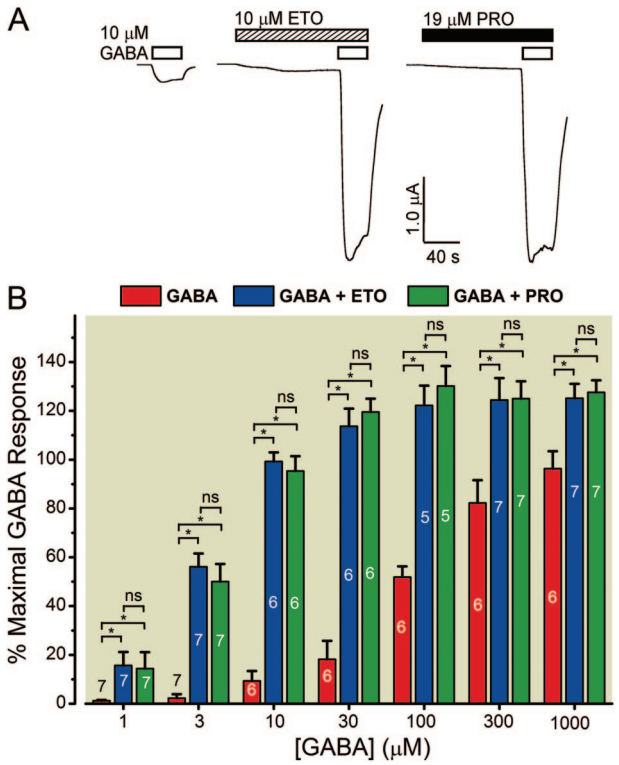

Fig. 2. Comparison of GABA modulation by 10 μM etomidate *versus* 19 μM propofol. (*A*) Three current traces from a single oocyte, illustrating how anesthetic modulation of receptor responses was assessed over a range of γ-aminobutyric acid (GABA) concentrations. After measuring GABA response, oocytes were preexposed to anesthetics (etomidate = ETO; propofol = PRO) before coapplication of anesthetic and GABA at the same concentration. Oocytes were washed for 15 min between ETO and PRO experiments. (*B*) Mean ± SD data from all experiments of this type. *Bar* color signifies experimental condition: GABA alone (*red*); GABA + 10 μM ETO (*blue*); GABA + 19 μM PRO (*green*). The numbers of oocytes is indicated for each condition within the *bar*. Significant enhancement was observed at all GABA concentrations, but no significant difference was observed between results for 10 μM etomidate and 19 μM propofol. * $P < 0.001$; ns $P > 0.05$. ETO = etomidate; GABA = γ-aminobutyric acid; PRO = propofol.

Because the sequential application of etomidate and propofol may have influenced the above results, we also assessed changes in GABA concentration-responses in additional sets of oocytes (n ≥ 5), where each oocyte was exposed to only one anesthetic. This resulted in two sets of control GABA concentration-responses, one for comparison with GABA plus 10 μM etomidate and another for GABA plus 19 μM propofol. These results, displayed in figure 3, show that the two control GABA concentration responses do not significantly differ [$F(4,172) = 1.39$; $P = 0.24$]. Moreover, comparison of logistic fits to normalized GABA concentration responses in the presence of 10 μM etomidate *versus* 19 μM propofol also identified no significant difference [$F(4,129) = 0.30$; $P = 0.88$]. The $\log EC_{50}$ shifts produced by 10 μM etomidate and 19 μM propofol were respectively

