The Benzodiazepine Diazepam Potentiates Responses of α1β2γ2L γ-Aminobutyric Acid Type A Receptors Activated by either γ-Aminobutyric Acid or Allosteric Agonists

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ABSTRACT

Background: The γ-aminobutyric acid (GABA) type A receptor is a target for several anesthetics, anticonvulsants, anxiolytics, and sedatives. Neurosteroids, barbiturates, and etomidate both potentiate responses to GABA and allosterically activate the receptor. We examined the ability of a benzodiazepine, diazepam, to potentiate responses to allosteric agonists.

Methods: The GABA type A receptors were expressed in human embryonic kidney 293 cells and studied using whole-cell and single-channel patch clamp. The receptors were activated by the orthosteric agonist GABA and allosteric agonists pentobarbital, etomidate, and alfaxalone.

Results: Diazepam is equally potent at enhancing responses to orthosteric and allosteric agonists. Diazepam EC_{50}s were 25 ± 4, 26 ± 6, 33 ± 6, and 26 ± 3 nM for receptors activated by GABA, pentobarbital, etomidate, and alfaxalone, respectively (mean ± SD, 5–6 cells at each condition). Mutations to the benzodiazepine-binding site (α1(H101C), γ2(R144C), γ2(R197C)) reduced or removed potentiation for all agonists, and an inverse agonist at the benzodiazepine site reduced responses to all agonists. Single-channel data elicited by GABA demonstrate that in the presence of 1 μM diazepam the prevalence of the longest open-time component is increased from 13 ± 7 (mean ± SD, n = 5 patches) to 27 ± 8% (n = 3 patches) and the rate of channel closing is decreased from 129 ± 28 s^{-1} to 47 ± 6 s^{-1} (mean ± SD)

Conclusions: We conclude that benzodiazepines do not act by enhancing affinity of the orthosteric site for GABA but rather by increasing channel gating efficacy. The results also demonstrate the presence of interactions between allosteric activators and potentiators, raising a possibility of effects on dosage requirements or changes in side effects.

What We Already Know about This Topic

- Whether benzodiazepines potentiate responses to allosteric agonists of the γ-aminobutyric acid type A receptor, an important target for anesthetic drugs, is unclear

What This Article Tells Us That Is New

- By using *in vitro* methods, the benzodiazepines did not act by enhancing affinity of the orthosteric site for γ-aminobutyric acid but rather by increasing channel gating efficacy; the results suggest that interactions between allosteric activators and potentiators may influence dosage requirements or secondary drug effects

THE γ-aminobutyric acid type A ($GABA_A$) receptor is a pentameric receptor whose activation underlies most rapid postsynaptic inhibition in the brain. The $GABA_A$ receptor is the major target for many anesthetics, anticonvulsants, anxiolytics, and sedatives that act to potentiate the function of the receptor and enhance the inhibitory tone in the brain.[1,2] In addition to potentiating responses to GABA, many of these drugs can also directly activate the receptor, although they do not interact with the GABA-binding (orthosteric) site on the receptor. Because several of these allosteric agonists are in clinical use, the possibility of additive or synergistic interactions among allosteric activators and potentiators is of interest both from a mechanistic perspective and from a more practical and clinical perspective.

Benzodiazepine agonists such as diazepam interact with a site in the extracellular domain of the receptor, at the subunit interface between the α and γ subunits.[3] Binding of a

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benzodiazepine agonist does not directly activate the $GABA_A$ receptor but potentiates the response to submaximal concentrations of GABA. In macroscopic whole-cell recordings, potentiation manifests as increased peak current.[4] In single-channel recordings, the frequency of channel opening events is increased in the presence of diazepam.[5] There is conflicting evidence about the mechanism of potentiation: some evidence supports the idea that it results from an increased affinity of the orthosteric site for GABA, so potentiation would only occur for agonists activating through the orthosteric site.[6,7] In contrast, there is also evidence that potentiation reflects an increase in channel opening efficacy, that would imply that responses to any agonist would be potentiated.[8–10]

We have examined the ability of a benzodiazepine to potentiate responses to three allosteric agonists: alfaxalone, pentobarbital, and etomidate. The three allosteric agonists are representatives of separate classes with distinct binding sites on the $GABA_A$ receptor. Alfaxalone is a neuroactive steroid that interacts with transmembrane regions of the α subunit.[11] It was in clinical use outside the United States for several years and is currently used in veterinary medicine under the name Alfaxan. Etomidate is in current use as an anesthetic. It interacts with transmembrane regions in the α and β subunits.[12] Pentobarbital is a representative barbiturate that has been used as an anticonvulsant, sedative, or to induce long-term comatose states. It is not known where barbiturates bind in the $GABA_A$ receptor, although mutations removing neurosteroid or etomidate actions do not remove pentobarbital potentiation.[13,14] Each of these drugs directly activates $GABA_A$ receptors, although alfaxalone and etomidate are less efficacious than pentobarbital or the transmitter GABA.

Our data demonstrate that diazepam potentiates currents elicited by all three allosteric agonists and the orthosteric agonist GABA. The concentration–response properties for diazepam are similar for all agonists tested, and the effects are sensitive to mutations shown to disrupt modulation of receptors activated by GABA. We infer that benzodiazepines modulate the $GABA_A$ receptor through changes in channel gating efficacy. Furthermore, the data demonstrate that potentiation can occur between an allosteric agonist and an allosteric potentiator. This raises the possibility that significant drug interactions may occur, with either desirable reductions in dosage requirements or undesirable enhancement of significant side effects.

