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Classic Benzodiazepines Modulate the Open–Close Equilibrium in $\alpha_1\beta_2\gamma_{2L}$ γ-Aminobutyric Acid Type A Receptors

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Background: **Classic benzodiazepine agonists induce their clinical effects by binding to a site on γ-aminobutyric acid type A ($GABA_A$) receptors and enhancing receptor activity. There are conflicting data regarding whether the benzodiazepine site is allosterically coupled to γ-aminobutyric acid binding *versus* the channel open–close (gating) equilibrium. The authors tested the hypothesis that benzodiazepine site ligands modulate $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptor gating both in the absence of orthosteric agonists and when the orthosteric sites are occupied.**

Methods: **$GABA_A$ receptors were recombinantly expressed in *Xenopus* oocytes and studied using two-microelectrode voltage clamp electrophysiology. To test gating effects in the absence of orthosteric agonist, the authors used spontaneously active $GABA_A$ receptors containing a leucine-to-threonine mutation at residue 264 on the α_1 subunit. To examine effects on gating when orthosteric sites were fully occupied, they activated wild-type receptors with high concentrations of a partial agonist, piperidine-4-sulfonic acid.**

Results: **In the absence of orthosteric agonists, the channel activity of α_1L264T$\beta_2\gamma_{2L}$ receptors was increased by diazepam and midazolam and reduced by the inverse benzodiazepine agonist FG7142. Flumazenil displayed very weak agonism and blocked midazolam from further activating mutant channels. In wild-type receptors activated with saturating concentrations of piperidine-4-sulfonic acid, midazolam increased maximal efficacy.**

Conclusions: **Independent of orthosteric site occupancy, classic benzodiazepines modulate the gating equilibrium in $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors and are therefore allosteric coagonists. A Monod-Wyman-Changeux coagonist gating model quantitatively predicts these effects, suggesting that benzodiazepines minimally alter orthosteric ligand binding.**

CLASSIC benzodiazepine agonists such as diazepam and midazolam are drugs that induce muscle relaxation, sedation, suppression of seizures, anxiolysis, and amnesia by enhancing the function of specific types of γ-aminobutyric acid type A ($GABA_A$) receptors.[1] $GABA_A$ receptors are inhibitory ligand-gated ion channels formed from five homologous subunits arranged around a gated chloride-selective pore. Multiple $GABA_A$ receptor subunit classes (α, β, γ, δ, ϵ, π, and ρ) and isoforms have been identified, each sharing the structural motif of the cys-loop ion channel superfamily: a large N-terminal extracellular domain, four transmembrane domains (M1 to M4), and a large cytoplasmic domain between M3 and M4.[2] Most synaptic $GABA_A$ receptors in mammalian brain are formed from α, β, and γ subunits with stoichiometry 2α:2β:1γ.[3] At the interfaces between α and β extracellular domains, each receptor-channel complex forms binding sites for the neurotransmitter γ-aminobutyric acid (GABA). When GABA binds these two orthosteric agonist sites, gating of the chloride channel is triggered.[4] Sedation and anxiolysis effects of benzodiazepine agonists are associated with another binding site on $GABA_A$ receptors, characterized by nanomolar affinity for its ligands. The amino acids that determine benzodiazepine binding are located at the interface between extracellular domains of the γ subunit and one of the two α subunits. The benzodiazepine site is a structural homolog of the GABA sites.[5,6]

Benzodiazepine agonist binding to $GABA_A$ receptors allosterically increases apparent GABA binding affinity and enhances electrophysiologic responses to low concentrations of GABA. Benzodiazepine site competitive antagonists block the enhancing effects of benzodiazepine agonists, whereas inverse agonists allosterically reduce GABA binding affinity and reduce electrophysiologic responses to GABA.[7,8] In principle, modulation of low GABA responses by benzodiazepine site ligands could be due either to altered GABA binding at the orthosteric sites or to altered gating efficacy (the transition from closed to open states) of agonist-bound receptors, or both.[9] In comparison to studies of general anesthetics that clearly affect the gating equilibrium of $GABA_A$ receptors,[10–12] experiments investigating gating modulation by classic benzodiazepines have provided conflicting results.[11,13–17] The absence of consistent evidence for gating modulation has led some to infer that benzodiazepines act by allosterically altering the microscopic binding of GABA.[11,16]

We have examined gating modulation of recombinantly expressed $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors by several high-affinity benzodiazepine site ligands: diazepam, midazolam, the competitive antagonist flumazenil, and the

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inverse agonist FG7142. Receptor-mediated chloride currents were monitored electrophysiologically in *Xenopus* oocytes using the two-microelectrode voltage clamp technique. To test for gating effects in the absence of GABA site agonists, we introduced a pore domain mutation, α_1L264T, that induces constitutive channel activation.[18,19] These spontaneously active mutant channels provide enhanced sensitivity for gating modulation; if benzodiazepines alter channel gating, the fraction of active channels, and hence chloride current, should change. We also tested midazolam for gating modulation in agonist-bound wild-type receptors using an apparently saturating concentration of the partial agonist piperidine-4-sulfonic acid (P4S). We compared the sensitivity of α_1L264T$\beta_2\gamma_{2L}$ *versus* $\alpha_1\beta_2\gamma_{2L}$ receptors to midazolam by measuring leftward shifts in GABA concentration responses.

Our results indicate that high-affinity benzodiazepine ligands allosterically alter the gating equilibrium of both agonist-bound and unbound $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. These results are explained by a Monod-Wyman-Changeux (MWC) allosteric coagonist mechanism.

Materials and Methods

Animal Care

Xenopus laevis maintenance and oocyte harvest procedures were approved by the Subcommittee on Research and Animal Care of the Massachusetts General Hospital, Boston, Massachusetts.

Drugs and Solutions

γ-Aminobutyric acid, dimethyl sulfoxide (99%), diazepam, midazolam (maleate salt), P4S, picrotoxin, and all buffers and salts were purchased from Sigma (Saint Louis, MO). FG7142 and flumazenil (Ro15-1788) were obtained from Tocris Cookson (Ellisville, MO), and propylene glycol was from Fisher Scientific (Fair Lawn, NJ).

Diazepam was dissolved in 100% propylene glycol to make a 1-mM stock solution, which was diluted in recording solution. The highest concentration of propylene glycol (1%) in electrophysiology experiments had no effect on resting leak currents or on GABA-activated currents. Midazolam was dissolved in double-distilled water (1 mM) and diluted in recording solution. FG7142 and flumazenil stock solutions (20 mM) were prepared in dimethyl sulfoxide and diluted into recording solution. The maximal concentration of dimethyl sulfoxide (0.05%) had no effect on resting leak currents or on GABA-activated currents. Picrotoxin (2 mM) was dissolved directly in recording solution by stirring for 30 min in the dark. GABA stock (1 M) in double-distilled water was stored at −80°C, and fresh aliquots were thawed each day and diluted into recording solution. P4S was dissolved directly in recording solution.