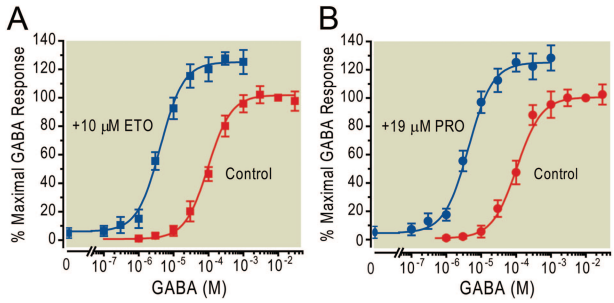

Fig. 3. Effects of 10 μM etomidate *versus* 19 μM propofol on GABA concentration-responses. Data points are mean ± SD measurements of peak currents in at least 5 oocytes per condition, normalized to responses elicited with 10 mM γ-aminobutyric acid (GABA) in the absence of anesthetics. *Lines* drawn through data represent nonlinear least-squares method fits to logistic functions (Equation 1, see Materials and Methods). Fitted parameters are reported as best fit (95% CI). (*A*) Control GABA responses (*red squares*) and responses in the presence of 10 μM etomidate (ETO; *blue squares*). Control: Maximum = 101% (98–102); EC_{50} = 105 μM (95–116 μM); Hill slope = 1.23 (1.10–1.35). With ETO: Maximum = 125% (121–129); EC_{50} = 4.3 μM (3.8–4.9 μM); Hill slope = 1.25 (1.07–1.42). The EC_{50} ratio (ETO/control) = 0.041 (0.034–0.048). (*B*) Control GABA responses (*red circles*) and responses in the presence of 19 μM propofol (PRO; *blue circles*). These data were obtained with different cells from those used for ETO. Control: Maximum = 102% (99–105); EC_{50} = 97 μM (86–109 μM); Hill slope = 1.3 (1.13–1.48). With PRO: Maximum = 125% (122–128); EC_{50} = 4.2 μM (3.6–4.8 μM); Hill slope = 1.3 (1.11–1.51). The EC_{50} ratio (PRO/control) = 0.043 (0.036–0.051). ETO = etomidate; GABA = γ-aminobutyric acid; PRO = propofol.

-1.388 ± 0.035 and -1.367 ± 0.039. These correspond to EC_{50} ratios (95% C.I.) of 0.041 (0.035–0.0480) for 10 μM etomidate and 0.043 (0.036–0.051) for 19 μM propofol. Given the large overlap in 95% CIs, the EC_{50} shifts produced by these two drug solutions are also indistinguishable.

In our allosteric model analysis of etomidate actions,[6] experiments that most determined the fitted number of anesthetic sites were etomidate-dependent reduction of GABA EC_{50} and enhancement of GABA EC5 responses. To provide a data set sufficient to constrain MWC coagonist model parameters for propofol and GABA actions on $GABA_A$ receptors, we quantified propofol-dependent direct activation and propofol-dependent enhancement of GABA EC2.5 (5 ± 1.5 μM). The results of these experiments, normalized to maximal GABA responses, are shown in figure 4.

Data from figures 3B (GABA concentration responses with and without 19 μM propofol) and 4B (propofol concentration-responses with and without EC2.5 GABA) were pooled and renormalized based on the assumption that 1 mM GABA + 19 μM propofol activates all channels. In the nonlinear least-squares method fit to Equation 2, we constrained L_0 to 50,000, a value based on earlier studies of wild-type $\alpha1\beta2\gamma2L$ $GABA_A$ receptors.[6,17,18] The resulting fitted parameters were $K_G = 209 + 43$ μM, $c = 0.0017 \pm 0.00018$,