Materials and Methods

Molecular Biology and Receptor Expression

The experiments were conducted on wild-type and mutant rat α1β2γ2L $GABA_A$ receptors. The α1(H101C), γ2(R144C), and γ2(R197C) mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene, San Diego, CA). To ascertain that only the desired mutation had been produced, the coding regions were fully sequenced. The complementary DNAs for the receptor subunits were subcloned into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA) in the T7 orientation.

For expression in human embryonic kidney 293 cells, we used a calcium phosphate precipitation–based transient transfection technique.[15] A total of 3 μg of complementary DNA in the ratio of 1:1:1 (α: β: γ) was mixed with 12.5 μl of 2.5 M $CaCl_2$ and dH_2O to a final volume of 125 μl. The solution was added slowly, without mixing, to an equal volume of 2× BES (*N,N-bis*(2-hydroxyethyl)-2-aminoethanesulfonic acid) buffered solution. The combined mixture was incubated at room temperature for 10 min followed by mixing the contents and an additional 15-min incubation. The precipitate was added to the cells in a 35-mm dish for overnight incubation at 37°C, followed by replacement of medium in the dish. The experiments were conducted in the course of the next 2 days after changing the medium.

Electrophysiological Recordings

Human embryonic kidney cells expressing $GABA_A$ receptors were identified using a bead-binding technique. The amino terminus of the α1 subunit was tagged with the FLAG epitope.[16] Surface expression of the FLAG epitope was determined using a mouse monoclonal antibody to the FLAG epitope (M2, Sigma-Aldrich, St. Louis, MO), which had been adsorbed to immuno-beads with a covalently attached goat anti-mouse IgG antibody (Dynal, Great Neck, NY).

The experiments were conducted using standard whole-cell voltage clamp and single-channel patch clamp techniques. The bath solution contained (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 D-glucose, and 10 HEPES; pH 7.4. In whole-cell recordings, the pipette solution contained (in mM): 140 CsCl, 4 NaCl, 4 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, and 10 HEPES; pH 7.4. In single-channel recordings, the pipette solution contained (in mM): 120 NaCl, 5 KCl, 10 $MgCl_2$, 0.1 $CaCl_2$, 20 tetraethylammonium, 5 4-aminopyridine, 10 D-glucose, and 10 HEPES; pH 7.4.

The agonist and modulator were applied through the bath using an SF-77B fast perfusion stepper system (Warner Instruments, Hamden, CT) in whole-cell experiments or added to the pipette solution in single-channel recordings. The recording and analysis of whole-cell currents were performed as described previously.[17] In most experiments, the cells were clamped at −60 mV. The currents were recorded using an Axopatch 200B amplifier (Molecular Devices, Union City, CA), low-pass filtered at 2 kHz and digitized with a Digidata 1320 series interface (Molecular Devices) at 10 kHz. The analysis of whole-cell currents was performed using the pClamp 9.0 software package (Molecular Devices).

The basic experiment consisted of applying a given concentration of agonist in the absence of diazepam, then again in the presence of diazepam. The effect of the modulator was evaluated from the ratio of the response plus diazepam to that minus diazepam (the response ratio). Analogous experiments

were conducted with methyl 6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate (DMCM). Concentration–response curves for diazepam were generated by applying a series of diazepam concentrations in the presence of a constant concentration of agonist (see Results for concentrations used). The data at a given diazepam concentration were averaged across all cells for a given receptor–agonist combination, and the averaged data were fit.

The recording and analysis of single-channel currents have been described in detail previously.[18,19] The pipette potential was held at +60 to +80 mV, which corresponds to an −120 to −100 mV potential difference across the patch membrane. Channel activity was recorded using an Axopatch 200B amplifier, low-pass filtered at 10 kHz, and acquired with a Digidata 1320 series interface at 50 kHz using pClamp software. The experiments were conducted in the cell-attached configuration.

The analysis of single-channel currents was performed using the QuB Suite (University at Buffalo, Buffalo, New York) and was limited to single-channel clusters, that is, episodes of activity arising from the activation of a single ion channel[20] or fragments of clusters. Clusters were selected by eye or by using a critical closed-time duration of 200–500 ms. Typically, a record from a patch contained 15–45 clusters. To determine the durations of intracluster open and closed intervals, the currents were digitally low-pass filtered at 1.5–2.5 kHz and idealized using the segmented-*k*-means algorithm (QuB Suite). The fitting program automatically applies a "missed event correction" to compensate for the effect of low-pass filtering. Data from each patch were fitted to a kinetic scheme incorporating three open states connected to a single, common closed state (to determine open-time parameters), or a scheme incorporating three closed state connected to a single open state (to determine closed-time parameters). The program determines the best-fitting values for transition rates into and out of the kinetic states. The mean duration of dwells in a state was calculated from the inverse of the rate for leaving the state. The fraction of events in a given state was calculated from the ratio of the rate for entering that state to the sum of the rates for entering states of equal conductance (*i.e.*, the fraction of openings in the OT3 state = [rate for entering OT3]/[sum of rates for entering OT1 + OT2 + OT3]).

Statistical Analysis

The effect of diazepam on macroscopic peak currents was evaluated from the ratio of the response plus diazepam to that minus diazepam (the response ratio). Statistical analysis was performed using the STATA (StataCorp, College Station, TX) software package or Excel (Microsoft, Redmond, WA). Two tests were performed. The first was to compare the response ratio to 1 (no effect) using a two-tailed paired *t* test (Excel). This test is equivalent to a one-sample *t* test to a hypothetical value of 1. This test is designed to determine whether a drug has a significant effect. The second was a one-way ANOVA for a given agonist applied to different mutations, with a Bonferroni *post hoc* correction (STATA).

