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Identification of Transduction Elements for Benzodiazepine Modulation of the $GABA_A$ Receptor: Three Residues Are Required for Allosteric Coupling

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Modulation of $GABA_A$ receptors by benzodiazepines (BZDs) is believed to involve two distinct steps: a recognition step in which BZDs bind and a conformational transition step in which the affinity of the receptor for GABA changes. Previously, using γ_2/α_1 chimeric subunits (χ), we demonstrated that although the N-terminal 167 γ_2 amino acid residues confer high-affinity BZD binding, other γ_2 domains couple BZD binding to potentiation of the GABA-mediated Cl^- current (I_{GABA}). To determine which γ_2 regions couple binding to potentiation, we generated χs with longer N-terminal γ_2 segments for voltage-clamp experiments in *Xenopus* oocytes. Chimeras containing greater than the N-terminal 167 γ_2 residues showed incremental gains in maximal potentiation for diazepam enhancement of I_{GABA}. Residues in $\gamma_2$199–236, $\gamma_2$224–236 (pre-M1), and particularly $\gamma_2$257–297 (M2 and surrounding loops) are important for BZD potentiation. For several positive BZD modulators tested, the same regions restored potentiation of I_{GABA}. In contrast, β-carboline inverse-agonism was unaltered in chimeric receptors, suggesting that structural determinants for positive and negative BZD allosteric modulation are different. Dissection of the $\gamma_2$257–297 domain revealed that three residues in concert, γ_2T281, γ_2I282 (M2 channel vestibule), and γ_2S291 (M2–M3 loop) are necessary to impart full BZD potentiation to chimeric receptors. Thus, these residues participate in coupling distant BZD-binding events to conformational changes in the $GABA_A$ receptor. The location of these novel residues provides insight into the mechanisms underlying allosteric coupling for other members of the ligand-gated ion channel superfamily.

Key words: GABA; $GABA_A$ receptor; benzodiazepines; benzodiazepine-binding site; allosteric coupling; chimeric subunits; mutagenesis; inverse agonist; positive modulation; γ subunit; α subunit; M2 domain; M2–M3 loop; Xenopus *oocytes*

$GABA_A$ receptors are the major inhibitory neurotransmitter receptors in the mammalian brain and are members of a ligand-gated ion channel (LGIC) superfamily (Ortells and Lunt, 1995), which also includes receptors for acetylcholine, glycine, and serotonin. The $GABA_A$ receptor gene family comprises several different classes and subtypes of receptor subunits including 6α, 4β, 3γ, 1δ, 1ϵ, and 1π (Barnard et al., 1998). $GABA_A$ receptors are pentameric proteins containing an integral chloride-selective channel with specific binding sites for GABA, benzodiazepines (BZDs), barbiturates, and steroids (Smith and Olsen, 1995). BZDs, clinically used for their anxiolytic and antiepileptic actions, exert their therapeutic effects by allosteric modulation of $GABA_A$ receptors (Sieghart, 1995). Positive BZD modulators increase the opening frequency of the Cl^- channel in the presence of GABA, whereas negative BZD modulators (e.g., β-carbolines) decrease the opening frequency (Rogers et al., 1994). Because the therapeutic clinical value of these drugs depends on their ability to exert positive modulation on I_{GABA}, we were interested in identifying the structural determinants underlying BZD efficacy in potentiating GABA-gated currents.

Evidence suggests that both the α and γ subunits play critical roles in BZD binding and potentiation. Using the agonist-binding site of the nicotinic ACh receptor as an archetype for pentameric LGIC receptors (Czajkowski et al., 1993), the BZD-binding site of the $GABA_A$ receptor has been modeled with the γ subunit apposed to an α subunit, and both subunits contributing to the binding site at the interface (Galzi and Changeux, 1994; Smith and Olsen, 1995). Although several studies have begun to identify amino acids in both γ and α subunits involved in BZD binding (for review, see Sigel and Buhr, 1997), little is known about the structural components involved in coupling BZD binding to BZD potentiation of GABA-gated current (I_{GABA}).

Our previous studies using γ_2/α_1 chimeric subunits demonstrated that chimeras (χ) containing the N-terminal 161 amino acid residues of γ_2 exhibit wild-type binding but drastically impaired potentiation of I_{GABA} by BZDs (Boileau et al., 1998). To further delineate the regions unique to the γ_2 subunit that confer BZD potentiation, we generated additional γ_2/α_1 chimeras, expanding the length of the N-terminal γ_2 portion and reducing the α_1 C-terminal portion (Fig. 1). Two main regions of the γ subunit, $\gamma_2$224–236 and $\gamma_2$257–297, improve the allosteric coupling of BZD binding to potentiation of I_{GABA}. The largest gain in potentiation is conferred by the $\gamma_2$257–297 region, which surrounds and includes the M2 transmembrane segment. Further investigation revealed that a triplet set of residues, γ_2T281, I282, and S291 underlie this function of the γ_2 subunit. Unlike positive modulatory BZD compounds, the negative modulator

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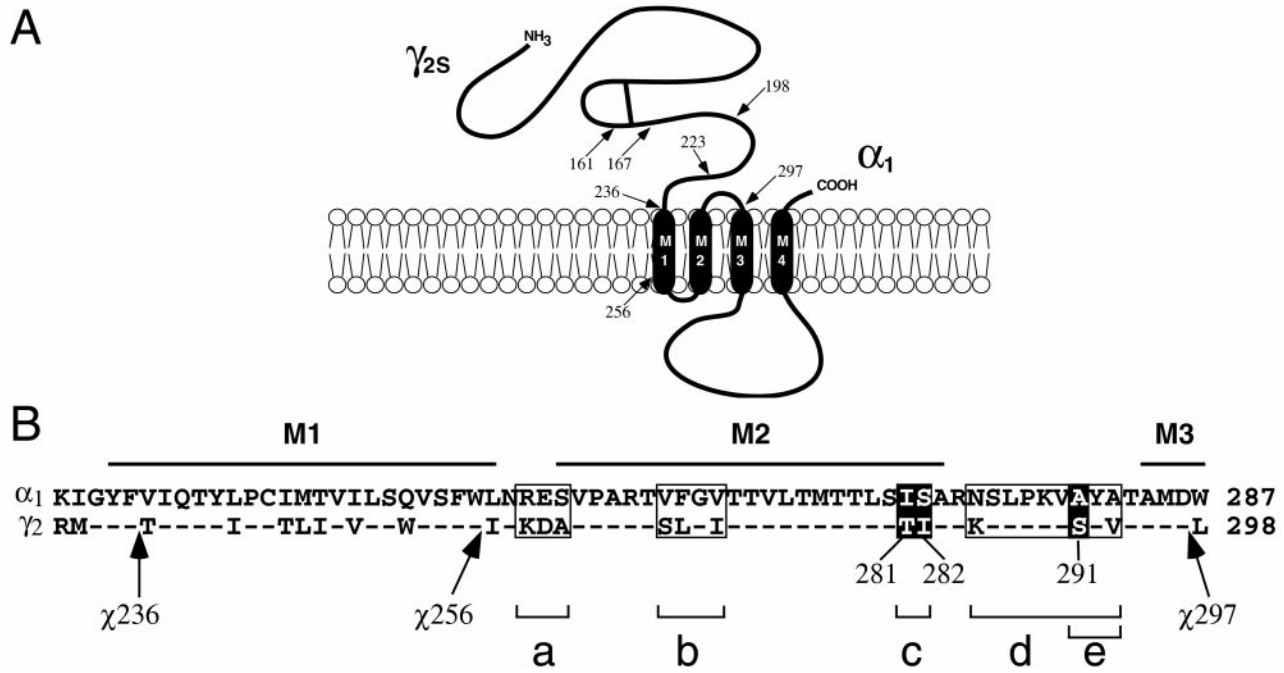