Molecular Biology

Plasmids containing complementary DNAs (cDNAs) for bovine α_1 and human β_2 and γ_{2L} $GABA_A$ receptor subunits were kindly provided by Dr. Paul Whiting (Merck Sharp & Dohme Research Labs, Essex, United Kingdom). The L264T mutation was introduced into the α_1 cDNA as previously described.[19] The presence of the mutation and the absence of stray mutations were confirmed by dideoxynucleotide sequencing. Messenger RNAs (mRNAs) were transcribed *in vitro* from linearized cDNA templates (Ambion Inc., Austin, TX), isolated using spin columns (Ambion Inc.), and stored at −80°C.

Oocyte Expression

Oocytes were harvested from female *Xenopus laevis* (Xenopus One, Ann Arbor, MI) as described previously.[10] Defolliculated stage V and VI oocytes were injected with 25–50 nl subunit mRNA mixtures with a weight/weight ratio of $1\alpha_1{:}1\beta_2{:}4\gamma_2$ to ensure efficient incorporation of γ_2 subunits and consistent sensitivity to classic benzodiazepines.[20,21] Oocytes were incubated for 3–6 days at 18°C in ND-96 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 0.5 U/ml penicillin and 5 μg/ml streptomycin.

Electrophysiology

Electrophysiologic recordings using the two-microelectrode oocyte voltage clamp technique were performed at room temperature (22°C). Oocytes were placed in a 0.02-ml flow chamber, impaled with borosilicate pipettes filled with 3 M KCl (0.5–2 MΩ), and voltage-clamped at −50 mV (model OC-725C; Warner Instrument Corp., Hamden, CT). Cells were superfused at a rate of 4–5 ml/min with ND-96 recording solution (without antibiotics) from glass syringe reservoirs *via* a polytetrafluoroethylene valve and tubing system. Delivery of superfusion solutions from reservoirs to the flow chamber was controlled by computer-activated solenoid valves. Currents were digitized at 100 Hz (Digidata 1200; Axon Instruments, Foster City, CA) and recorded on a personal computer running commercial software (Clampex8; Axon Instruments).

Experimental Protocols

Because α_1L264T$\beta_2\gamma_{2L}$ receptors are very sensitive to GABA, particular care was taken to eliminate GABA and other orthosteric agonists from the apparatus during investigation of the direct effects of benzodiazepines. The potent $GABA_A$ receptor inhibitor picrotoxin (2 mM) was used to measure the spontaneously active currents. After complete washout of picrotoxin (10 min), benzodiazepine solutions were applied to oocytes, and benzodiazepine-elicited currents (I^{BZ}) were normalized to the spontaneous picrotoxin-sensitive current (I^{PTX}). Benzodiazepine applications varied in duration from 15 to

120 s, depending on the rate of current activation for the specific study solution, followed by a washout of at least 5 min in ND-96. Separate experiments on a different set of oocytes were performed to determine the ratio of I^{PTX} to maximal GABA-activated current (I_{max}^{GABA}).

Midazolam effects on P4S efficacy in wild-type $GABA_A$ receptors were studied as follows: In one set of oocytes, P4S concentration responses were measured to determine the concentration range associated with maximal receptor activation (3–10 mM). Every other sweep was activated with 1 mM P4S for normalization. These oocytes were then exposed to 1 μM midazolam for 10 s before activation with 1 μM midazolam plus 10 mM P4S for 30 s. A second set of oocytes was used to compare the maximal efficacy of 10 mM P4S (I_{max}^{P4S}) to that of 1 mM GABA (I_{max}^{GABA}).

γ-Aminobutyric acid concentration–response studies in oocytes expressing wild-type or mutant receptors were performed in the absence and presence of midazolam. The GABA concentration ranges used for wild-type (up to 1 mM) and mutant (up to 100 μM) receptors were based on previous studies.[10,19] Currents were activated with GABA for 15–30 s followed by a 3- to 5-min washout. For normalization of peak currents, every other sweep was activated with maximal GABA. After GABA responses were recorded in the absence of midazolam, a second set of experiments in the same oocyte was performed in the presence of midazolam (added to both ND-96 wash and GABA solutions).

Data Analysis and Statistics

Analysis of current traces was conducted off-line.

Concentration–Response Analysis. After leak correction, currents were normalized to the average of pretest and posttest control responses in the same oocyte (elicited with 2 mM picrotoxin, 1 mM P4S, or maximal GABA). These values were renormalized relative to maximal GABA responses based on the independent determinations of I^{PTX}/I_{max}^{GABA} and $I_{max}^{P4S}/I_{max}^{GABA}$ ratios.

Leak-corrected and normalized concentration responses in individual oocytes were fitted by nonlinear least squares with Hill (logistic) equations of the general form

$$\frac{I^X}{I_{max}^{GABA}} = \frac{I_{max}^X}{I_{max}^{GABA}} \times \left(\frac{1}{1 + (EC_{50}/[X])^n}\right), \quad (1)$$

where X is the varied activating ligand (GABA or a benzodiazepine agonist), $I_{max}^{GABA}$is the maximally evoked current, EC_{50} is the concentration of X eliciting half of its maximal effect, and n is the Hill coefficient of activation. For GABA concentration–response studies, we defined EC_{50}^0 as the half-effect GABA concentration in the absence of other ligands and EC_{50}^{MDZ} in the presence of midazolam. Because GABA EC_{50}s varied more than twofold from oocyte to oocyte, GABA EC_{50} left-shift ratios (EC_{50}^{MDZ}/EC_{50}^0) were calculated for individual oocytes.

Normalized current data were also used to estimate the overall open probability (P_{open}) of both wild-type and mutant receptors. To estimate P_{open}, we combined the ligand-stimulated receptor currents (I^X, where X represents either GABA or benzodiazepine agonist) and the basal spontaneous (picrotoxin-sensitive) current (I^{PTX}). The total current was normalized to the sum of the basal current and the maximal stimulated GABA current ($I_{max}^{GABA} + I^{PTX}$). Finally, estimated P_{open} values were renormalized assuming that P_{open} in the presence of maximal GABA is 0.85 for wild-type $\alpha_1\beta_2\gamma_{2L}$ and 1.0 for α_1L264T$\beta_2\gamma_{2L}$ (based on previous macrocurrent and single-channel studies).[10,18] For wild-type currents, where I^{PTX} is too small to measure, this simply required multiplying $I^X/I_{max}^{GABA} \times 0.85$. In a generalized format, estimated P_{open} (P_{open}^{est}) was calculated as follows:

$$P_{open}^{est} = P_{open}^{max} \times \left(\frac{I^X + I^{PTX}}{I_{max}^{GABA} + I^{PTX}}\right) = P_{open}^{max} \times \left(\frac{\frac{I^X}{I_{max}^{GABA}} + \frac{I^{PTX}}{I_{max}^{GABA}}}{1 + \frac{I^{PTX}}{I_{max}^{GABA}}}\right), \quad (2)$$

where all currents are used as positive amplitudes. Logistic fits were also performed on the data displayed as P_{est}^{open} using equations of the form

$$\frac{I^X + I^{basal}}{I_{max}^{GABA} + I^{basal}} = \frac{I_{max}^X}{I_{max}^{GABA} + I^{basal}} \times \left(\frac{1}{1 + (EC_{50}/[X])^n}\right) + \frac{I^{basal}}{I_{max}^{GABA} + I^{basal}}, \quad (3)$$

where X represents the variable stimulating ligand (GABA or a benzodiazepine) and I^{basal} represents either the spontaneous current (I^{PTX}) or when midazolam was present during GABA concentration responses, $I^{PTX} + I^{MDZ}$.