The concentration–response curves were fitted to the equation: $Y([\text{diazepam}]) = 1 + (Y_{max} - 1)[\text{diazepam}]^n/([\text{diazepam}]^n + EC_{50}{}^n)$, where Y_{max} is the maximal potentiating effect in the presence of diazepam, EC_{50} is the concentration producing the half-maximal effect, and n is the Hill coefficient. The fitting was conducted using the program NFIT (The University of Texas Medical Branch at Galveston). The fitting program returns uncertainty estimates on the best-fitting parameter values. To equalize the apparent maximal effect and enable focus on the midpoints of the curves, the data are plotted in a normalized form. Normalization was conducted through the following equation: Normalized potentiation = ([peak current in the presence of diazepam/peak current in the absence of diazepam] − 1)/([maximal peak current in the presence of diazepam/peak current in the absence of diazepam] − 1). Statistical analysis of single-channel parameters was performed using a two-tailed *t* test (Excel). All data for the reported conditions are included.

Drugs and Solutions

The drugs used in the study were obtained from Sigma-Aldrich or Tocris (Ellisville, MO). The stock solutions of diazepam and DMCM (both at 10 mM) and GABA (at 500 mM) were made in bath solution and stored at −20°C. The stock solution of pentobarbital was made at 1 mM in bath solution and stored at +4°C. The stock solution of etomidate was made at 20 mM in dimethyl sulfoxide and stored at +4°C. The stock solution of alfaxalone was made at 10 mM in dimethyl sulfoxide and stored at room temperature. The stock solutions were further diluted on the day of experiment.

Results

Diazepam Potentiates $GABA_A$ Receptors Activated by Orthosteric and Allosteric Agonists

We examined the ability of the benzodiazepine diazepam to modulate currents from the $\alpha1\beta2\gamma2L$ $GABA_A$ receptor. The receptors were activated by the transmitter GABA, or the allosteric agonists pentobarbital, etomidate, or alfaxalone. Because the degree of potentiation can depend on the degree of activation, that is, relative potentiation is reduced when higher concentrations of agonist are used, we selected the concentrations of agonists so that the control responses corresponded to less than 5% of the maximal response in the presence of the same agonist, that is, GABA, pentobarbital, or etomidate. The exception was alfaxalone, which is a low-efficacy agonist and which was used at a saturating concentration (10 μM). In all experiments, only a single type of agonist was used to prevent contamination of responses caused by incomplete washout.

We found that diazepam potentiated receptors activated by the orthosteric agonist GABA and receptors activated by