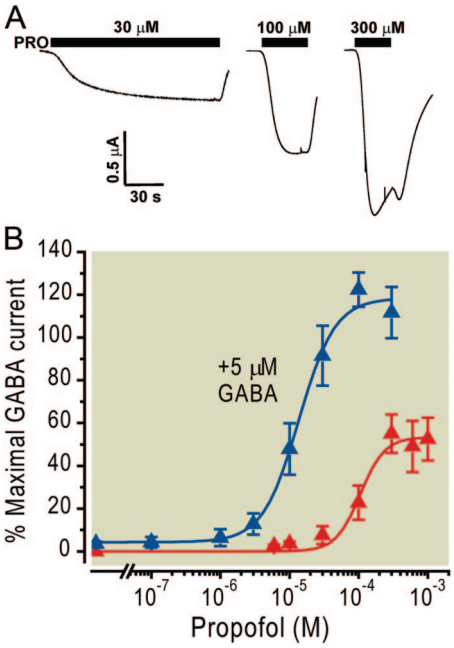

Fig. 4. Propofol direct activation and enhancement of low GABA responses. (*A*) Example traces of γ-aminobutyric acid type A ($GABA_A$) receptor-mediated currents stimulated with propofol (PRO). The *bars* over the traces indicate the period of PRO exposure. Slow activation is observed at 30 μM PRO, and "surge" currents after exposure to 300 μM and higher PRO concentrations indicate a second inhibitory effect. (*B*) Summary data for both PRO-direct activation (*red triangles*) and PRO enhancement of GABA EC2.5 (approximately 5 μM) responses (*blue triangles*). Data points are mean ± SD measurements of peak currents normalized to responses elicited with 10 mM GABA in the absence of anesthetics (n ≥ 4). *Lines* drawn through data represent nonlinear least squares fits to logistic functions (Equation 1, see Materials and Methods). Fitted parameters are reported as best fit (95% CI). PRO-direct activation: Maximum = 53 (50–57); EC_{50} = 106 μM (86–138 μM); Hill slope = 2.5 (1.8–3.5). PRO enhancement of GABA EC2.5: Maximum = 118% (114–126); EC_{50} = 13.6 μM (12.2–16.4 μM); Hill slope = 1.65 (1.4–2.10). GABA = γ-aminobutyric acid.

K_P = 97 ± 56 μM, d = 0.020 ± 0.023, and n = 2.8 ± 0.79. The model is shown overlaid on the data in figure 5.

Given the large uncertainty in the parameters for propofol efficacy and number of sites, we investigated the sensitivity of these fitted values in two ways. First, we examined the chi-square for the global fit while constraining the number of propofol sites (n) to different values. The chi-square test analysis showed that models with multiple propofol sites were clearly better than a one-site model, but there was a very shallow minimum near n = 3, with only 20% change in the chi-square tes as n varied between 2 and 5. We also investigated how the fitted parameters for the number of propofol sites changed as we varied the constrained L_0 value two-fold up or down. When L_0 was 25,000, the fitted value for n was 2.3 ± 0.50, while L_0 constrained to 100,000 resulted in n = 2.7 ± 0.67.

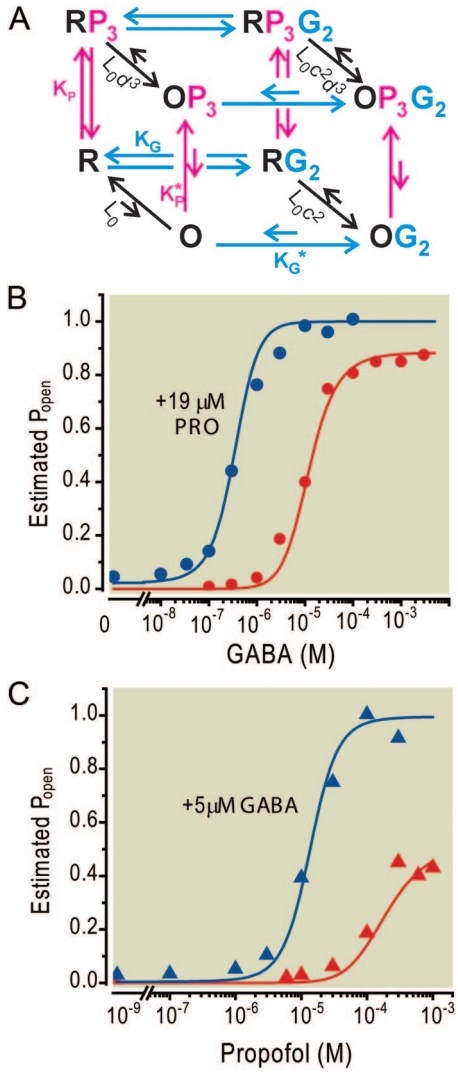

Fig. 5. A Monod-Wyman-Changeux (MWC) coagonist model for propofol and GABA actions. (*A*) A MWC equilibrium coagonist model, with two functional channel states, R (closed) and O (open). After constraining the number of γ-aminobutyric acid and propofol sites, the model has five free parameters (Equation 2, see Materials and Methods). The basal equilibrium between these states (R/O) in the absence of ligands is L_0. γ-Aminobutyric acid (G) binds to two equivalent sites. K_G is the dissociation constant for γ-aminobutyric acid at closed receptors and $K_G^* = cK_G$ is the dissociation constant for γ-aminobutyric acid at open receptors. Propofol (P) binds to three equivalent sites. K_P is the dissociation constant for propofol at closed receptors and $K_P^* = dK_P$ is the dissociation constant for propofol at open receptors. (*B* and *C*) show the MWC model together with P_{open} estimates from figs. 3B and 4B, respectively. Estimated P_{open} was calculated by correcting data normalized to maximal γ-aminobutyric acid (GABA) responses for the apparent efficacy of GABA. Equation 2 was fitted to average P_{open} values with L_0 constrained at 50,000. The *lines* overlying the data represent the best fit (± SD): K_G = 209 ± 43 μM; c = 0.0017 ± 0.00018; K_P = 97 ± 56 μM; d = 0.019 ± 0.023; n = 2.8 ± 0.79. γ-aminobutyric acid = G and GABA; O = open functional channel of MWC equilibrium coagonist model; R = closed functional channel of MWC equilibrium coagonist model; propofol = P and PRO.