Analysis Using the Monod-Wyman-Changeux Model. Open probability in the MWC coagonist model, which assumes two equivalent GABA sites and a single benzodiazepine site, is defined by the following equation[9,22]:

$$P_{open} = \frac{1}{1 + L_0\left(\frac{1 + [GABA]/K_G}{1 + [GABA]/cK_G}\right)^2\left(\frac{1 + [BZ]/K_{BZ}}{1 + [BZ]/dK_{BZ}}\right)}, \quad (4)$$

where L_0 is the basal gating equilibrium constant ([closed]/[open] for unliganded receptors), K_G is the equilibrium dissociation constant for GABA binding to closed receptors, c is the GABA efficacy for each of the two orthosteric sites (the ratio of dissociation constants in open *vs.* closed receptors), K_{BZ} is the equilibrium dissociation constant for benzodiazepine binding to closed receptors, and d is the benzodiazepine efficacy.

The MWC allosteric model was fitted by nonlinear least squares to subsets of P_{open}^{est} values derived from electrophysiologic data. The two independent L_0 parameters for the mutant and wild-type receptor models were constrained during fitting. L_0 for mutant receptors was calculated based on mutant basal activity results ($L_0^{mut} = I_{max}^{GABA}/I^{PTX} = 9.1$). L_0 for wild-type receptors ($L_0^{wt} = 40{,}000$) was calculated based on the model's predicted relation between GABA EC_{50} and L_0^{mut} for mutations such as α_1L264T that selectively affect the gating equilibrium[18,19]:

$$L_0^{wt} = L_0^{mut} \times \left(\frac{EC_{50}^{wt}}{EC_{50}^{mut}}\right)^2. \quad (5)$$

Binding and efficacy parameters for both GABA and benzodiazepines were assumed to be global parameters (the same in both mutant and wild-type models). Using P_{open}^{est} values calculated from direct activation results in mutant receptors, benzodiazepine binding and efficacy parameters were fitted to equation 4 with $L_0^{mut} = 9.1$ and GABA = 0. K_G and c were fitted to wild-type GABA concentration–response P_{open}^{est}values using equation 4 with $L_0^{wt} = 40{,}000$ and BZ = 0.

Predicted midazolam effects on GABA concentration responses for both wild-type and mutant models were calculated with equation 4 using the appropriate L_0 values and midazolam and GABA values varied to simulate experimental conditions.

Nonlinear least-square fits and model calculations were performed using Origin 6.0 software (Microcal Inc., Northampton, MA). Fitted values are given as mean ± SD.

Statistical Analyses. The Student *t* test was used to statistically establish minimally active concentrations of benzodiazepines and to compare responses elicited by P4S and those elicited by P4S plus midazolam. The Wilcoxon test was applied to compare GABA EC_{50}s in the absence *versus* presence of midazolam. A *P* value of less than 0.05 was considered to be statistically significant.

Results

Constitutive Activity in GABA$_A$ Receptors

Oocytes expressing α_1L264T$\beta_2\gamma_{2L}$ $GABA_A$ receptors displayed high holding currents when voltage clamped at −50 mV in ND-96. Picrotoxin (2 mM) caused an apparently outward current in oocytes expressing α_1L264T$\beta_2\gamma_{2L}$ $GABA_A$ receptors (fig. 1A), which represents inhibition of the current through constitutively active chloride channels.[10,18] Higher concentrations of picrotoxin did not further inhibit mutant channel activity. For all oocytes tested, the picrotoxin-sensitive leak current (I^{PTX}) averaged 11 ± 3.9% (n = 15) of the maximal inward current evoked by 1 mM GABA (I_{max}^{GABA}).

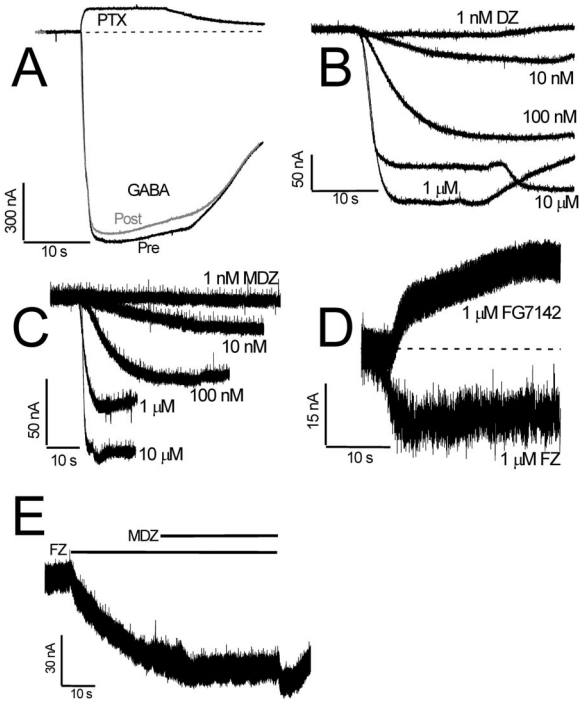

Fig. 1. Currents through constitutively active mutant α_1L264T$\beta_2\gamma_{2L}$ γ-aminobutyric acid type A ($GABA_A$) receptors are modulated by benzodiazepine site ligands. (*A*) Currents from an oocyte expressing α_1L264T$\beta_2\gamma_{2L}$ $GABA_A$ receptors display constitutive activity in the absence of agonists. An apparently outward current is observed in the presence of 2 mM picrotoxin (PTX), which inhibits active channels. Inward currents from the same oocyte elicited with 1 mM γ-aminobutyric acid (GABA) before and after the picrotoxin exposure are also shown. (*B*) Diazepam elicits inward currents from an oocyte expressing α_1L264T$\beta_2\gamma_{2L}$ $GABA_A$ receptors. Traces are labeled with the diazepam (DZ) concentration. At 10 μM, diazepam initially elicits a smaller current than 1 μM diazepam, and a "surge" current is observed before deactivation. (*C*) Midazolam elicits inward currents from an oocyte expressing α_1L264T$\beta_2\gamma_{2L}$ $GABA_A$ receptors. Traces are labeled with the midazolam (MDZ) concentration. (*D*) FG7142, a benzodiazepine inverse agonist, reduces the activity of α_1L264T$\beta_2\gamma_{2L}$ $GABA_A$ receptors (apparent outward current), whereas flumazenil (FZ) elicits small inward currents. (*E*) Flumazenil (1 μM) elicits a small inward current and blocks further activation by 100 nM midazolam.