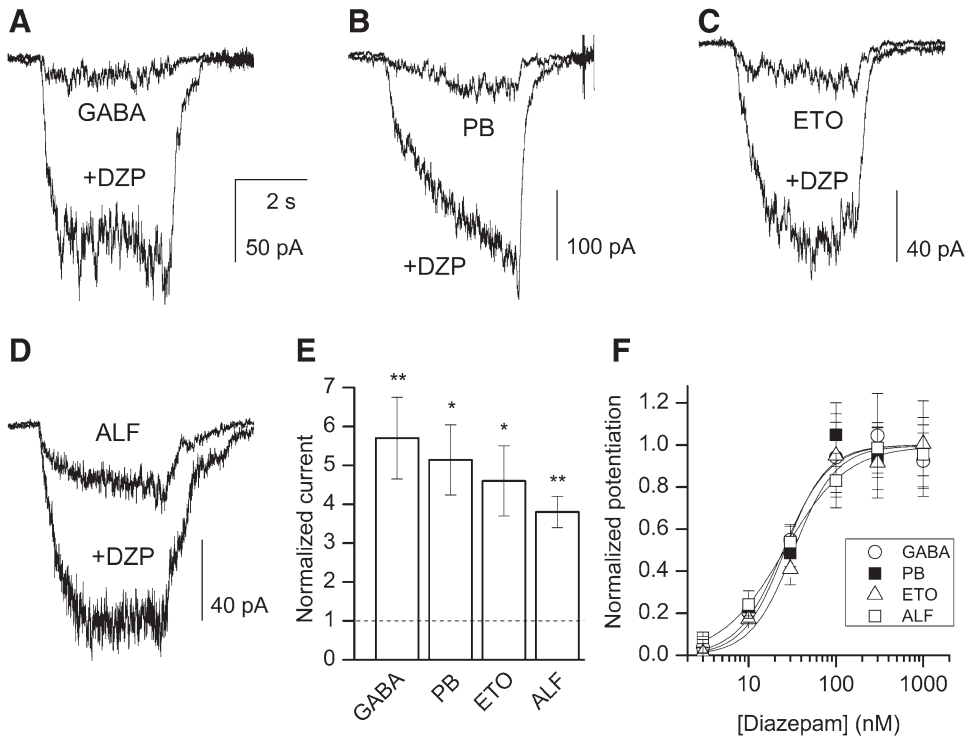


Fig. 1. Diazepam (DZP) potentiates receptors activated by orthosteric and allosteric agonists. (*A–D*) Sample traces from human embryonic kidney cells expressing rat α1β2γ2L γ-aminobutyric acid type A ($GABA_A$) receptors. The receptors were activated by 0.5 μM GABA (*A*), 60 μM pentobarbital (PB; *B*), 1 μM etomidate (ETO; *C*), or 10 μM alfaxalone (ALF; *D*) in the presence and absence of 1 μM DZP. (*E*) Summary of the data. The graph shows the potentiating effect of 1 μM DZP (mean ± SE. from 5 to 6 cells for each agonist). The symbols summarize results of a paired *t* test with comparison to no effect. **P* < 0.05; ***P* < 0.01. ANOVA analysis indicated that the response ratio did not differ among treatments (*P* > 0.05 for all comparisons, Bonferroni *post hoc* correction). (*F*) Concentration–response curves for DZP. The receptors were activated by 0.5 μM GABA, 60 μM PB, 1 μM ETO, or 10 μM alfaxalone in the presence and absence of 3-1000 nM DZP. The data points give mean ± S.E. from five to six cells for each agonist. The mean data were fitted with the following equation: $Y([DZP]) = 1 + (Y_{max} - 1)\,[DZP]^n/([DZP]^n + EC_{50}{}^n)$, where Y_{max} equals to the maximal potentiating effect in the presence of DZP, EC_{50} is the concentration producing the half-maximal effect, and n is the Hill coefficient. Curve fitting was performed using the program NFIT (The University of Texas Medical Branch at Galveston). The data are plotted in a normalized form to equalize the apparent maximal effect and focus on differences in the midpoints of the curves. Normalization was conducted through the following equation: Normalized potentiation = ([peak current in the presence of DZP/peak current in the absence of DZP] – 1)/([maximal peak current in the presence of DZP/peak current in the absence of DZP] – 1). The fitted values for Y_{max} in these experiments were 6.1 ± 0.2, 5.3 ± 0.3, 4.6 ± 0.2, and 3.9 ± 0.1 (best fitting values ± calculated uncertainty) for receptors activated by GABA, PB, ETO, and alfaxalone, respectively. The EC_{50} estimates were 25 ± 4, 26 ± 6, 33 ± 6, and 26 ± 3 nM for receptors activated by GABA, PB, ETO, and ALF, respectively. Overall, the data indicate that DZP similarly modulates receptors activated by orthosteric and allosteric agonists.

the allosteric agonists (fig. 1, A–D). The peak responses in the presence of 1 μM diazepam were 5.7 ± 1.1 times of control (mean ± SE, six cells) for receptors activated by 0.5 μM GABA, 5.1 ± 0.9 times of control (five cells) for 60 μM pentobarbital, 4.6 ± 0.9 times of control for 1 μM etomidate (five cells), and 3.8 ± 0.4 times of control for 10 μM alfaxalone (five cells) (fig. 1E).

To determine whether the concentration–response properties for diazepam are similar in receptors activated by orthosteric and allosteric agonists, we measured potentiation by 3–1000 nM diazepam. The concentration–response curves indicate that the diazepam concentration–response relationship does not depend on the nature of the agonist (fig. 1F). Diazepam EC_{50}s were 25 ± 4 nM for GABA, 26 ± 6 nM for pentobarbital, 33 ± 6 nM for etomidate, and 26 ± 3 nM for receptors activated by alfaxalone. The data indicate that diazepam virtually identically potentiates α1β2γ2L $GABA_A$ receptor activated by orthosteric and allosteric agonists.

The Inverse Benzodiazepine Agonist DMCM Similarly Modulates $GABA_A$ Receptors Activated by Orthosteric and Allosteric Agonists

We next examined the pharmacology of the benzodiazepine site. DMCM acts as a convulsant in rodents and has been shown to inhibit $GABA_A$ receptors in many preparations. The effect is mediated by the classic benzodiazepine site. We probed whether DMCM similarly acts on receptors activated by orthosteric and allosteric agonists.

We found that DMCM inhibited α1β2γ2L receptors activated by GABA or the allosteric agonists. The peak

responses in the presence of 1 μM DMCM were 0.43 ± 0.06 times control (four cells) when the receptors were activated by GABA, and 0.61 ± 0.05 times (five cells), 0.41 ± 0.06 times (five cells), or 0.60 ± 0.08 times (four cells) in the presence of pentobarbital, etomidate, or alfaxalone, respectively. Sample traces are shown in figure 2A, and the summary of the data is given in figure 2B.

We infer that the pharmacological properties of the benzodiazepine site are similar when the receptor is activated by the transmitter GABA or the allosteric agonists pentobarbital, etomidate, or alfaxalone.

Mutations to the Classic Benzodiazepine-binding Site Affect Modulation of Receptors Activated by Orthosteric and Allosteric Agonists

To gain more insight into the nature of the interaction site mediating the effect of diazepam on receptors activated by allosteric agonists, we introduced mutations to the α1 and γ2 subunits, previously shown to reduce or eliminate potentiation by benzodiazepines.