Figure 1. Chimeric γ_2/α_1 subunits and mutants. *A,* Chimeras (χ) used in this study contain 5′ γ_2 and 3′ α_1 sequences and are named for the γ_2 amino acid residue where the crossover with α_1 occurs (*arrows*) in the mature rat protein sequence. For example, χ161 contains γ_2 residues from position 1 to 161 and α_1 residues in the remainder of the subunit. Chimeric crossovers depicted are for χ161, χ167, χ198, χ223, χ236, χ256, and χ297. *B,* Shown are aligned α_1 and γ_2 protein sequence segments containing the putative transmembrane domains M1, M2, and the beginning of M3, with the relative crossover positions of χ236, χ256, and χ297 indicated (*arrows*). Mutants were constructed in the background of χ236. Numbering for χ and γ_2 subunits is identical. *Boxes* indicate blocks of mutations constructed simultaneously: box *a* represents the substitution of α_1 RES residues to the aligned γ_2 residues KDA (RES→KDA); box *b* corresponds to VFGV→SLGI; box *c* corresponds to IS→TI; box *d* corresponds to NAA→KSV; box *e* corresponds to a subset of box *d*, AA→SV. Residues highlighted in *black* are important for positive BZD modulation of I_{GABA}, and their positions in γ_2 are indicated below them.

3-carbomethoxy-4-ethyl-6,7-dimethoxy-β-carboline (DMCM) exhibits full wild-type inhibition for all chimeric receptors.

MATERIALS AND METHODS

Molecular cloning. Chimeras are named for the last γ_2 amino acid residue before the crossover with α_1 in the mature rat protein sequence. Thus, numbering for χ and γ_2 subunits is identical. For chimeras used in this study (Fig. 1), the γ_2 and α_1 amino acid residues at the crossovers are "γD161/αA149"(χ161), "γL167/αK155" (χ167), "γL198/αN188" (χ198), "γM223/αT213" (χ223), "γF236/αV226" (χ236), "γW256/αL246" (χ256), and "γD297/αW287" (χ297). χ161 and χ167 were generated by a targeted random chimera production method (Boileau et al., 1998). All others (Fig. 1*A*) were produced by recombinant PCR using overlapping complementary oligonucleotides designed to create a γ/α crossover at the desired amino acid junction. Using a γ_2 sense oligonucleotide and an antisense γ/α junction oligonucleotide with γ_2 cDNA as a template, upstream PCR fragments with γ_2 5′ and an α_1 3′ overhang were generated. Simultaneously, using a sense junction oligonucleotide and an α_1 antisense oligonucleotide with α_1 cDNA as template, downstream PCR fragments with γ_2 5′ overhang and α_1 3′ sequences were also prepared in separate PCR reactions. Upstream and downstream PCR fragments were then combined and amplified to create γ/α DNA cassette fragments that were subcloned into χ167 cDNA using *Afl*II and *Nco*I, thus replacing the cassette region of χ167 with the new chimeric junction sequences.

Mutant subunit fragments were generated using the same recombinant PCR method and subcloned into the background of the χ236 subunit to generate different combinations of mutants (Fig. 1*B*). Single and multiple mutants were named according to the α_1 to γ_2 substitutions made in χ236. For example, the "χ236-RES→KDA" mutant is χ236 with α_1 Arg-Glu-Ser (RES) residues replaced by the aligned γ_2 sequence at positions 259–261, Lys-Asp-Ala (KDA). The "χ236-A291S" mutant replaces α_1 Ala with γ_2 Ser291 in χ236; the "χ236-I281T + A291S" mutant is χ236 with an I281T and an A291S mutation. The chimeric and mutant subunits were subcloned in pGH19 vector (Liman et al., 1992; Robertson et al., 1996) for expression in *Xenopus* oocytes. All chimeras were verified by restriction digest and double-stranded DNA sequencing using standard techniques (Sambrook et al., 1989).

Expression of rat $GABA_A$ receptors in Xenopus *oocytes.* Capped cRNA coding for the wild-type and chimeric subunits was synthesized by *in vitro* transcription from *Nhe*I-linearized cDNA template in pGH19 using the mMessage mMachine T7 kit (Ambion). Oocytes from *Xenopus laevis* were prepared as previously described (Boileau et al., 1998) and injected with 28 nl of mRNA (10–200 pg/nl/subunit) mixed in a ratio of 1:1 (α:β), or 1:1:≥20 (α:β:γ or α:β:χ). Excess molar ratios of γ or χ cRNA were injected to ensure expression of these subunits in the receptor complex (Boileau et al., 1998). Oocytes were stored at 17–19°C in recording solution supplemented with 100 μg/ml gentamycin and 100 μg/ml BSA, and were used for electrophysiological experiments 2–14 d after injection. Total amount of cRNA was scaled to yield maximal GABA-induced currents of ~3–8 μA for $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2\chi$. cRNA concentrations were calculated by UV absorption and corroborated by comparison to RNA standards on 1.5% agarose gels.

Voltage-clamp analysis. Oocytes under two-electrode voltage-clamp (V_{hold} = −80 mV) were perfused continuously with ND96 recording solution containing (in mM) 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 5 HEPES, pH 7.4 at a rate of 5 ml/min. In general, drugs and reagents were dissolved in ND96. Stock drug solutions (1000–10,000×) were made in dimethylsulfoxide. No differences in currents were observed with the vehicle. Maximal current (~3–8 μA) for all receptors was achieved with 10 mM GABA. For some chimeras and mutants, GABA EC_{50} was estimated by examining the ratio of response to 1 μM versus 10 mM GABA. GABA (1 μM) elicits currents corresponding to EC_5 for both $\alpha_1\beta_2\chi$ (range, EC_{2-7}) and $\alpha_1\beta_2\gamma_2$ ($EC_{4.8 \pm 1.7}$) receptors. Using a standard Hill equation to model the GABA dose responses and the determined 1 μM/10 mM GABA current ratio, we calculate that the chimeric and mutant receptors show no more than a twofold shift in GABA EC_{50} from wild-type receptors (EC_{50} = 12 μM). Greater than twofold shifts are detectable in our assay.