Discussion

The major finding of our experiments is that the linkage between propofol direct activation of $GABA_A$ receptors and its modulation of GABA-induced activation (left shift) is identical to that observed with etomidate. This result is consistent with the hypothesis that both propofol actions are mediated by the same binding sites, rather than different sites separately mediating agonism and GABA modulation. In addition, we found that MWC coagonism can quantitatively account for both propofol actions. The fitted propofol dissociation constant (K_P = 97 μM) is higher than values we have fitted for etomidate (K_E = 35–79 μM), and the fitted efficacy value for propofol (d = 0.02) is larger than those for etomidate (d = 0.0077–0.0096),[6,19,20] suggesting that propofol affinity for open receptors (dK_P = 1.9 μM) is weaker than that for etomidate (dK_E = 0.27–0.76 μM).

It is conceivable that independent $GABA_A$ receptor sites mediate propofol-induced agonism and modulating effects, but it is extremely unlikely that the relative effects of propofol at separate agonist and GABA-modulating sites happen by chance to exactly match those we observed with our selected etomidate solution. Furthermore, the allosteric coagonist model accounts for both direct activation and GABA modulation over a wide range of propofol concentrations (fig. 5), not just at the one concentration we studied in detail.

The idea that a single class of sites mediates both propofol modulation and direct activation of $GABA_A$ receptors is consistent with many prior studies. For example, a strong correlation between GABA modulation and direct-activation potencies is apparent for a large series of propofol analogs, including 13 compounds that display neither activity.[21] Furthermore, mutations at both β2N265[13,22] and β2M286[13] in $\alpha1\beta2\gamma2$L $GABA_A$ receptors reduce or eliminate both direct propofol activation and GABA modulation. However, in $GABA_A$ receptors with different subunit composition, a βM286W mutation reduces GABA modulation by propofol without abolishing direct activation.[3,13,22] We have reported similar results for etomidate actions in $\alpha1\beta2\gamma2$L receptors containing the α1M236W mutation.[20,23] These observations remain consistent with allosteric coagonist models, because αM236W and βM286W mutations also produce spontaneous receptor gating,[20] which sensitizes receptors to direct activation by both agonists and allosteric coagonists.[6,17,20]

Other prior studies also reinforce strong parallels between the actions of propofol and etomidate on $GABA_A$ receptors. Both drugs enhance the agonist efficacy of piperidine-4-sulfonic acid, a partial $GABA_A$ receptor agonist.[6,24] Single amino acid mutations on $GABA_A$ receptor β subunits, at positions 265 and 286, similarly alter molecular sensitivity to both propofol and etomidate.[13,22,25,26] Knock-in mice containing β3N265M mutations display dramatically reduced sensitivity to both propofol- and etomidate-induced loss-of-righting reflexes and suppression of nociceptive reflexes.[27] These observations, combined with the results of this study, indicate that propofol and etomidate affect $GABA_A$ receptors through similar molecular mechanisms, and perhaps at nearby sites. Nonetheless, a high concentration of propofol only partially inhibits $GABA_A$ receptor photolabeling by azietomidate, indicating that the sites where these drugs bind do not fully overlap.[28]

Our results are in good accord with other studies of propofol effects at human $GABA_A$ receptors expressed in oocytes.[26] The majority of other electrophysiological studies have examined propofol effects on rodent $GABA_A$ receptors.[13,21,29] We also observed that both onset of $GABA_A$ receptor activation by propofol and its washout from oocytes weres slower than with etomidate. In addition, onset and offset of agonism by both these drugs is slower in oocytes than with GABA, despite their use at similar concentrations. The most likely explanation for these observations is that propofol binds more avidly than etomidate to lipids and proteins inside the oocyte, and these binding sites act to buffer the intramembrane concentration of drug when the extracellular solution changes. Similar buffering effects have been described to explain the slow onset and offset of neuroactive steroid actions in cultured cells.[30–32] GABA, as a charged molecule that acts at the extracellular structures of the receptor, equilibrates more rapidly when its concentration is changed.