Direct Activation of α_1L264T$\beta_2\gamma_{2L}$ $GABA_A$ Receptors by Classic Benzodiazepine Agonists

In the absence of GABA or other orthosteric agonists, diazepam reversibly induced inward currents in oocytes expressing α_1L264T$\beta_2\gamma_{2L}$ $GABA_A$ receptors (fig. 1B). Diazepam-induced current magnitudes were concentration dependent. In some oocytes, 1 nM diazepam caused a detectable deviation from basal activity, but on average, this was not significantly different from holding current ($P = 0.19$), whereas at 10 nM ($P = 0.001$) and at all higher concentrations studied ($P < 0.001$), diazepam consistently evoked inward currents. Midazolam also directly activated α_1L264T$\beta_2\gamma_{2L}$ $GABA_A$ receptors in a concentration-dependent manner (fig. 1C). At 10 nM ($P = 0.003$) and at higher concentrations ($P < 0.001$), midazolam consistently evoked inward currents.

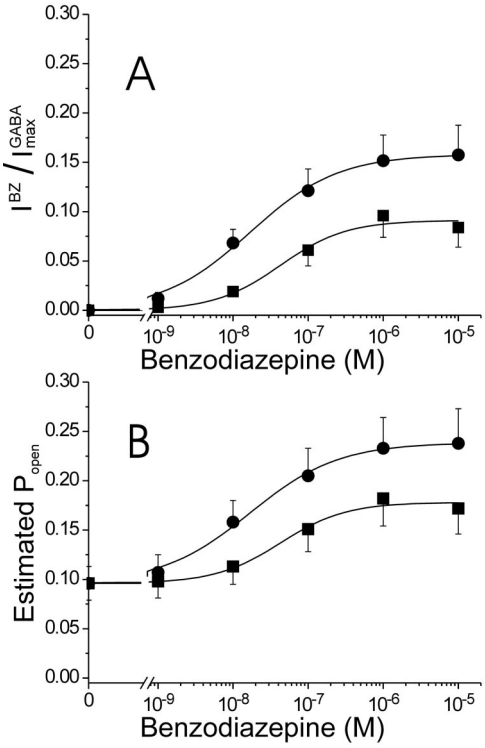

Fig. 2. Benzodiazepine agonist concentration responses in $\alpha_1L264T\beta_2\gamma_{2L}$ γ-aminobutyric acid type A receptors. (*A*) Data points represent averaged normalized data (mean ± SE) from oocyte currents elicited with diazepam (*squares*; n = 5) and midazolam (*circles*; n = 5). *Lines* through data represent logistic fits to equation 1 (Materials and Methods). For diazepam, $I^{DZ}_{max}/I^{GABA}_{max}$ = 0.091 ± 0.0052, EC^{DZ}_{50} = 40 ± 14 nM, and n = 1.0 ± 0.26. For midazolam, $I^{MDZ}_{max}/I^{GABA}_{max}$ = 0.16 ± 0.004, EC^{MDZ}_{50}= 17 ± 2.7 nM, and n = 0.80 ± 0.071. (*B*) Estimated P_{open} values (equation 2, Materials and Methods) were calculated from the same data shown in *A* and are plotted on identical axes for comparison. Note that estimated P_{open} includes both the spontaneous (picrotoxin-sensitive) current and the current elicited with benzodiazepines. Although the baseline activity (P_0 = 0.096) and overall scaling are altered, logistic fits (equation 3) derived EC_{50}s and Hill slope values almost identical to those from *A*. For diazepam, EC^{DZ}_{50} = 40 ± 17 nM and n = 1.0 ± 0.32. For midazolam, EC^{MDZ}_{50} = 17 ± 2.9 nM and n = 0.70 ± 0.080.

Effects of FG7142 and Flumazenil on $\alpha_1L264T\beta_2\gamma_{2L}$ $GABA_A$ Receptors

In the absence of agonist, the benzodiazepine inverse agonist FG7142 elicited apparently outward currents in oocytes expressing $\alpha_1L264T\beta_2\gamma_{2L}$ $GABA_A$ receptors (fig. 1D), indicating reduced channel activity (n = 8). Reduced mutant channel activation was consistently observed at 10 nM FG7142 (P = 0.03) and at higher concentrations ($P < 0.001$). Flumazenil weakly activated inward currents in oocytes expressing $\alpha_1L264T\beta_2\gamma_{2L}$ $GABA_A$ receptors (n = 8; fig. 1D). Flumazenil at 10 nM (P = 0.005) and higher concentrations ($P < 0.005$) consistently increased mutant channel activity. When oocytes expressing $\alpha_1L264T\beta_2\gamma_{2L}$ $GABA_A$ receptors were exposed to 10 μM flumazenil followed by 10 μM flumazenil plus 100 nM midazolam, flumazenil alone activated a small inward current, but no further activation was observed after the addition of 100 nM midazolam, which alone elicited more than three times the current stimulated by flumazenil (fig. 1E; n = 3). Therefore, flumazenil blocked midazolam activation of mutant channels.

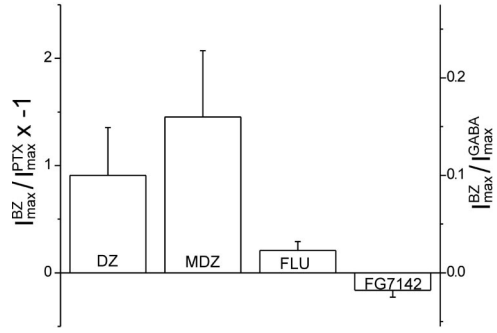

Fig. 3. Relative efficacies of benzodiazepine site ligands in $\alpha_1L264T\beta_2\gamma_{2L}$ γ-aminobutyric acid type A receptors. The bar chart depicts maximal efficacies (mean ± SD, n ≥ 3), scaled relative to both the maximal picrotoxin-inhibited current (I^{PTX}_{max}; *left ordinate axis*) and the maximal γ-aminobutyric acid (GABA)–activated current (I^{GABA}_{max}; *right ordinate axis*). FG7142, an inverse agonist, seems to have a negative efficacy because it reduces channel activity. DZ = diazepam; FLU = flumazenil; MDZ = midazolam.

Potency and Efficacy of Benzodiazepine Site Ligands in $\alpha_1L264T\beta_2\gamma_{2L}$ $GABA_A$ Receptors

Figure 2A displays average leak-corrected normalized responses from direct benzodiazepine activation experiments in oocytes expressing $\alpha_1L264T\beta_2\gamma_{2L}$ $GABA_A$ receptors. The same raw data were used in equation 2 (Materials and Methods) to derive estimated P_{open} values, shown in figure 2B. The major difference between figures 2A and B is that the latter explicitly incorporates the spontaneous currents observed in experiments with mutant receptors. Data in both panels of figure 2 were fitted with Hill equations (equation 1 for fig. 2A and equation 3 for fig. 2B; Materials and Methods). The EC_{50}s from Hill fits (n = 5 for each drug) were 40 ± 14 nM for diazepam and 17 ± 2.7 nM for midazolam. Maximal direct activation was seen at diazepam or midazolam concentrations of 1–10 μM. Maximum average activation by diazepam was 9% of I^{GABA}_{max}, whereas maximum activation by midazolam was approximately 16% of I^{GABA}_{max} (fig. 3).