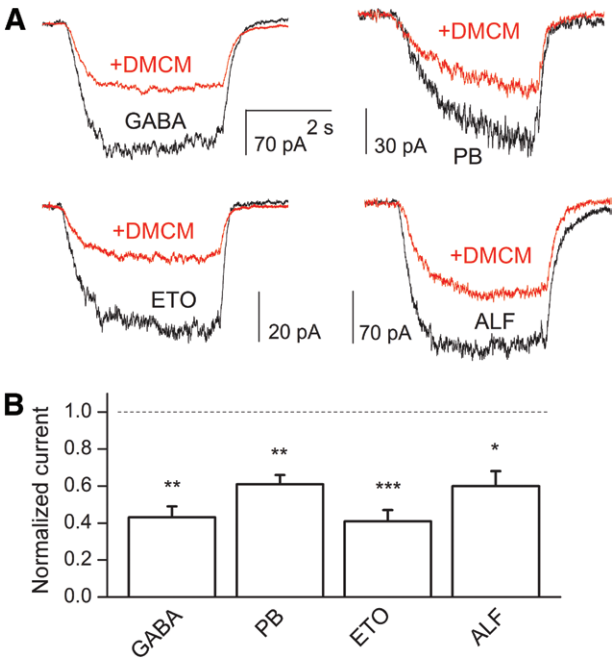


Fig. 2. Receptors activated by orthosteric and allosteric ligands are similarly modulated by the inverse benzodiazepine agonist, methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM). (*A*) Sample traces from human embryonic kidney cells expressing rat α1β2γ2L γ-aminobutyric acid type A ($GABA_A$) receptors. The receptors were activated by 1 μM GABA, 100 μM pentobarbital (PB), 1 μM etomidate (ETO), or 10 μM alfaxalone (ALF) in the presence and absence of 1 μM DMCM. (*B*) Summary of the data on the inhibitory effects of DMCM. The graph shows mean ± S.E. from four to five cells in each condition. The symbols summarize results of a paired *t* test with comparison to no effect. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. ANOVA analysis indicated that the response ratio did not differ among treatments (*P* > 0.05 for all comparisons, Bonferroni *post hoc* correction). Overall, the data indicate that DMCM similarly affects receptors activated by orthosteric and allosteric agonists.

We used the following mutations: γ2(R197C), γ2(R144C), and α1(H101C). The γ2(R197C) and γ2(R144C) residues are located in Loops E and F, respectively, and have been shown to strongly reduce maximal potentiation by several benzodiazepine modulators without affecting their affinity.[21,22] The α1(H101C) residue is located in Loop A of the α1 subunit and has been shown to disrupt modulation by benzodiazepines,[23] likely through effect on receptor affinity to diazepam.[3]

We tested whether these mutations affect diazepam modulation of receptors activated by allosteric agonists. Receptors containing the mutated subunits were activated by GABA, or pentobarbital, etomidate, or alfaxalone, and exposed to 1 μM diazepam. The agonist concentrations at which diazepam effects were examined were selected as described earlier.

The findings demonstrate that the mutations strongly reduced or eliminated potentiation by diazepam (fig. 3). When the receptors contained the γ2(R197C) mutation, diazepam-induced potentiation was significantly reduced or eliminated. No modulation was observed when the receptors were activated by 0.2 μM GABA (1.1 ± 0.1 times control, six cells; *P* > 0.5 that the response ratio differs from 1) or 50 μM pentobarbital (0.9 0.05, five cells; *P* > 0.05). Receptors activated by 10 μM alfaxalone or 1 μM etomidate were weakly potentiated (1.1 ± 0.02, *P* < 0.05 and 1.2 ± 0.03, *P* < 0.01, respectively).

For receptors containing the γ2(R144C) mutation, the mean current in the presence of diazepam was 0.95 ± 0.03 times control (five cells; *P* > 0.2) when the receptors were activated by 0.2 μM GABA, and 1.0 ± 0.09 (five cells, *P* > 0.8), 0.89 ± 0.06 (five cells, *P* > 0.1), and 1.03 ± 0.05 (five cells, *P* > 0.6) of control for 50 μM pentobarbital, 1 μM etomidate, and 10 μM alfaxalone, respectively.

Potentiation of α1(H101C)β2γ2 receptors was similarly reduced. The mean current in the presence of diazepam was 1.21 ± 0.05 times control (five cells, *P* < 0.05) when the receptors were activated by 1 μM GABA. When diazepam was coapplied with allosteric agonists, the peak responses were 1.09 ± 0.04 (five cells, *P* > 0.09), 0.91 ± 0.06 (six cells, *P* > 0.2), and 0.98 ± 0.03 (six cells, *P* > 0.5) of control for 100 μM pentobarbital, 6 μM etomidate, and 10 μM alfaxalone, respectively.

For each of the agonists studied, potentiation for the mutated receptors was significantly less than for wild type ($P < 4 \times 10^{-4}$ for comparison to wild type, one-way ANOVA with Dunnett correction).

Overall, we confirm that the mutations diminish potentiation of receptors activated by GABA. Furthermore, the data demonstrate that the mutations similarly act on receptors activated by allosteric agonists. We infer from the data that the same coupling mechanism underlies potentiation in the presence of GABA and allosteric agonists.