The presence of multiple allosteric propofol sites per receptor is supported by our data. The Hill slope for propofol direct activation is 2.5 (fig. 4), and the best fit for the number of equivalent propofol sites in the global MWC model is 2.8 (fig. 5). These values lead us to conclude that there are at least two propofol sites per receptor, and perhaps more. Our modeling is most consistent with the presence of three equivalent propofol sites, but does not strongly favor this value relative to models with two, four, or five sites. In comparison, our modeling of etomidate actions clearly showed a best fit with two sites per receptor.[6] One factor contributing to the uncertainty in the propofol model is that propofol at high concentrations inhibits $GABA_A$ receptors, which may result in underestimating propofol efficacy and overestimating its affinity (etomidate also inhibits $GABA_A$ receptors, but less potently than propofol). While our model assumes noninteracting equivalent sites, distinct propofol sites may interact with each other allosterically and may not contribute equally to receptor gating. Moreover, MWC models with higher numbers of sites are intrinsically more difficult to distinguish, as the contributions of each site to receptor gating becomes smaller.

This uncertainty may be short-lived. A propofol-based anesthetic photolabel has recently been synthesized[33] and another is being developed (written personal communication from Keith W. Miller, Ph.D., Professor of Anesthesia, Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Boston, Massachusetts, in February 2011). In parallel, cultured cell-lines have been developed that produce high levels of homogeneous purified $GABA_A$ receptors suitable for photolabeling experiments.[34]

The combination of these new technologies may reveal, in the near future, both the number of propofol sites on $GABA_A$ receptors and their locations.

The MWC coagonist model provides a formal framework for future experiments aimed at understanding how and where propofol produces its beneficial actions *via* $GABA_A$ receptors, which may help guide development of improved clinical anesthetics. Reframing anesthetics as coagonists also provides insights into how anesthetics affect neuronal circuits through different types of $GABA_A$ receptors. Specifically, important behavioral effects of anesthetics may be mediated by extrasynaptic $GABA_A$ receptors formed from α, β, and δ subunits, that display tonic low-level gating activity (extrasynaptic GABA is approximately 0.5–1 μM), and that may be spontaneously active.[35] Assuming that their propofol sites are similar to those on synaptic receptors, allosteric coagonism predicts that extrasynaptic receptors will be directly activated and/or significantly modulated by low, clinically relevant propofol concentrations, resulting in significantly reduced neuronal excitability. In contrast, clinical propofol concentrations only modestly increase the peak activity of synaptic $GABA_A$ receptors in response to presynaptic release of brief high (more than 1 mM) GABA concentrations. Instead, propofol prolongs activation of synaptic receptors, likely influencing the frequency response of neuronal circuits. Moreover, with a predicted K_P near 100 μM and clinical concentrations in the low micromolar range, another implication of our model is that propofol may occupy only a small fraction of its $GABA_A$ receptor sites when producing its central nervous system effects.

In conclusion, quantitative comparisons of direct $GABA_A$ receptor agonism and GABA-induced receptor activation in the presence of both propofol and etomidate show that the relationship between these measurements is the same for both drugs. This finding strongly favors the hypothesis that propofol-binding sites on these receptors mediate both effects, rather than separate sites independently mediating each effect. Furthermore, quantitative analysis of propofol effects using a MWC coagonist model indicates the presence of multiple propofol-binding sites per $GABA_A$ receptor, with three sites being the value most consistent with this class of model.