Direct effects of both FG7142 and flumazenil on mutant receptor currents were concentration dependent, but because of their low efficacy, EC_{50}s and Hill slopes for these drugs could not be accurately determined. Maximal effects of both FG7142 and flumazenil were seen at concentrations of 1–10 μM. For FG7142, the average maximum outward current was 1.8% of I^{GABA}_{max}, whereas flumazenil elicited maximal inward currents that were approximately 2% of I^{GABA}_{max} (fig. 3).

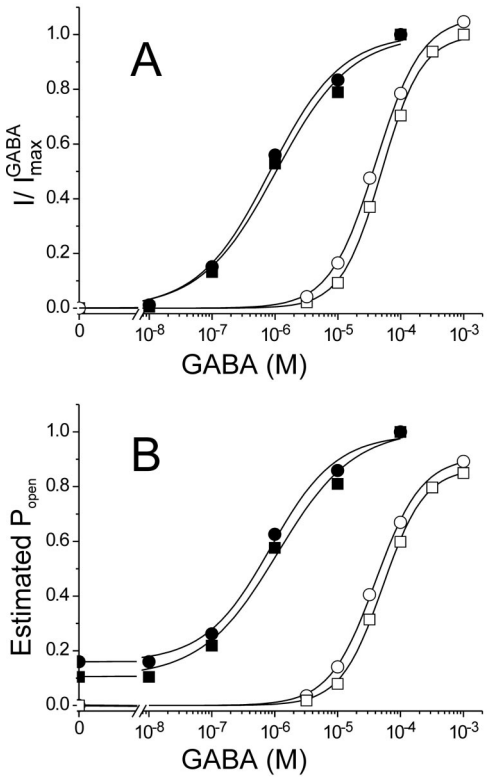

Fig. 4. Midazolam induces similar left shifts in γ-aminobutyric acid (GABA) concentration-responses of both wild-type $\alpha_1\beta_2\gamma_{2L}$ and mutant α_1L264T$\beta_2\gamma_{2L}$ GABA type A receptors. (*A*) Normalized current measurements from two representative oocytes—one expressing wild-type receptors (*open symbols*) and one expressing mutant receptors (*solid symbols*)—are plotted against GABA concentration. In each oocyte, GABA responses were measured both in the absence (*squares*) and presence (*circles*) of 10 nM midazolam. Maximal currents in this wild-type oocyte (and others) were slightly enhanced (approximately 5%) by midazolam. *Lines* drawn through data represent logistic fits with equation 1 (Materials and Methods). Wild-type control (*open squares*): EC_{50}^{0} = 52 ± 1.6 μM, n = 1.4 ± 0.06. Wild-type plus midazolam (*open circles*): EC_{50}^{MDZ} = 40 ± 1.7 μM, n = 1.2 ± 0.05. Mutant control (*solid squares*): EC_{50}^{0}= 1.1 ± 0.17 μM, n = 0.7 ± 0.14. Mutant plus midazolam (*solid circles*): EC_{50}^{MDZ} = 0.85 ± 0.12 μM, n = 0.8 ± 0.13. The $EC_{50}^{MDZ}/EC_{50}^{0}$ ratios for wild-type and mutant data from these oocytes are both 0.77. (*B*) Estimated P_{open} values were calculated (equation 2, Materials and Methods) from the data in *A* and redrawn on identical axes for comparison. In the data for the oocyte expressing mutant receptors (*solid symbols*), both the basal activity (P_0 = 0.105) and direct activation by midazolam ($P_{0,MDZ}$ = 0.16) are evident. Logistic fits (equation 3, Materials and Methods) to estimated P_{open} values derive EC_{50}s and Hill slopes almost identical to those from *A*. Wild-type control (*open squares*): EC_{50}^{0} = 52 ± 1.6 μM, n = 1.4 ± 0.06. Wild-type plus midazolam (*open circles*): EC_{50}^{MDZ} = 40 ± 1.7 μM, n = 1.2 ± 0.05. Mutant control (*solid squares*): EC_{50}^{0} = 1.1 ± 0.37 μM, n = 0.7 ± 0.14. Mutant plus midazolam (*solid circles*): EC_{50}^{MDZ} = 0.86 ± 0.19 μM, n = 0.8 ± 0.13.

Leftward Shifts by Midazolam in Wild-type and Mutant Receptors

Midazolam modulation of $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors was assessed by measuring leftward shifts in GABA concentration responses. Results from individual oocytes expressing wild-type receptors displayed variable GABA EC_{50}s, averaging 66 ± 36.2 μM (± SD; n = 14). In the presence of 10 nM midazolam, a consistent and significant (P = 0.043) leftward shift of the GABA response curve was observed. An example for one oocyte is shown in figure 4 (open symbols). The average wild-type $EC_{50}^{MDZ}/EC_{50}^{0}$ ratio at 10 nM midazolam was 0.77 ± 0.10 (n = 5). Left-shift ratios ($EC_{50}^{MDZ}/EC_{50}^{0}$) at 100 nM (n = 3) and 1 μM (n = 6) midazolam averaged 0.50 ± 0.055 and 0.42 ± 0.10, respectively.

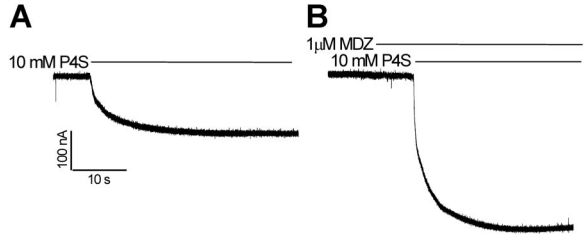

Fig. 5. Midazolam increases the maximal efficacy of the partial agonist piperidine-4-sulfonic acid (P4S) in wild-type γ-aminobutyric acid type A receptors. Two current traces from a single oocyte expressing $\alpha_1\beta_2\gamma_{2L}$ γ-aminobutyric acid type A receptors are shown. (*A*) A maximal control current elicited with 10 mM P4S. (*B*) preexposure of the oocyte to 1 μM midazolam (MDZ) elicits no apparent current, but subsequent addition of 10 mM P4S elicits a current that is over twice as large as the control current.