BZD potentiation or β-carboline inhibition I_{GABA} were recorded at 1 μM GABA (EC_5). Potentiation is defined as $(I_{GABA + DRUG}/I_{GABA}) - 1)$,

where $I_{GABA + DRUG}$ is the current response in the presence of the drug tested, and I_{GABA} is the control GABA-induced current. For single concentration experiments, BZD site ligands were tested at concentrations eliciting maximal effects on I_{GABA} in wild-type $\alpha_1\beta_2\gamma_2$ receptors. If differences in potentiation for mutant receptors were caused entirely by a shift in GABA affinity, the Hill equation predicts that a sixfold shift in GABA affinity, and only to the left, would be required to account for a 50% reduction in maximal potentiation. This would result in a 1 μM/10 mM GABA ratio corresponding to $EC_{27–32}$ (Hill coefficient varied from 1 to 2), well out of our observed range. Standard two-electrode voltage-clamp recording was performed using a GeneClamp 500 (Axon Instruments, Foster City, CA) interfaced to a computer with an IT-16 A/D device (Instrutech). Electrodes were filled with 3 M KCl and had a resistance of 0.5–1.5 MΩ.

Data acquisition and analysis were performed using AxoData, AxoGraph (Axon Instruments), and Prism software (Graphpad). Statistical comparisons of potentiation data employed one-way ANOVA with Dunnet and Tukey post-tests for multiple independent samples using Prism software.

RESULTS

Regions of the γ_2 subunit responsible for BZD binding can be separated from domains required for coupling BZD binding to potentiation of I_{GABA} (Boileau et al., 1998). This "uncoupling" of high-affinity BZD binding and potentiation is apparent in the γ/α chimera χ161, which binds BZDs with wild-type affinity when expressed with wild-type α_1 and β_2 subunits, but displays drastically impaired diazepam modulation of I_{GABA}. In an effort to identify regions of the γ_2 subunit required for full BZD potentiation, several additional chimeras (Fig. 1*A*) were constructed with longer γ_2 N-terminal domains. Chimeras used here, named for the γ_2 amino acid where the crossovers occur, are χ161, χ167, χ198, χ223, χ236, χ256, and χ297 (see Materials and Methods). These chimeras were expressed in *Xenopus* oocytes and screened by two-electrode voltage clamp to test for restoration of allosteric modulation of I_{GABA} with several structurally diverse BZD-binding site ligands. The expression, BZD radioligand binding, and diazepam modulation of GABA-gated currents for $\alpha_1\beta_2\chi$161 and $\alpha_1\beta_2\chi$167 receptors have been described previously (Boileau et al., 1998).

Different domains alter BZD EC_{50} and potentiation

Chimeric cRNA was coinjected with wild-type α_1 and β_2 subunit cRNA into oocytes and tested for diazepam potentiation of I_{GABA} with 1 μM GABA. This concentration corresponded to EC_5 for GABA for both wild-type and chimeric receptors. Both maximal potentiation and EC_{50} for diazepam modulation of I_{GABA} were measured. Dose–response curves and traces for diazepam potentiation of the GABA response for selected chimera-containing and wild-type receptors are depicted in Figure 2. At concentrations >1 μM diazepam, potentiation of wild-type receptors begins to depress (Fig. 2, *dashed line*; Amin et al., 1997). The decrease may be attributable to channel block and/or BZD blockage of the GABA-binding site. Receptors containing χ161 and χ167 exhibited low potentiation, whereas chimeras with increasingly longer N-terminal γ_2 segments exhibited incremental gains in maximal potentiation (Table 1, Fig. 2). Full potentiation was restored with χ297, which contains γ_2 residues up to the beginning of the M3 transmembrane domain. For maximal potentiation, data from chimeric and wild-type receptors yielded the series $\alpha\beta \ll \alpha\beta\chi161 \approx \alpha\beta\chi167 < \alpha\beta\chi198 \approx \alpha\beta\chi223 < \alpha\beta\chi236 \approx \alpha\beta\chi256 \ll \alpha\beta\chi297 \approx \alpha\beta\gamma$.

Differences in EC_{50} for diazepam potentiation of I_{GABA} between chimeric and wild-type receptors were also measured (Fig. 2, *inset;* Table 1). Diazepam EC_{50} values for receptors containing χ223 (120 ± 43 nM), χ236 (164 ± 29 nM), χ256 (73 ± 26 nM), and χ297 (98 ± 5 nM) were not significantly different from those for wild-type $\alpha_1\beta_2\gamma_2$ receptors (64 ± 15 nM). However, the EC_{50} values for diazepam were significantly higher ($p < 0.01$) for receptors containing χ161, χ167, and χ198 (310 ± 45, 437 ± 87, and 340 ± 118, respectively). A significant decrease in diazepam EC_{50} occurred between χ198 and χ223 (Fig. 2, *inset*). It was also noted that χ198 displays unusually fast desensitization to application of either GABA or GABA plus diazepam. The reason for this is unclear, and was not pursued. Interestingly, although the diazepam EC_{50} for $\alpha_1\beta_2\chi$256 receptors was similar to wild-type $\alpha_1\beta_2\gamma_2$ receptors, the maximal potentiation was reduced by ~60% compared to wild-type receptors (Fig. 2; see Fig. 4). The most significant gain of potentiation occurred between χ256 and χ297 (Fig. 1); $\alpha_1\beta_2\chi$297 receptors exhibit potentiation and EC_{50} for diazepam indistinguishable from wild-type receptors.