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References

1. Alkire MT, Hudetz AG, Tononi G: Consciousness and anesthesia. Science 2008; 322:876–80
2. Franks NP: Molecular targets underlying general anaesthesia. Br J Pharmacol 2006; 147(Suppl 1):S72–81
3. Krasowski MD, Koltchine VV, Rick CE, Ye Q, Finn SE, Harrison NL: Propofol and other intravenous anesthetics have sites of action on the γ-aminobutyric acid type A receptor distinct from that for isoflurane. Mol Pharmacol 1998; 53: 530–8
4. Sanna E, Murgia A, Casula A, Biggio G: Differential subunit dependence of the actions of the general anesthetics alphaxalone and etomidate at γ-aminobutyric acid type A receptors expressed in *Xenopus laevis* oocytes. Mol Pharmacol 1997; 51:484–90
5. Meera P, Olsen RW, Otis TS, Wallner M: Etomidate, propofol and the neurosteroid THDOC increase the GABA efficacy of recombinant $\alpha4\beta3\delta$ and $\alpha4\beta3$ $GABA_A$ receptors expressed in HEK cells. Neuropharmacology 2009; 56:155–60
6. Rüsch D, Zhong H, Forman SA: Gating allosterism at a single class of etomidate sites on $\alpha1\beta2\gamma2L$ $GABA_A$ receptors accounts for both direct activation and agonist modulation. J Biol Chem 2004; 279:20982–92
7. Husain SS, Ziebell MR, Ruesch D, Hong F, Arevalo E, Kosterlitz JA, Olsen RW, Forman SA, Cohen JB, Miller KW: 2-(3-Methyl-3H-diaziren-3-yl)ethyl 1-(1-phenylethyl)-1H-imidazole-5-carboxylate: A derivative of the stereoselective general anesthetic etomidate for photolabeling ligand-gated ion channels. J Med Chem 2003; 46:1257–65
8. Yang J, Uchida I: Mechanisms of etomidate potentiation of GABAA receptor-gated currents in cultured postnatal hippocampal neurons. Neuroscience 1996; 73:69–78
9. O'Shea SM, Williams CA, Jenkins A: Inverse effects on gating and modulation caused by a mutation in the M2–M3 Linker of the $GABA_A$ receptor γ subunit. Mol Pharmacol 2009; 76: 641–51
10. Feng HJ, Macdonald RL: Multiple actions of propofol on $\alpha\beta\gamma$ and $\alpha\beta\delta$ $GABA_A$ receptors. Mol Pharmacol 2004; 66:1517–24
11. Zhong H, Rusch D, Forman SA: Photo-activated azi-etomidate, a general anesthetic photolabel, irreversibly enhances gating and desensitization of γ-aminobutyric acid type A receptors. Anesthesiology 2008; 108:103–12
12. Lam DW, Reynolds JN: Modulatory and direct effects of propofol on recombinant $GABA_A$ receptors expressed in xenopus oocytes: Influence of α- and γ2-subunits. Brain Res 1998; 784:179–87
13. Siegwart R, Krähenbühl K, Lambert S, Rudolph U: Mutational analysis of molecular requirements for the actions of general anaesthetics at the γ-aminobutyric acidA receptor subtype, $\alpha1\beta2\gamma2$. BMC Pharmacology 2003; 3:13
14. Hosie AM, Wilkins ME, da Silva HM, Smart TG: Endogenous neurosteroids regulate $GABA_A$ receptors through two discrete transmembrane sites. Nature 2006; 444:486–9
15. Li GD, Chiara DC, Sawyer GW, Husain SS, Olsen RW, Cohen JB: Identification of a $GABA_A$ receptor anesthetic binding site at subunit interfaces by photolabeling with an etomidate analog. J Neurosci 2006; 26:11599–605
16. Rüsch D, Musset B, Wulf H, Schuster A, Raines DE: Subunit-dependent modulation of the 5-hydroxytryptamine type 3 receptor open-close equilibrium by n-alcohols. J Pharmacol Exp Ther 2007; 321:1069–74
17. Rüsch D, Forman SA: Classic benzodiazepines modulate the open-close equilibrium in $\alpha1\beta2\gamma2L$ γ-aminobutyric acid type A receptors. Anesthesiology 2005; 102:783–92
18. Chang Y, Weiss DS: Allosteric activation mechanism of the $\alpha1\beta2\gamma2$ γ-aminobutyric acid type A receptor revealed by mutation of the conserved M2 leucine. Biophys J 1999; 77:2542–51
19. Desai R, Ruesch D, Forman SA: γ-amino butyric acid type A receptor mutations at β2N265 alter etomidate efficacy while preserving basal and agonist-dependent activity. Anesthesiology 2009; 111:774–84
20. Stewart D, Desai R, Cheng Q, Liu A, Forman SA: Tryptophan mutations at azi-etomidate photo-incorporation sites on α1 or β2 subunits enhance $GABA_A$ receptor gating and reduce etomidate modulation. Mol Pharmacol 2008; 74:1687–95