To compare midazolam modulation of $\alpha_1\beta_2\gamma_{2L}$ and α_1L264T$\beta_2\gamma_{2L}$ $GABA_A$ receptors, we also assessed leftward shifts in the presence of 10 nM midazolam in oocytes expressing α_1L264T$\beta_2\gamma_{2L}$ $GABA_A$ receptors. In oocytes (n = 5) expressing mutant channels, GABA EC_{50}^{0} was 1 μM ± 0.45 μM. In the presence of 10 nM midazolam, a significant (P = 0.04) leftward shift was consistently observed (fig. 4). The $EC_{50}^{MDZ}/EC_{50}^{0}$ ratio for mutant receptors averaged 0.67 ± 0.22. Therefore, in both wild-type and mutant $GABA_A$ receptors, the leftward shifts caused by 10 nM midazolam were similar.

Midazolam Effects on P4S Efficacy in $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ Receptors

The partial orthosteric agonist P4S elicited inward currents in oocytes expressing wild-type $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors. Concentration-dependent activation by P4S showed an EC_{50} of 290 ± 18 μM and a Hill slope of 0.94 ± 0.039 (n = 5; data not shown). There was less than a 5% increase in current response when P4S was increased from 3 to 10 mM. Therefore, 10 mM P4S (34 × EC_{50}) was maximally activating in wild-type $\alpha_1\beta_2\gamma_{2L}$ receptors and presumably occupies essentially all of the orthosteric agonist sites. Maximal P4S currents averaged 38 ± 3.4% (n = 3) of maximal currents elicited with 1 mM GABA. Currents elicited by 1 μM midazolam plus 10 mM P4S were significantly ($P < 0.01$) larger compared with peak currents elicited by 10 mM P4S alone (fig. 5). Midazolam increased maximal P4S efficacy by a factor of 2.5 ± 0.55 (n = 5).

Discussion

We tested the hypothesis that ligand binding to the high-affinity benzodiazepine site formed at the interface of $GABA_A$ receptor α and γ subunits is coupled to the channel open–closed gating equilibrium (gating allosterism). Electrophysiologic concentration–response data from peak currents in oocytes expressing $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors were previously shown to mirror results obtained using smaller voltage clamped HEK293 cells, where currents were elicited with millisecond concentration jumps.[10] This is because $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptor currents desensitize slowly when the γ_2 subunit is overexpressed[20] and because we use relatively rapid solution exchange in our custom-built oocyte flow chamber.

Our electrophysiologic experiments assessed the effects of benzodiazepine site ligands on gating in receptors that either had no orthosteric ligand bound or were agonist bound. We found that the classic benzodiazepine agonists diazepam and midazolam directly enhance the open probability of constitutively activated mutant $GABA_A$ receptors in the absence of orthosteric agonists, whereas the benzodiazepine inverse agonist FG7142 reduced spontaneous channel openings (figs. 1 and 3). In addition, flumazenil, which is classified as a competitive benzodiazepine antagonist, seems to be a very weak partial agonist at the benzodiazepine site. These data unambiguously indicate that benzodiazepine site ligands allosterically alter gating in the mutant $GABA_A$ receptors in the absence of orthosteric agonists.

To address the mechanism of benzodiazepine action in agonist-bound receptors, we used saturating concentrations of the orthosteric partial agonist P4S in wild-type $GABA_A$ receptors. We found that midazolam increases the maximal efficacy of P4S (fig. 5). Because $GABA_A$ receptor single-channel conductance is unchanged by classic benzodiazepines[13,14] and orthosteric site occupancy cannot increase at saturating P4S concentrations, our result indicates that gating of P4S-bound $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors is allosterically enhanced in the presence of midazolam.

Walters *et al.*[23] reported that, in addition to its actions at the high-affinity α/γ site, very high concentrations (> 10 μM) of diazepam can enhance the function of $GABA_A$ receptors *via* one or more low-affinity sites, which are not affected by flumazenil. We found that flumazenil completely blocks further direct activation of α_1L264T$\beta_2\gamma_{2L}$ $GABA_A$ receptors by the full benzodiazepine agonist midazolam (fig. 1E). Therefore, the low affinity sites described by Walters *et al.* do not mediate direct benzodiazepine activation under the conditions used in our experiments. Both flumazenil and other benzodiazepine actions we observed in mutant receptors are due to interactions at the high-affinity α/γ interfacial site.

The leftward shifts in GABA concentration responses caused by 10 nM midazolam were similar in both wild-type and mutant receptors (fig. 4). These results indicate that sensitivity to benzodiazepines is comparable in wild-type receptors and those containing the α_1L264T mutation. Furthermore, the efficacy of midazolam was similar in both unbound and agonist-bound $GABA_A$ receptors. In α_1L264T$\beta_2\gamma_{2L}$ receptors, channel activity was increased approximately 160% above constitutive by 1–10 μM midazolam, indicating approximately a 2.6-fold increase in the open probability. In wild-type receptor currents, 1 μM midazolam also increased the efficacy of P4S approximately 2.5-fold.

Allosteric coagonism, as depicted in figure 6A, is a mechanism that elegantly accounts for the gating effects by benzodiazepines in both unbound and agonist-bound $GABA_A$ receptors. In this equilibrium scheme, based on classic MWC allosterism,[22] there are only two types of receptors, open and closed, and both GABA binding and benzodiazepine binding are coupled to the open–closed equilibrium *via* differential binding affinities. The scheme is highly constrained and defined by only five equilibrium parameters: L_0 (the basal equilibrium between closed [R] and open [O] states), K_G (the closed state microscopic dissociation constant for GABA binding to its two equivalent orthosteric sites), c (GABA efficacy, defined as the ratio of dissociation constants in open *vs.* closed receptors), K_{BZ} (the closed state microscopic dissociation constant for benzodiazepine binding), and d (benzodiazepine efficacy). In this and other MWC mechanisms, agonists are ligands that selectively bind to the open state and therefore have efficacy values less than 1.0, whereas inverse agonists bind more tightly to the closed state (efficacy > 1.0), and competitive antagonists have efficacy $= 1.0$.

We[10] and others[18] have previously shown that common GABA binding (K_G) and efficacy (c) values but different L_0 values can be used to nearly quantitatively account for the GABA concentration responses in both wild-type and α_1L264T$\beta_2\gamma_{2L}$ receptors. Based on the equal left shifts seen at 10 nM midazolam (fig. 4), we also surmised that the underlying benzodiazepine binding (K_{BZ}) and efficacy (d) parameters are common to both wild-type and mutant receptors. We estimated binding and efficacy parameters for diazepam and midazolam by fitting equation 4 (with GABA $= 0$; Materials and Methods) to estimated P_{open} values derived from direct activation of α_1L264T$\beta_{2\gamma 2L}$ receptors (fig. 2B). In these fits, L_0^{mut} was constrained to a value of 9.1, based on the average ratio of maximal GABA-activated current to picrotoxin-inhibited current in oocytes expressing α_1L264T$\beta_2\gamma_{2L}$ $GABA_A$ receptors (9.1 ± 3.0). These fits are shown in figure 6B, and the fitted parameters are reported in the legend of figure 6. Our analysis with the MWC mechanism suggests that midazolam is an agonist that binds approximately threefold more tightly to open