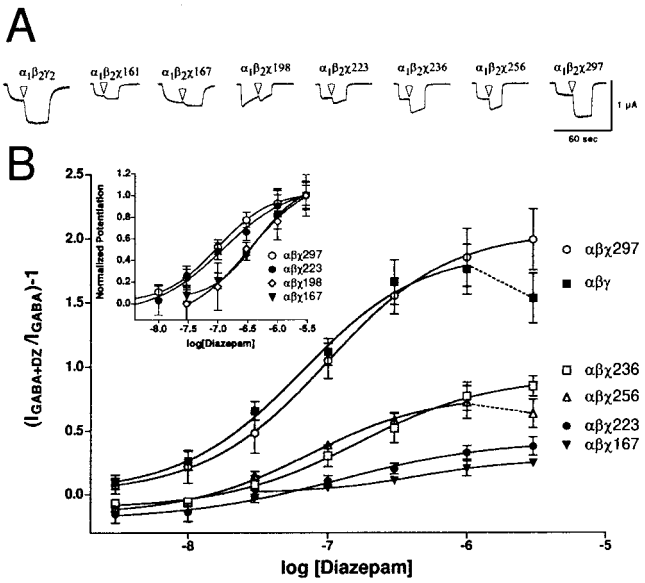

Figure 2. Diazepam potentiation of I_{GABA} for $\alpha_1\beta_2\chi$ receptors. *A,* Trace recordings from cells injected with $\alpha_1\beta_2\gamma_2$ (*leftmost*), and chimeric constructs. Cells were voltage-clamped at −80 mV and perfused with ND96 recording solution or ND96 with 1 μM GABA or 1 μM GABA plus 1 μM diazepam (transition to diazepam-containing solutions indicated by *white arrowheads*). Cells were washed with ND96 recording solution for 5–20 min between drug applications. $\alpha_1\beta_2\chi$198 exhibits unusually fast desensitization properties. Note that chimeras show incrementally larger potentiation up to $\alpha_1\beta_2\chi$297, which is similar to wild-type $\alpha_1\beta_2\gamma_2$. *B,* Oocytes injected with wild-type $\alpha_1\beta_2\gamma_2$ (1:1:20) and $\alpha_1\beta_2\chi$ (1:1:≥20) cRNA mixtures were treated with a range of diazepam concentrations in the presence of GABA and further analyzed. A potentiation response ratio was determined by dividing the peak current for $\alpha_1\beta_2\gamma_2$ (■), $\alpha_1\beta_2\chi$167 (▼), $\alpha_1\beta_2\chi$198 (◇), $\alpha_1\beta_2\chi$223 (●), $\alpha_1\beta_2\chi$236 (□), $\alpha_1\beta_2\chi$256 (△), and $\alpha_1\beta_2\chi$297 (○) exposed to 1 μM GABA plus diazepam (*DZ*) by the response to 1 μM GABA alone. Data were fitted to a curve described by the equation $Y = Min + (Max - Min)/(1 + 10^{((\mathrm{LogEC_{50}} - X)\cdot n_H)})$, where *Max* is the maximal potentiation, *Min* is the potentiation at the lowest drug concentration tested, *X* is the logarithm of diazepam concentration, EC_{50} is the half-maximal potentiation response, and n_H is the Hill coefficient. Data points represent mean potentiation from four or more cells from two or more batches of oocytes. Error bars indicate SD. The parameters from the curve fits are presented in Table 1. *Inset,* A plot of the same data after normalizing to the maximum diazepam potentiation for each receptor.

Positive BZD modulators use the γ257–297 domain

To explore whether potentiation or inhibition by other drugs acting at the BZD site would require similar regions of the γ_2 subunit, the chimeras were tested with several different BZD-

Table 1. Summary of dose–response data for diazepam potentiation of chimeric and wild-type receptors

	Diazepam potentiation		
	Max. Pot	EC_{50}(nM)	$EC_{50(mut)}/EC_{50(wt)}$
$\alpha_1\beta_2\gamma_2$	1.91 ± 0.17	64 ± 15	1.0
$\alpha_1\beta_2$	0.03 ± 0.03	–	–
$\alpha_1\beta_2\chi161$	0.26 ± 0.01	310 ± 45	4.8
$\alpha_1\beta_2\chi167$	0.28 ± 0.02	437 ± 87	6.8
$\alpha_1\beta_2\chi198$	0.52 ± 0.18	340 ± 118	5.3
$\alpha_1\beta_2\chi223$	0.42 ± 0.14	120 ± 43	1.9
$\alpha_1\beta_2\chi236$	0.94 ± 0.11	164 ± 29	2.6
$\alpha_1\beta_2\chi256$	0.77 ± 0.02	73 ± 26	1.1
$\alpha_1\beta_2\chi297$	2.05 ± 0.03	98 ± 5	1.5
$\alpha_1\beta_2\chi236$-I281T+A291S	1.05 ± 0.04	165 ± 17	2.6
$\alpha_1\beta_2\chi236$-S282I+A291S	1.09 ± 0.19	187 ± 32	2.9
$\alpha_1\beta_2\chi236$-IS→TI	1.07 ± 0.06	31 ± 5	0.5
$\alpha_1\beta_2\chi236$-A291S	1.67 ± 0.19	144 ± 37	2.2
$\alpha_1\beta_2\chi236$-IS→TI+A291S	2.07 ± 0.16	109 ± 20	1.7

Dose–response data for wild-type and chimeric subunit combinations for diazepam potentiation of 1 μM GABA responses are tabulated. Means and SDs for maximum potentiation and EC_{50} values were calculated from dose–response data (Figs. 2, 6). Data for each receptor were obtained from ≥6 oocytes from ≥2 batches.

binding site ligands. Surprisingly, the β-carboline DMCM, an inverse agonist at the BZD site, inhibited I_{GABA} in all chimeric receptors to the same extent as wild-type $\alpha_1\beta_2\gamma_2$ receptors (Fig. 3). In contrast, the positive modulatory BZD site ligands tested showed significant differences in potentiation between chimeric receptors (Fig. 4, Table 2). The differences between chimeric receptors for positive modulation could be caused by inefficient expression of the chimera in a receptor complex, yielding a mixture of $\alpha_1\beta_2$ and $\alpha_1\beta_2\chi$ receptors that would appear to have reduced potentiation. However, the observation that each chimera responds to DMCM akin to $\alpha_1\beta_2\gamma_2$ receptors, and not $\alpha_1\beta_2$ receptors, allays this concern.

Classical 1,4-benzodiazepines such as diazepam and water-soluble flurazepam exhibited similar increments in potentiation of I_{GABA} in chimeras with increasingly larger N-terminal segments (Fig. 4*A*). The strong positive modulators midazolam (a triazolobenzodiazepine, Fig. 4*B*) and zolpidem (an imidazopyridine, Fig. 4*C*), and the partial agonist CL218,872 (a triazolopyridazine, Fig. 4*D*) also showed increments in potentiation from χ167 to χ297, indicating a common pattern of residues required for positive BZD allosteric modulation.

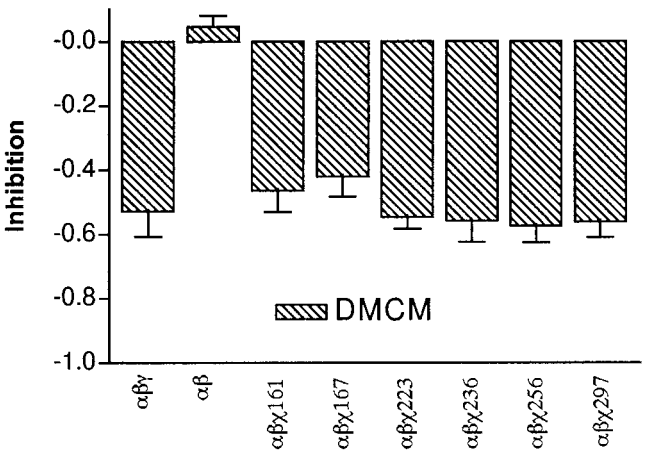

Figure 3. Chimeric receptors are indistinguishable from wild-type receptors for negative modulation by DMCM. Negative modulation of 1 μM GABA responses by the β-carboline DMCM (1 μM) is graphed for wild-type $\alpha_1\beta_2\gamma_2$, $\alpha_1\beta_2\chi161$, $\alpha_1\beta_2\chi167$, $\alpha_1\beta_2\chi223$, $\alpha_1\beta_2\chi236$, $\alpha_1\beta_2\chi256$, and $\alpha_1\beta_2\chi297$ chimeric receptors. No significant difference in the inhibition of the GABA current was measured between wild-type and chimeric receptors.