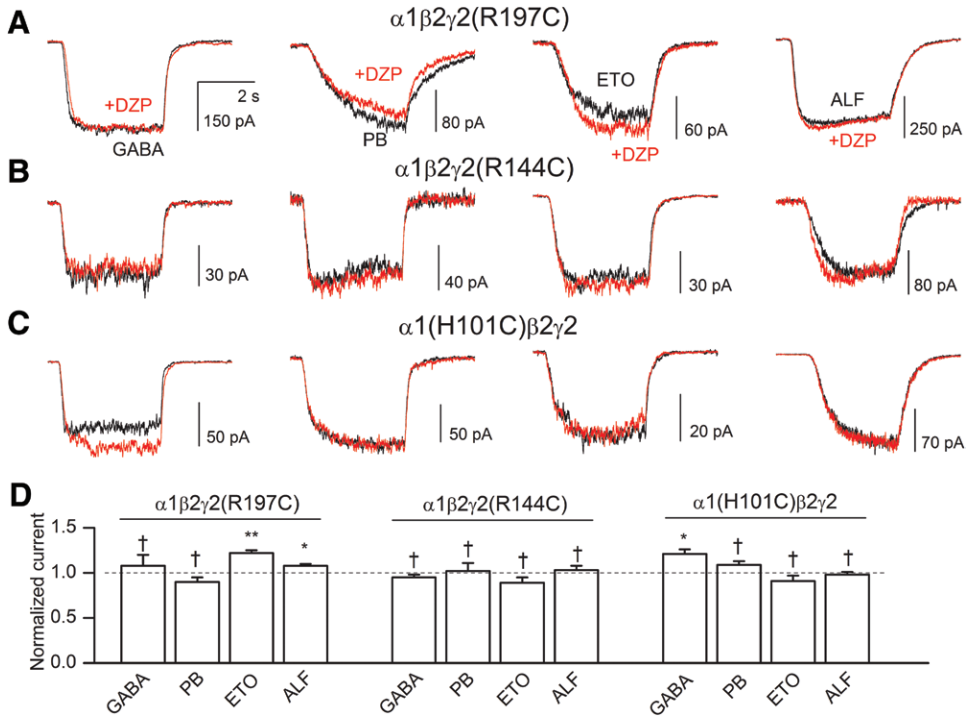


Fig. 3. Selected mutations similarly affect diazepam (DZP) potentiation of receptors activated by orthosteric and allosteric agonists. (*A*) Sample current traces from human embryonic kidney cells expressing α1β2γ2(R197C) receptors. The receptors were activated by 0.2 μM γ-aminobutyric acid (GABA), 50 μM pentobarbital (PB), 1 μM etomidate (ETO), or 10 μM alfaxalone (ALF) in the presence and absence of 1 μM DZP. (*B*) Sample current traces from cells expressing α1β2γ2(R144C) receptors. The receptors were activated by 0.2 μM GABA, 50 μM PB, 1 μM ETO, or 10 μM ALF in the presence and absence of 1 μM DZP. (*C*) Sample current traces from cells expressing α1(H101C)β2γ2 receptors. The receptors were activated by 1 μM GABA, 100 μM PB, 6 μM ETO, or 10 μM ALF in the presence and absence of 1 μM DZP. (*D*) Summary of the data. The graph shows the effect of 1 μM DZP (mean ± S.E. from four to six cells for each agonist). The symbols summarize results of a paired *t* test with comparison to no effect. $^*P < 0.05$; $^{**}P < 0.01$; †$P > 0.05$. ANOVA analysis indicated that the response ratio did not differ among treatments ($P > 0.05$ for all comparisons, Bonferroni *post hoc* correction).

Effect of Diazepam on Single-channel Currents Elicited by GABA

Intermediate-to-high (20 μM and above) concentrations of GABA elicit clear single-channel clusters in cells expressing α1β2γ2L receptors.[24] The advantage of studying channel activity in clusters is the certainty that the currents originate from a single ion channel protein.[20] Thus, even in the absence of a commonly accepted activation model, mechanistic insight can be gained by examining the effect of a drug on the intracluster open and closed times.

We recorded single-channel currents from human embryonic kidney cells expressing α1β2γ2L receptors activated by 50 μM GABA in the presence and absence of 1 μM diazepam. This concentration of GABA was chosen because it is close to the half-maximally effective concentration for single-channel activation.[24] Accordingly, it produces clearly apparent clusters of activity and either increases or decreases in activation can be assessed. Sample recordings are shown in figure 4, and the open- and closed-time parameters are summarized in tables 1–2. The intracluster open-time components were best-fitted by a reaction scheme with three kinetically distinct open states. Diazepam did not affect the properties of the two briefer open-time components OT1 and OT2. However, the prevalence of the longest open-time component, OT3, was enhanced in the presence of diazepam (table 1). The intracluster closed times were fitted with three kinetically distinct closed states. The application of diazepam had no effect on the mean durations of closed times but significantly reduced the rate of entry into the longest-lived closed-time component, CT3 (table 2).

The kinetic change that underlies the potentiating effects observed in macroscopic, whole-cell recordings is the reduction in the rate of entry into the longest-lived closed-time component. The increase in %OT3 has a smaller overall effect on macroscopic currents.[25] In our previous work, we have associated the CT3 component with the activation pathway, that is, dwells in the mono- and unliganded states.[24] In this model, the finding that diazepam does not affect the mean duration of CT3 indicates that the affinity of the closed receptor to GABA is unchanged. The reduction in the rate of entry to CT3 is indicative of an effect by diazepam on channel closing.

Discussion

We have presented data showing that the prototypic benzodiazepine agonist diazepam is capable of potentiating α1β2γ2 $GABA_A$ receptors activated by the allosteric