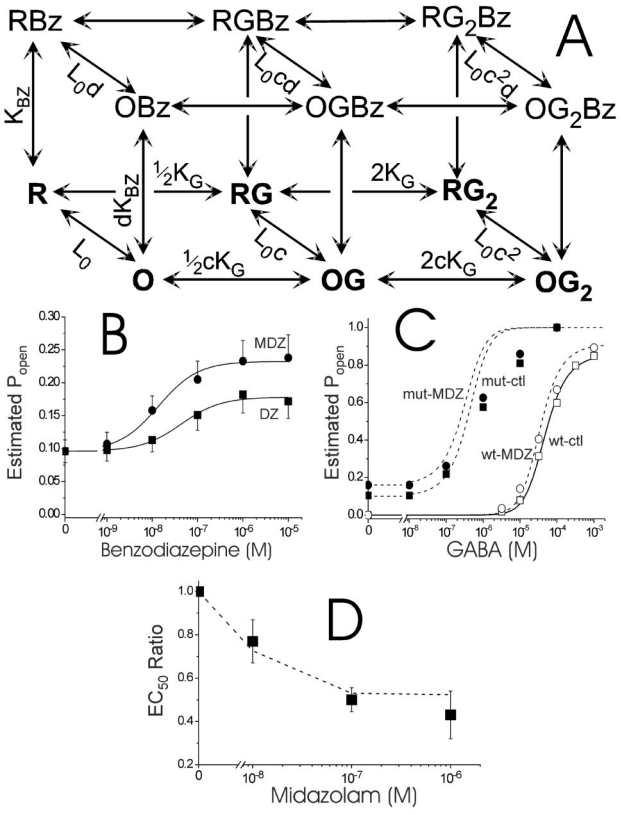

Fig. 6. Monod-Wyman-Changeux coagonist mechanism for classic benzodiazepine and γ-aminobutyric acid (GABA) modulation of GABA type A receptors. (*A*) A schematic of the mechanism shows 12 states and their equilibria, which are defined by five equilibrium parameters. The fundamental two-state gating equilibrium is defined by L_0 = [R]/[O]. Ligands modulate the open-closed equilibrium by selectively binding to one of the two states. GABA binds to two equivalent orthosteric sites, each with a microscopic dissociation constant K_G, and the efficacy of GABA is defined by c, the affinity ratio in open *versus* closed receptors. Benzodiazepines bind to a single allosteric site, with a microscopic dissociation constant K_{BZ} and an efficacy of d, defined analogously to c. (*B*) Averaged data for diazepam and midazolam direct activation of mutant GABA type A currents is redrawn from fig. 2B. *Lines* through data represent fits with equation 4 (holding GABA = 0 and L_0^{mut} = 9.1; Materials and Methods). Diazepam (*squares*): K_{DZ} = 45 ± 13 nM, d = 0.5 ± 0.21. Midazolam (*circles*): K_{MDZ} = 16 ± 3.3 nM, d = 0.34 ± 0.095. (*C*) Modeling the effects of midazolam on GABA concentration responses. Data points are redrawn from fig. 4B. The *solid line* through wild-type control data (wt-ctl; *open squares*) was fitted with equation 4 (holding BZ = 0 and L_0^{wt} = 40,000; Materials and Methods). The fitted K_G = 88 ± 9.7 μM, and c = 0.0020 ± 0.00026. The *dashed lines* were calculated using equation 4 and the independently fitted GABA and midazolam global parameters, plus L_0 appropriate to the type of receptor. Wild-type plus 10 nM midazolam (wt-MDZ): L_0^{wt} = 40,000, K_G = 88 μM, c = 0.002, K_{MDZ} = 16 nM, d = 0.34. Mutant control (mut-ctl) and mutant plus 10 nM midazolam (mut-MDZ; *lines*): L_0^{mut} = 9.1, K_G = 88 μM, c = 0.002, K_{MDZ} = 16 nM, d = 0.34, BZ = 0 or 10 nM. (*D*) The fitted model accurately predicts midazolam-induced leftward shifts in wild-type GABA concentration responses. *Solid squares* represent average (± SD) EC_{50} ratios ($EC_{50}^{MDZ}/EC_{50}^{0}$) from oocytes (n ≥ 3) expressing wild-type GABA type A receptors. The *dashed line* represents EC_{50} ratios calculated from simulated GABA concentration–response data calculated using equation 4 with the parameters from the wild-type model in *C*. Simulated data were analyzed using logistic fits (equation 1, Materials and Methods) to derive EC_{50}s.

versus closed receptors at its single high-affinity site (d = 0.34), whereas diazepam shows only a twofold preference for open *versus* closed receptors (d = 0.5).

Previous estimates of the basal gating equilibrium constant L_0 for wild-type $\alpha_1\beta_2\gamma_{2L}$ receptors are in the range of 0.5–1 × 10^5.[10,18,19] Based on the predicted inverse square-root relation between L_0 and EC_{50} for mutations such as α_1L264T that selectively alter receptor gating (equation 5, Materials and Methods),[9,19,24] we calculated L_0^{wt} from the L_0^{mut} (9.1) and GABA EC_{50}s for wild-type (66 μM) and mutant (1 μM) receptors. The resulting L_0^{wt} value of 0.4 × 10^5 is close to the previous estimates and indicates that the basal open probability of unliganded wild-type receptors [$P_0 = (L_0 + 1)^{-1}$] is approximately 2.5 × 10^{-4}.

To provide rough K_G and c values, we fitted equation 4 (with BZ = 0; Materials and Methods) to wild-type estimated P_{open} values from figure 4B. The fit is shown as the solid line in figure 6C. Altogether, we derived six parameters for our model from different subsets of our data: the distinct L_0 parameters for the mutant and wild-type models and the four global parameters K_{MDZ}, d, K_G, and c.

The MWC coagonist model was then used to predict the impact of midazolam on GABA concentration responses. First, we calculated the wild-type GABA concentration response in the presence of 10 nM midazolam (fig. 6C, wild type–midazolam, dashed line). In close agreement with experimental results, the model predicts an $EC_{50}^{MDZ}/EC_{50}^{0}$ ratio of 0.7 at 10 nM midazolam and an EC_{50} ratio near 0.5 at both 100 nM and 1 μM midazolam (fig. 6D). The specific model shown in figure 6C is derived from GABA concentration–response data in a single oocyte, whereas the leftward shift induced by midazolam in the model is entirely independent of the GABA binding and efficacy parameters. In the presence of high midazolam concentrations, the wild-type model $P_{open} = (L_0 d + 1)^{-1} \approx 7.1 \times 10^{-5}$, which is nearly a tripling of the basal spontaneous P_0 but is too low to measure with macrocurrent electrophysiologic methods, which have a maximal sensitivity range (signal/noise) of approximately three orders of magnitude. Thus, the lack of evidence for direct activation in wild-type synaptic $GABA_A$ receptors is a predictable consequence of the low efficacy of benzodiazepine gating effects.