Table 2 summarizes the effects of various BZD site ligands on the potentiation of I_{GABA} for chimeric receptors compared to wild-type $\alpha_1\beta_2\gamma_2$ receptors. For all positive modulators tested, a correlation between recovery of potentiation and length of the chimeric γ_2 sequence emerges. Small increases in potentiation are observed between χ167 and χ223 (~10–15%), and between χ223 and χ236 (~10–25%) for all of the positive modulators tested. Whether a significant recovery of potentiation occurred between χ167 and χ198 or between χ198 and χ223 depended on the drug tested. The largest increases in potentiation (50–60%) for all of the positive modulatory BZDs tested occurred between χ256 and χ297, indicating that amino acid residues surrounding and/or including M2 are required for full potentiation of I_{GABA} by these drugs.

In addition, we tested the activity of the imidazobenzodiazepine antagonist Ro15–1788 (flumazenil; Fig. 4*B*) in wild-type and chimera-containing receptors. Ro15–1788 (1 μM) was effective at blocking the midazolam-induced (1 μM) potentiation of I_{GABA} in all chimeric receptors (Fig. 4*B*, *hatched bars*). At micromolar concentrations, Ro15–1788 acts as a weak positive modulator of GABA activation (Fig. 4*B*, *open bars*; Mihic et al., 1997). The pattern of potentiation in wild-type and chimeric receptors was similar to that seen for other positive modulators. After subtracting out the minor background Ro15–1788-induced potentiation, the midazolam-induced potentiation is inhibited by Ro15–1788 to the same extent for wild-type and all chimeric receptors (91.5 ± 3.7%).

Mutations conferring positive modulation of I_{GABA}

Because the largest gain in BZD potentiation is mediated by the γ_2I257-D297 domain, we focused on identifying the amino acid residue or residues in this region that contribute to BZD modulation of I_{GABA}. To determine whether $\alpha_1\beta_2\chi236$ receptors are altered in their ability to bind BZDs, we performed radioligand-binding assays. The K_D for [^{3}H]flunitrazepam binding in $\alpha_1\beta_2\chi236$ receptors (3 ± 1 nM; $n = 2$) was similar to the values obtained for $\alpha_1\beta_2\chi161$ (11.3 ± 1.7 nM) and $\alpha_1\beta_2\gamma_2$ (9.9 ± 0.8 nM; Boileau et al., 1998). Several mutants were constructed in a χ236 background, corresponding to nonidentical residues between γ_2 and α_1 in the M1–M2 loop, the M2 transmembrane domain, and the M2–M3 loop (Fig. 1*B*). The first set of such mutants with α_1 amino acid residues substituted to the homologous γ_2 residues were χ236-RES→KDA (Fig. 1*B, box a*), χ236-RES→KDA + NAA→KSV (*box a + box d*), χ236-VFGV→SLGI (*box b*), χ236-VFGV→SLGI + NAA→KSV (*box b + box d*), χ236-IS→TI (*box c*), and χ236-IS→TI + AA→SV (*box c + box e*). Of these, only χ236-IS→TI + AA→SV (*box c + box e*) exhibited full potentiation of I_{GABA} by diazepam. All single-, double-, and triple-mutant combinations of the substitutions present in χ236-IS→TI + AA→SV (*box c + box e*) were generated and tested with 1 μM diazepam for potentiation of I_{GABA} (Fig. 5). Of these, only the triple mutant combination χ236-IS→TI + A291S (Fig. 1*B*, *black outlined residues*) restored full potentiation to χ236; all values for diazepam potentiation of I_{GABA} by mutant receptors