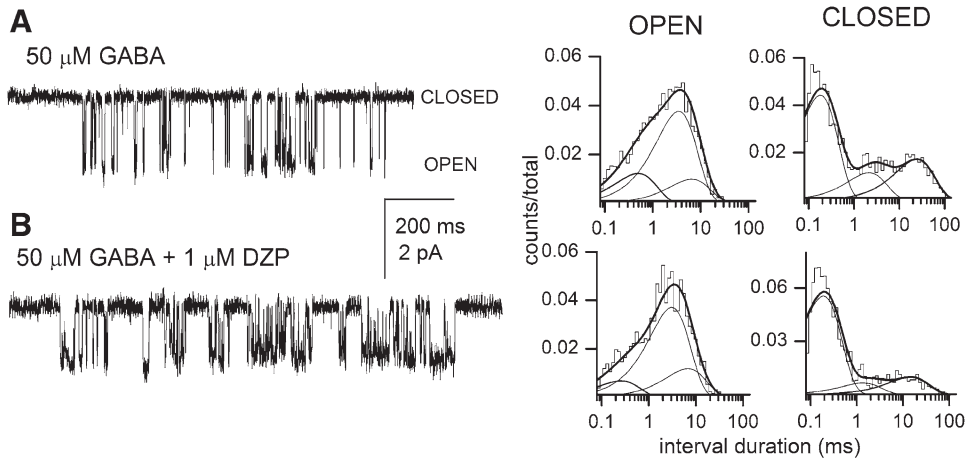


Fig. 4. Diazepam (DZP) modulates single-channel currents elicited by γ-aminobutyric acid (GABA). (*A*) A sample single-channel cluster from a human embryonic kidney cell expressing α1β2γ2L $GABA_A$ receptors and exposed to 50 μM GABA in the cell-attached configuration. Channel openings are shown as downward deflections. The *open* and *closed-time histograms* for all data from this patch are shown next to the current trace. Both *open-* and *closed-time histograms* were fitted to a sum of three exponentials. The open times were 0.46 ms (21%), 3.2 ms (63%), and 6.0 ms (16%). The closed times were 0.16 ms (61%), 2.0 ms (16%), and 22 ms (23%). (*B*) A sample single-channel cluster in the presence of 50 μM GABA + 1 μM DZP, and the corresponding duration histograms. The open times were 0.24 ms (12%), 2.8 ms (68%), and 6.3 ms (20%). The closed times were 0.17 ms (78%), 1.3 ms (9%), and 14 ms (13%). The summary of the single-channel data is presented in table 1.

agonists pentobarbital, etomidate, and alfaxalone. The concentration–response relationships for diazepam are similar in the presence of the allosteric agonists and the transmitter GABA. The benzodiazepine inverse agonist DMCM inhibits currents elicited by orthosteric and allosteric agonists. Furthermore, mutations previously shown to disrupt benzodiazepine potentiation of receptors activated by GABA also disrupt potentiation of receptors activated by the allosteric agonists. We conclude that benzodiazepines have a significant action to increase the efficacy of gating for all agonists, rather than to increase the affinity at a particular site. However, we cannot rule out the possibility that there are additional, less significant effects on the orthosteric agonist site.

Our data show that the EC_{50}s for diazepam are 26–33 nM for receptors activated by pentobarbital, etomidate, or alfaxalone. This is similar to the diazepam EC_{50} estimates for receptors activated by GABA, obtained by us (25 nM) and others (32–59 nM[4]) and the EC_{50}s for potentiation of spontaneous currents (40–72 nM[8,9]).

The effects of mutations (α1(H101C), γ2(R144C), γ2(R197C)) known to diminish potentiation of GABA-activated receptors by benzodiazepines also reduced potentiation of receptors activated by allosteric agonists. The α1H101 residue is an integral component of the benzodiazepine-binding pocket and likely comes into contact with C7-Cl of the diazepam molecule.[3] Mutation of the histidine residue to cysteine significantly reduces affinity of the receptor to diazepam.[3] The γ2(R144C) and γ2(R197C) mutations have been shown to disrupt signal transduction, without affecting the affinity of the site to benzodiazepines.[21,22,26] The similarity in the effects of mutations was indeed striking (fig. 3), providing further proof that a common interaction site mediates the effects of diazepam and indicative that the same signal transduction mechanism is used with the orthosteric and allosteric agonists.

A previous study found that diazepam increases the opening frequency of single-channel currents in native $GABA_A$ receptors in cultured mouse spinal neurons activated by low concentrations (2 μM) of GABA.[5] Although the significance

Table 1. The Summary of Single-channel Kinetic Analysis of the Open-time Distributions from the α1β2γ2L Receptor under Control Conditions and in the Presence of Diazepam

Agonist, Modulator	OT1, ms	Fraction OT1	OT2, ms	Fraction OT2	OT3, ms	Fraction OT3	n
50 μM GABA	0.30 ± 0.06	0.20 ± 0.04	3.0 ± 0.6	0.67 ± 0.06	6.8 ± 2.9	0.13 ± 0.07	5
+ 1 μM DZP	0.29 ± 0.04†	0.19 ± 0.07†	2.7 ± 0.4†	0.54 ± 0.12†	5.5 ± 0.8†	0.27 ± 0.08*	3

The intracluster open-time histograms were fitted to a sum of three exponentials. The table gives the mean durations ± SD (OT1-3) and average relative contributions ± SD (fraction OT1-3) for the three open-time components, and the number of patches under each condition (n). Statistical analysis was performed using two-tailed *t* test (Excel, Microsoft, Redmond, WA). The significance levels apply to comparison to control condition (50 μM GABA). The data for 50 μM GABA are from Li *et al*.[32]

* $P < 0.05$; † $P > 0.05$.

DZP = diazepam; GABA = γ-aminobutyric acid; OT1 = briefest open-time component; OT2 = intermediate open-time component; OT3 = longest open-time component.