21. Krasowski MD, Jenkins A, Flood P, Kung AY, Hopfinger AJ, Harrison NL: General anesthetic potencies of a series of propofol analogs correlate with potency for potentiation of γ-aminobutyric acid (GABA) current at the $GABA_A$ receptor but not with lipid solubility. J Pharmacol Exp Ther 2001; 297:338–51
22. Krasowski MD, Nishikawa K, Nikolaeva N, Lin A, Harrison NL: Methionine 286 in transmembrane domain 3 of the $GABA_A$ receptor β subunit controls a binding cavity for propofol and other alkylphenol general anesthetics. Neuropharmacology 2001; 41:952–64
23. Forman SA: Clinical and molecular pharmacology of etomidate. ANESTHESIOLOGY 2011; 114:695–707
24. O'Shea SM, Wong LC, Harrison NL: Propofol increases agonist efficacy at the GABA(A) receptor. Brain Res 2000; 852: 344–8
25. Siegwart R, Jurd R, Rudolph U: Molecular determinants for the action of general anesthetics at recombinant $\alpha2\beta3\gamma2$ γ-aminobutyric acid(A) receptors. J Neurochem 2002; 80:140–8
26. Jonsson Fagerlund M, Sjödin J, Krupp J, Dabrowski MA: Reduced effect of propofol at human $\alpha1\beta2$(N289M)γ2 and $\alpha2\beta3$(N290M)γ2 mutant $GABA_A$ receptors. Br J Anaesth 2010; 104:472–81
27. Jurd R, Arras M, Lambert S, Drexler B, Siegwart R, Crestani F, Zaugg M, Vogt KE, Ledermann B, Antkowiak B, Rudolph U: General anesthetic actions *in vivo* strongly attenuated by a point mutation in the $GABA_A$ receptor β3 subunit. FASEB J 2003; 17:250–2
28. Li GD, Chiara DC, Cohen JB, Olsen RW: Numerous classes of general anesthetics inhibit etomidate binding to γ-aminobutyric acid type A $GABA_A$ receptors. J Biol Chem 2010; 285: 8615–20
29. Trapani G, Latrofa A, Franco M, Altomare C, Sanna E, Usala M, Biggio G, Liso G: Propofol analogues. Synthesis, relationships between structure and affinity at $GABA_A$ receptor in rat brain, and differential electrophysiological profile at recombinant human $GABA_A$ receptors. J Med Chem 1998; 41: 1846–54
30. Akk G, Shu HJ, Wang C, Steinbach JH, Zorumski CF, Covey DF, Mennerick S: Neurosteroid access to the $GABA_A$ receptor. J Neurosci 2005; 25:11605–13
31. Chisari M, Eisenman LN, Krishnan K, Bandyopadhyaya AK, Wang C, Taylor A, Benz A, Covey DF, Zorumski CF, Mennerick S: The influence of neuroactive steroid lipophilicity on $GABA_A$ receptor modulation: Evidence for a low-affinity interaction. J Neurophysiol 2009; 102:1254–64
32. Akk G, Covey DF, Evers AS, Steinbach JH, Zorumski CF, Mennerick S: The influence of the membrane on neurosteroid actions at $GABA_A$ receptors. Psychoneuroendocrinology 2009; 34(Suppl 1):S59–66
33. Hall MA, Xi J, Lor C, Dai S, Pearce R, Dailey WP, Eckenhoff RG: m-Azipropofol (AziPm) a photoactive analogue of the intravenous general anesthetic propofol. J Med Chem 2010; 53:5667–75
34. Dostalova Z, Liu A, Zhou X, Farmer SL, Krenzel ES, Arevalo E, Desai R, Feinberg-Zadek PL, Davies PA, Yamodo IH, Forman SA, Miller KW: High-level expression and purification of Cys-loop ligand-gated ion channels in a tetracycline-inducible stable mammalian cell line: $GABA_A$ and serotonin receptors. Protein Sci 2010; 19:1728–38
35. Belelli D, Harrison NL, Maguire J, Macdonald RL, Walker MC, Cope DW: Extrasynaptic $GABA_A$ receptors: Form, pharmacology, and function. J Neurosci 2009; 29:12757–63