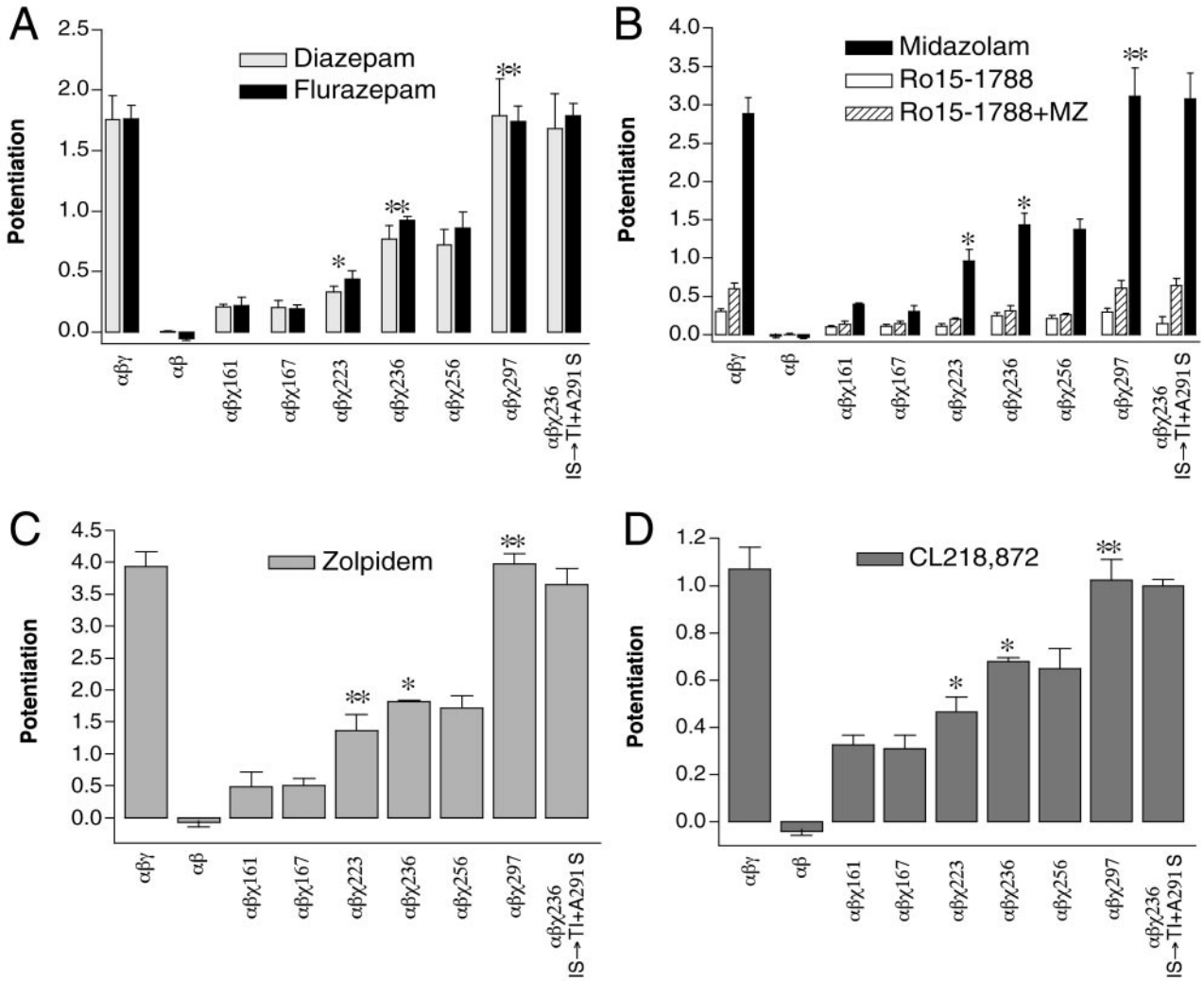

Figure 4. Positive BZD-site modulators potentiate the GABA responses of chimeric receptors to different extents depending on the amount of γ_2 subunit in each receptor. Potentiation of 1 μM GABA responses is graphed for wild-type $\alpha_1\beta_2\gamma_2$, $\alpha_1\beta_2\chi161$, $\alpha_1\beta_2\chi167$, $\alpha_1\beta_2\chi223$, $\alpha_1\beta_2\chi236$, $\alpha_1\beta_2\chi256$, $\alpha_1\beta_2\chi297$, and "$\alpha_1\beta_2\chi236$-IS→TI + A291S" chimeric receptors using 1 μM diazepam, 1–3 μM flurazepam (*A*), 1–3 μM midazolam (*B*), 10 μM zolpidem (*C*), and 1 μM CL218,872 (*D*). Also depicted in *B* is inhibition of the midazolam (*MZ*) potentiation by 1 μM Ro15–1788 (*hatched bars*) for chimeric and wild-type receptors. *Open bars* indicate the potentiation of I_{GABA} by 1 μM Ro15–1788. *Asterisks* indicate level of significance (*$p < 0.01$; **$p < 0.001$) comparing mean potentiation for that chimera with the preceding chimera in the series (*left* to *right*). Error bars indicate SD.

depicted in Figure 5 are significantly different ($p < 0.01$) from those for $\chi297$ except for the quadruple mutant $\chi236$-IS→TI + AA→SV (*box c* + *box e*) and the triple mutant $\chi236$-IS→TI + A291S.

Diazepam dose–response curves for the components of $\chi236$-IS→TI + A291S, namely $\chi236$-IS→TI, $\chi236$-A291S and the other two double mutants $\chi236$-I281T + A291S and $\chi236$-S282I + A291S demonstrate that all three mutations are necessary for full potentiation for diazepam (Fig. 6). Summary data for maximal potentiation and EC_{50} values are shown in Table 1. Although $\chi236$-A291S alone did restore some of the potentiation to $\chi236$, it is still significantly less than for $\chi297$ ($p < 0.01$). The triple mutant also exhibited wild-type maximal potentiation for other BZD-positive modulators (Fig. 4, Table 2).

DISCUSSION

We previously reported that chimeric subunits with γ_2 sequence in the first 161 amino acid residues and α_1 sequence in the remainder exhibit high-affinity BZD binding, but impaired BZD modulation of I_{GABA} (Boileau et al., 1998). Efficient coupling of BZD binding to potentiation of I_{GABA} is therefore mediated, at least in part, by γ_2 amino acid residues that are not directly involved in the binding of BZDs. In this study, we identified two novel γ_2 regions, $\gamma_2$224–236 (pre-M1) and $\gamma_2$257–297 (M2 and surrounding loops) that are necessary for full restoration of coupling between high-affinity BZD binding and full BZD potentiation of I_{GABA} by several disparate BZD positive allosteric modulators (Figs. 2, 4). In addition, a threefold shift in EC_{50} is observed between $\alpha_1\beta_2\chi198$ and $\alpha_1\beta_2\chi223$ receptors (Table 1). This change in diazepam EC_{50} may account for some or all of the increased potentiation observed in $\alpha_1\beta_2\chi223$ receptors as compared to $\alpha_1\beta_2\chi167$ receptors (Figs. 2, 4).

For all the BZD positive allosteric modulators tested, the largest recovery in BZD potentiation of I_{GABA} (50–60%) is conferred by one or more γ_2 residues in and/or surrounding the M2 region between $\chi256$ and $\chi297$. For this region, a change in diazepam EC_{50} cannot explain the complete restoration in the

Table 2. Recovery of BZD potentiation of I_{GABA} in chimeric receptors

	Diazepam	Flurazepam	Midazolam	Zolpidem	CL218,872
$\alpha_1\beta_2\chi$161	12 ± 2%	12 ± 4%	14 ± 1%	12.2 ±6%	30 ± 5%
	(4)	(4)	(3)	(7)	(3)
$\alpha_1\beta_2\chi$167	12 ± 4%	11 ± 2%	11 ± 3%	13 ± 3%	29 ± 6%
	(6)	(4)	(4)	(6)	(4)
$\alpha_1\beta_2\chi$198	15 ± 3%	24 ± 5%	20 ± 2%	13 ± 2%	27 ± 3%
	(4)	(3)	(4)	(5)	(5)
$\alpha_1\beta_2\chi$223	19 ± 4%	25 ± 4%	33 ± 6%	35 ± 7%	44 ± 7%
	(6)	(5)	(3)	(5)	(5)
$\alpha_1\beta_2\chi$236	44 ± 8%	52 ± 4%	50 ± 6%	46 ± 3%	64 ± 6%
	(6)	(5)	(4)	(30)	(3)
$\alpha_1\beta_2\chi$256	41 ± 9%	49 ± 8%	48 ± 6%	44 ± 6%	61 ± 9%
	(4)	(5)	(5)	(4)	(7)
$\alpha_1\beta_2\chi$297	108 ± 21%	99 ± 10%	108 ± 15%	101 ± 7%	95 ± 12%
	(6)	(8)	(6)	(8)	(7)
$\alpha_1\beta_2\chi$236-IS→TI+A291S	96 ± 20%	102 ± 9%	107 ± 14%	93 ± 8%	93 ± 8%
	(8)	(4)	(4)	(4)	(3)

Chimeric receptors were tested for modulation of I_{GABA} in *Xenopus* oocytes by a variety of BZD-site ligands and normalized to wild-type receptor potentiation. Percentage of recovery is defined as $(I_{GABA\,+\,DRUG}/I_{GABA})$-1 for $\alpha_1\beta_2\chi$ receptors divided by that measured for $\alpha_1\beta_2\gamma_2$ receptors × 100. Drug concentrations used were 1 μM diazepam, 1-3 μM flurazepam, 1-3 μM midazolam, 10 μM zolpidem, and 1 μM CL218,872 in the presence of 1 μM GABA. Data are means ± SD. *n* indicates the number of oocytes from ≥2 batches tested.