Table 2. The Summary of Single-channel Kinetic Analysis of the Closed-time Distributions from the α1β2γ2L Receptor under Control Conditions and in the Presence of Diazepam

Agonist, Modulator	CT1, ms	k_1, s^{-1}	CT2, ms	k_2, s^{-1}	CT3, ms	k_3, s^{-1}	n
50 μM GABA	0.15 ± 0.01	276 ± 100	1.4 ± 0.3	57 ± 16	13.6 ± 4.0	129 ± 28	5
+ 1 μM DZP	0.17 ± 0.02†	313 ± 59†	1.1 ± 0.3†	61 ± 25†	10.8 ± 2.7†	47 ± 6**	3

The intracluster closed-time histograms were fitted to a sum of three exponentials. The table gives the mean durations ± SD (CT1-3), and the rates of entry into the state (k_{1-3}) for the three closed-time components, and the number of patches under each condition (n). Statistical analysis was performed using two-tailed *t* test (Excel, Microsoft, Redmond, WA). The significance levels apply to comparison to control condition (50 μM GABA). The data for 50 μM GABA are from Li *et al*.[32]

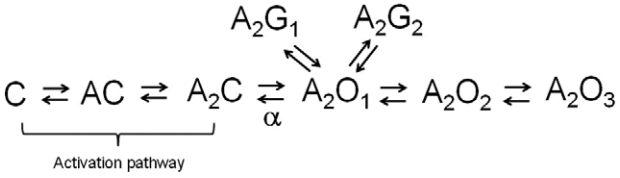
** $P < 0.01$; † $P > 0.05$.

CT1 = briefest closed-time component; CT2 = intermediate closed-time component; CT3 = longest closed-time component; DZP = diazepam; GABA = γ-aminobutyric acid.

of changes in opening frequency is unclear because of multiple receptors in the patches and possible contributions from desensitization, it was proposed that diazepam enhances receptor affinity to GABA *via* changes in the agonist association rate constant.

We used a higher concentration of GABA (50 μM) to study the kinetic effects of diazepam. At 50 μM GABA (and above), channel openings are condensed into high-frequency episodes of activity, that is, single-channel clusters.[20] Neighboring clusters are separated by dwells in long-lived desensitized states, so the effects on desensitization can be discarded. The open and closed events within a cluster represent transitions between various di-, mono-, and unliganded states, so the properties of intracluster events can be related to receptor affinity and channel gating, even in the absence of a commonly accepted activation scheme for the $GABA_A$ receptor.

Our data demonstrate that 1 μM diazepam induces a significant reduction in the rate of entry into the longest intracluster closed-time component (CT3) without affecting its mean duration. We previously associated CT3 with dwells in the activation pathway.[24] In a simple activation scheme[19]:



where C, AC, and A_2C states represent un-, mono, and diliganded closed states, respectively, A_2O_i represents the three open states and A_2G_i corresponds to various brief closed states outside the activation pathway, CT3 is associated with dwells in the C, AC, and A_2C states. Specifically, the duration of CT3 is dependent on the binding and unbinding rates of agonist (A). A lack of changes in the mean duration of CT3 suggests that diazepam does not modulate closed receptor affinity to GABA (*i.e.*, rates for agonist association and dissociation are unaltered). The reduced rate of entry into CT3 can be associated with a reduction in the closing rate constant, α. It is interesting that changes in α underlie, in part, potentiation observed in the presence of neuroactive steroids.[19,27] The increase in the fraction of OT3 (table 1) can be accounted for by an increase in the rate of transition from A_2O_2 to A_2O_3.

A recent study proposed that diazepam acts by facilitating $GABA_A$ receptor preactivation, a transitional state that precedes channel opening.[28] If this interpretation is correct, our results would imply that the transitional states that underlie preactivation must be the same in receptors activated by orthosteric and allosteric agonists. However, we did not see changes in single-channel kinetics that we associate with a change in rates connecting states before channel opening (states C, AC, and A_2C in the scheme) nor in the transition from A_2C to A_2O_1. More work is required to resolve this apparent difference; for example, it is possible that an effect on transitions into or out of A_2O_3 (in our scheme) could be influenced and result in observations similar to those made by Gielen *et al*.[28]

It is perhaps not surprising that a benzodiazepine enhances gating by each of these agonists, if we believe that a $GABA_A$ receptor reaches a similar open-channel state, with similar immediately preceding transitional states, no matter how it is activated. However, the binding sites for allosteric agonists are proposed to be in the membrane-spanning region of the receptor, relatively close to the channel and far from both the GABA-binding site and the benzodiazepine-binding site. Furthermore, it is known that allosteric agonists can produce conformational changes in the benzodiazepine-binding site and that these changes often differ from those produced by GABA or by other allosteric agonists.[29,30] Other regions of the receptor, including some residues in the transmembrane domain and in regions proposed to couple the extracellular (GABA-binding) and transmembrane regions also apparently undergo different conformation changes during activation by GABA or allosteric agonists.[31,32] These observations suggest that activation by allosteric agonists is conformationally distinct from activation by GABA. Possibly, occupation of the benzodiazepine-binding site by diazepam results in a conformational change that is propagated to the transmembrane domain and hence has a global effect to enhance open probability.

The results indicate the possibility of significant interactions among allosteric agents at the $GABA_A$ receptor. In particular, therapeutic use of benzodiazepines may affect

the clinical dosage requirements during general anesthesia. There have been relatively few studies of interactions between benzodiazepines and GABAergic anesthetics in a clinical setting. Studies involving propofol and the benzodiazepine midazolam have noted that administration of midazolam reduces the dose of propofol needed to induce anesthetic endpoints such as sedation, hypnosis, and antinociception.[33–35] The current work provides a potential mechanistic explanation to these clinical findings.

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