When L_0^{mut} was used in the coagonist model (fig. 6C), it correctly simulated both the low GABA EC_{50} and the significant spontaneous activity of α_1L264T$\beta_2\gamma_{2L}$ receptors (fig. 6C, mutant–control, dashed line). The mutant model with 10 nM midazolam (fig. 6C, mutant–midazolam, dashed line) also correctly predicts both enhanced opening probability at 0 GABA (*i.e.*, direct midazolam activation) and a decrease in EC_{50} to 0.7 × control. Therefore, despite the highly constrained nature of this simple MWC coagonist model, it is able to closely simu-

late our results in both the absence and the presence of GABA, for both wild-type and mutant channels, using four global parameters and a single additional basal gating parameter for each type of receptor.

The MWC model does not simulate the GABA concentration responses of mutant receptors as well as it does for wild-type receptors (fig. 6C). Specifically, the model predicts that the Hill slope of the GABA concentration responses for the mutant should be between 1.3 and 1.6, whereas our oocyte experiments consistently demonstrated much lower slopes near 0.8. Reduced Hill slopes could be due to rapid desensitization or inhibitory effects at high GABA, or negative GABA binding cooperativity. Currents elicited from rapidly superfused cell membrane patches expressing α_1L264T$\beta_2\gamma_{2L}$ $GABA_A$ receptors desensitize very slowly[19] and do not show surge currents at high GABA, making the first two possibilities unlikely. Adding a variable parameter for GABA binding cooperativity would enable our model to fit the mutant Hill slope better, but this modification would not alter our conclusions regarding the mechanism of benzodiazepine actions. Similarly, the model only addresses the positive gating effects of benzodiazepines and therefore does not explain the surge currents observed with high diazepam (fig. 1B), which indicate the presence of a low-affinity inhibitory action.

We have recently described a similar MWC coagonist mechanism for *R*(+)-etomidate, a potent intravenous anesthetic that acts selectively at $GABA_A$ receptors.[10] We found that etomidate modulation was consistent with the presence of two equivalent sites on $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, each with a dissociation constant of 36 μM and an efficacy factor of 0.0077 (a 130-fold open-state preference). As a result of its two sites and greater efficacy, etomidate enhances GABA-elicited responses much more than midazolam or diazepam, and high etomidate concentrations can directly activate wild-type $GABA_A$ receptors. Comparing the distinct coagonist sites for etomidate and classic benzodiazepines on $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, the benzodiazepine site shows high affinity and low efficacy, whereas the etomidate sites display low affinity and high efficacy.

Our results agree with previous data supporting a gating effect for classic benzodiazepines. One previous study[17] has shown that maximal efficacy of a partial $GABA_A$ receptor agonist, 5-(4-piperidyl)isoxazol-3-ol (4-PIOL), is enhanced by benzodiazepine agonists. Thompson *et al.*[25] described another constitutively active $GABA_A$ receptor mutant that is directly activated by benzodiazepine agonists *via* a site where flumazenil competes for occupancy. Benzodiazepine enhancement of extrasynaptic "tonic" $GABA_A$ receptor currents in cultured neurons has also been reported.[26,27] It has been suggested that these tonic neuronal chloride currents are stimulated by GABA spillover from synaptic release,[27] but direct gating by benzodiazepines at spontaneously active receptors provides an alternative explanation for these observations. Several reports have previously concluded that flumazenil acts as a weak partial benzodiazepine agonist.[28–30] Boileau and Czajkowski[31] previously suggested that benzodiazepines act as coagonists, based on indirect structure–function evidence. Serfozo and Cash[32] suggested that benzodiazepine agonists may promote gating of $GABA_A$ receptors bound by only one GABA molecule. The MWC coagonist mechanism implies that enhancement is independent of the number of GABA molecules bound.

Given the low impact on the gating equilibrium by benzodiazepines in comparison to general anesthetics such as etomidate or pentobarbital, it is not surprising that studies comparing their actions on $GABA_A$ receptors have concluded that different coupling mechanisms were at work. That general anesthetics stabilize $GABA_A$ receptor open states is supported by evidence that these drugs enhance the efficacy of partial orthosteric agonists,[10–12] lengthen the average single-channel open time of receptors,[33,34] and significantly slow deactivation of patch macrocurrents.[35,36] Single-channel studies of benzodiazepines on neuronal $GABA_A$ receptors did not detect any increase in average channel lifetime.[13,37] Heterogeneous $GABA_A$ receptor species in neurons makes interpreting these results difficult. Moreover, with GABA present at low concentrations that likely favor occupation of one GABA site (*i.e.*, the RG state in fig. 6A), benzodiazepines may primarily promote opening directly from this state (*via* the OGBz state), which might be characterized by brief openings. This idea is supported by other experiments[32,38] and could explain why single $GABA_A$ receptor channels seem to open more frequently in the presence of benzodiazepine agonists but without increased mean open times.

Kinetic studies using rapid patch superfusion have given conflicting results on whether classic benzodiazepine agonists prolong deactivation. Krampfl *et al.*[15] studied $\alpha_1\beta_2\gamma_{2L}$ receptors in excised patches from HEK293 cells and observed slowed deactivation in the presence of diazepam. A similar study by Lavoie and Twyman[16] using $\alpha_2\beta_1\gamma_2$ receptors reported no slowing of deactivation by diazepam. In another study of $\alpha_2\beta_1\gamma_2$ receptors expressed in HEK293 cells, O'Shea *et al.*[11] reported that midazolam produced a leftward shift of P4S responses without increasing maximal response, in contrast with our results and others[17] using $\alpha_1\beta_2\gamma_{2L}$. These discordant observations suggest that benzodiazepines may couple to both GABA binding and gating steps and that allosteric coupling to the benzodiazepine site may differ in $\alpha_1\beta_2\gamma_2$ *versus* $\alpha_2\beta_1\gamma_2$ receptors. The data for $\alpha_1\beta_2\gamma_2$ receptors consistently favors a gating mechanism, and the ability of our MWC coagonist model to accurately predict benzodiazepine effects under a variety of experimental conditions suggests that binding allosteric interactions between the GABA and benzodi-

azepine sites are minimal. Further studies are needed to determine whether the mechanism of benzodiazepine modulation is altered for other $GABA_A$ receptor subunit mixtures.

In summary, our results demonstrate that high-affinity classic benzodiazepines modulate $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors *via* allosteric coupling to channel gating. An MWC allosteric coagonist mechanism quantitatively accounts for the modulation of GABA-activated currents in both wild-type receptors and spontaneously active mutant receptors. The same mechanism also accounts for the direct activation of spontaneously active $GABA_A$ receptors by benzodiazepine agonists and the lack of similar observations in wild-type synaptic receptors. Classic benzodiazepines are high-affinity but low-efficacy $GABA_A$ receptor coagonists, in contrast to many general anesthetic compounds that act on the same receptors with low affinity but high efficacy.

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