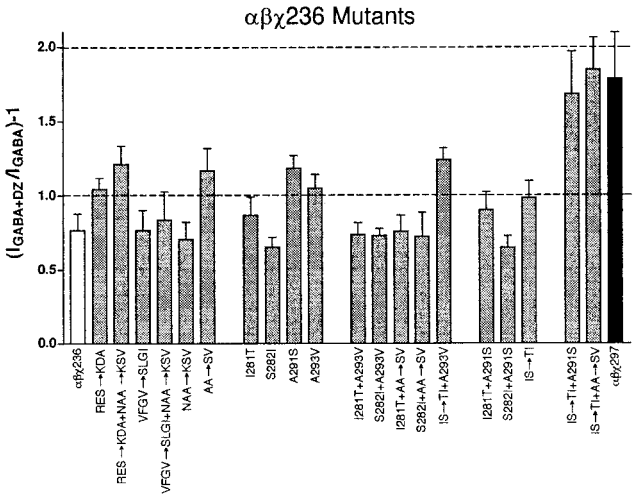

Figure 5. Diazepam potentiation of chimeric and chimeric mutant receptors. Potentiation of 1 μM GABA current using 1 μM diazepam (*DZ*) is graphed for $\alpha_1\beta_2\chi$236 (*white*), mutants, and mutant combinations made in the background of $\alpha_1\beta_2\chi$236 receptors (*gray*) and $\alpha_1\beta_2\chi$297 (*black*) chimeric receptors. Potentiation is defined as $(I_{GABA\,+\,DZ}/I_{GABA}) - 1)$, where $I_{GABA\,+\,DZ}$ is the current response in the presence of diazepam, and I_{GABA} is the control GABA-induced current. *Dashed lines* at 1.0 and 2.0 on the ordinate axis are shown for ease of comparison. Only the $\alpha_1\beta_2\chi$236-IS→TI + A291S and $\alpha_1\beta_2\chi$236-IS→TI + AA→SV chimeric receptors show no significant difference from $\alpha_1\beta_2\chi$297 receptors.

ability of diazepam to potentiate I_{GABA} in $\alpha_1\beta_2\chi$297 receptors as compared to $\alpha_1\beta_2\chi$256 receptors because the diazepam EC_{50} values for $\alpha_1\beta_2\chi$297 and $\alpha_1\beta_2\chi$256 receptors are not significantly different from each other or wild-type receptors (Table 1). The differences in levels of potentiation for chimeric receptors are also not caused by inefficient expression of the chimeric subunit, as evidenced by the equivalence of DMCM inhibition of I_{GABA} for each chimeric receptor and $\alpha_1\beta_2\gamma_2$ receptors (Fig. 3). Thus, DMCM inhibition of I_{GABA} serves as a useful benchmark and control for chimeric insertion into the receptor complex. Finally, the differences in potentiation are not attributable to differences in GABA dose responses. Previously, we established that $\alpha_1\beta_2\chi$161 and $\alpha_1\beta_2\chi$167 receptors have EC_{50} values for GABA similar to $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2$ receptors (Boileau et al., 1998). In addition, we have calculated that the EC_{50} values for all of the chimeric receptors are shifted by less than twofold compared to wild-type receptors (see Materials and Methods), which could not alone account for the decrease in maximal potentiation observed. Thus, we conclude that the $\gamma_2$257–297 region contains structural determinants required for coupling BZD binding to BZD potentiation of I_{GABA}.

Because DMCM inhibits $\alpha_1\beta_2\chi$167 receptors to a similar extent as wild-type $\alpha_1\beta_2\gamma_2$ receptors (Fig. 3), negative modulation of the GABA-gated current by β-carboline-binding to the BZD site must be transduced through different structural elements, i.e., using γ_2 residues located within the first 167 amino acid residues, with or without downstream γ_2 residues that are conserved in the α_1 and γ_2 subunits. Because we are comparing α to γ subunits for differences in BZD function, we cannot detect amino acid residues important for processes that are conserved between the two subunit subtypes.

The specific residues in the $\gamma_2$257–297 region that underlie BZD potentiation of I_{GABA} were identified. Of the twelve nonidentical residues between χ256 and χ297, the residues γ_2T281, I282, and S291 are, in combination, necessary to confer wild-type potentiation of I_{GABA} by positive BZD modulators (Figs. 4–6). Although we cannot rule out the possibility that these mutations somehow serve to relieve a conformational dysfunction in the structure of the χ236 subunit and do not play a direct role in BZD actions, we think this unlikely. Wild-type responses to DMCM and equivalent values for competitive displacement by Ro15–1788 for all the chimeric receptors suggest that the overall structures of the chimeras are not severely disrupted. Equilibrium binding values for [^{3}H]flunitrazepam to $\alpha_1\beta_2\chi$161, $\alpha_1\beta_2\chi$167, and $\alpha_1\beta_2\chi$236 receptors were similar to wild-type receptors, indicating that the BZD-binding site is intact. Further support for our conclusion that γ_2T281, I282 and S291 are compulsory γ_2 ele-

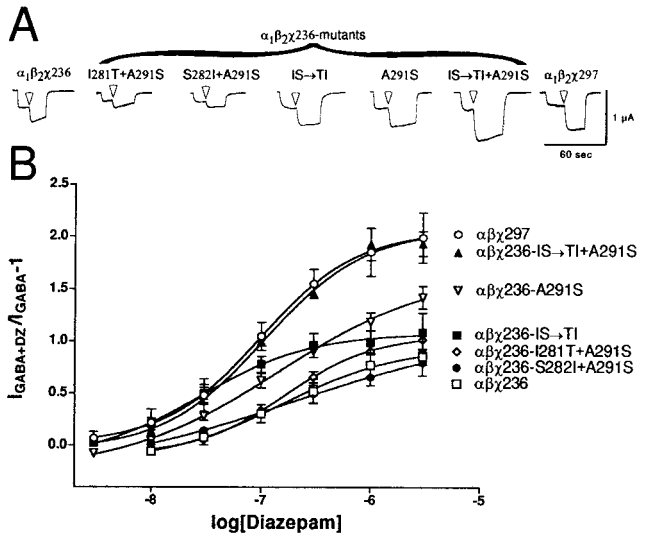

Figure 6. Diazepam potentiation of I_{GABA} for mutant $\alpha_1\beta_2\chi$236 receptors. *A,* Trace recordings from cells injected with wild-type α_1, β_2, and either χ236 (*left*), mutant χ236 (*middle*), or χ297 (*right*). Cells were voltage-clamped at −80 mV and perfused with ND96 recording solution, or ND96 with 1 μM GABA or 1 μM GABA plus 1 μM diazepam (transition to diazepam-containing solutions indicated by *white arrowheads*). Cells were washed with ND96 recording solution for 5–20 min between drug applications. Note that only the $\alpha_1\beta_2\chi$236-IS→TI + A291S mutant chimeric receptors show potentiation as large as $\alpha_1\beta_2\chi$297 receptors. *B,* Oocytes injected with chimeric and chimeric mutant cRNA mixtures were treated with a range of diazepam concentrations in the presence of GABA and further analyzed. A potentiation response ratio was determined by dividing the peak current for $\alpha_1\beta_2\chi$297 (○), $\alpha_1\beta_2\chi$236-IS→TI + A291S (▲), $\alpha_1\beta_2\chi$236-A291S (▼), $\alpha_1\beta_2\chi$236-IS→TI (■), $\alpha_1\beta_2\chi$236-I281T + A291S (◇), $\alpha_1\beta_2\chi$236-S282I + A291S (●), and $\alpha_1\beta_2\chi$236 (□) exposed to 1 μM GABA plus diazepam (*DZ*) by the response to 1 μM GABA alone. Data were fitted to a curve described by the equation $Y = Min + (Max - Min)/(1 + 10^{((\mathrm{LogEC_{50}} - X)\cdot n_H)})$, where *Max* is the maximal potentiation, *Min* is the potentiation at the lowest drug concentration tested, *X* is the logarithm of diazepam concentration, EC_{50} is the half-maximal potentiation response, and n_H is the Hill coefficient. Data points represent mean potentiation from six or more cells from two or more batches of oocytes. Error bars indicate SD. The parameters from the curve fits are presented in Table 1.

ments for BZD activity derives from the fact that all three residues are conserved in all known γ subunits cloned from various species but vary in other subunit subtypes. These identified residues, however, are not the sole γ_2 determinants controlling BZD potentiation. Certainly other γ_2 amino acid residues also play a role, particularly residues in the pre-M1 regions (e.g., $\gamma_2$224–236), which have yet to be identified, and/or amino acid residues that are conserved between the γ and α subunits.

Alterations in the ability of positive BZD modulators to potentiate I_{GABA} can arise from several possible sources, including alterations at the BZD-binding site (Colquhoun, 1998), disruptions of the coupling (transduction of BZD binding to potentiation) machinery, and/or modifications of the ion channel pore itself. An example of a mutation that alters BZD "efficacy" of several BZD ligands is the γ_2 subunit mutation T142S (Mihic et al., 1994); both a competitive antagonist at the BZD-binding site (Ro15–1788) and a weak negative modulator (Ro15–4513) take on the character of weak positive modulators. Because we previously demonstrated that high-affinity BZD binding is localized to the N-terminal 161 γ_2 amino acid residues (Boileau et al., 1998), the novel regions and residues identified in this study control BZD potentiation by influencing the coupling machinery and/or the ion channel pore itself rather than the BZD-binding site. For the chimeric receptors described in this study, strong positive BZD modulators behave like weak modulators or BZD antagonists. Additionally, we observe that $\alpha_1\beta_2\chi$161, $\alpha_1\beta_2\chi$167, and $\alpha_1\beta_2\chi$236 receptors exhibit wild-type, high-affinity radioligand binding. Together, these observations suggest that we are measuring disruptions in coupling rather than binding. Because γ_2T281 and I282 are likely to line the water-accessible surface of the Cl^- channel based on homology with the α_1 subunit (Xu and Akabas, 1996), it is tempting to speculate that this region controls BZD potentiation by affecting the ion channel. These γ_2 residues may influence ion channel gating, possibly facilitating channel opening by GABA when BZDs are present.

Structurally, allosteric coupling between GABA and BZD-binding sites could occur exclusively in the N-terminal extracellular domains of the receptor, from one binding site to the other. The Monod–Wyman–Changeux allosteric model predicts that ligand-binding events cause allosteric transitions that change the state of the receptor, which result in changes in the binding sites (Monod et al., 1965). Our chimeric receptors have either weakened or deleted allosteric transitions that are restored by γ_2 segments distant from the presumed binding sites, and in particular by the combination of the channel-lining residues γ_2T281 and I282 coupled with the M2-M3 loop residue γ_2S291. Thus, allosteric coupling between the GABA and BZD-binding sites requires transduction through transmembrane or intracellular regions and then back out to the extracellular binding regions, subsequently affecting the kinetics of GABA binding at one or both cooperative GABA-binding sites.

Our results demonstrate that two M2 residues (γ_2 T281, I282) and a M2–M3 extracellular loop residue (γ_2 S291) are required for full, wild-type BZD potentiation of I_{GABA} by a variety of BZD ligands. It is interesting that these γ_2 residues map to corresponding regions as residues in other $GABA_A$ receptor subunits and the homologous glycine receptor believed to be involved in "direct" channel gating by agonists. In the $GABA_A$ receptor, when the M2 central leucine is substituted with threonine in the human β_1 subunit, GABA activation is abolished, even though binding with the GABA agonist [^{3}H]muscimol is unaltered (Tierney et al., 1996). In the glycine receptor, mutation of α_1 R271 (in the M2–M3 loop) to leucine or glutamine converts glycinergic agonists β-alanine and taurine from agonists to competitive antagonists for glycine (Rajendra et al., 1995). Our results that residues in M2 and the M2–M3 loop are necessary for coupling BZD binding to BZD potentiation suggests that not only are the BZD and GABA-binding sites structurally conserved (Olsen et al., 1996), but the regions of the receptor involved in the coupling of binding to their functional effects are also conserved. We speculate that the BZD-binding site may in reality be a very low-affinity GABA-binding site that over evolutionary time has acquired the ability to bind BZDs and when the γ_2 M2 region is present, BZDs may act as coagonists and increase channel opening frequency.

Mutations within the M2 region and surrounding loops in the $GABA_A$ receptor also affect the actions of other $GABA_A$ receptor modulators. Mutations in two specific M2 amino acid residues, α_1S267 or the aligned β_1S265, affect the ability of ethanol, enflurane, etomidate, loreclezole, and furosemide to modulate the $GABA_A$ receptor (Wingrove et al., 1994; Belelli et al., 1997; Mihic et al., 1997; Thompson et al., 1999). Both of these residues align with γ_2S280 (Fig. 1*B*). The position of these M2 residues relative to γ_2T281 and I282 identified in this study suggest that

this region of M2 may not contain the binding site or sites for anesthetics and other related compounds, but instead may act as a common pathway for mediating the coupling between binding and the functional effects of these drugs. Mutations in either M2 or the M2–M3 loop in the glycine receptor also alter the efficacy of modulators and agonists (Rajendra et al., 1995; Lynch et al., 1997). Thus, for LGIC receptors we hypothesize that the extracellular end of the channel and the nearby M2–M3 extracellular loop are transduction domains that help couple distant ligand-binding events to the channel gate.

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