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Mechanism of action of benzodiazepines on $GABA_A$ receptors

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1 Wild-type and mutant $\alpha1\beta2\gamma2$ $GABA_A$ receptors were expressed in *Xenopus laevis* oocytes and examined using the two-electrode voltage clamp.

2 Dose–response relationships for GABA were compared in the absence and presence of 1 μM diazepam (DZP) or methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate (DMCM). The dose–current relationships yielded EC_{50}'s (concentration for half-maximal activation) of 41.0 ± 3.0, 21.7 ± 2.7, and 118.3 ± 6.8 μM for GABA, GABA plus DZP, and GABA plus DMCM, respectively.

3 DZP- and DMCM-mediated modulation were examined in $GABA_A$ receptors in which the β-subunit carries the L259S mutation. This mutation has been shown to produce spontaneous opening and impart a leftward shift in the dose–response relationship. In this case, neither DZP nor DMCM produced a significant alteration in the GABA dose–response relationship with GABA EC_{50}'s of 0.078 ± 0.005, 0.12 ± 0.03, and 0.14 ± 0.004 μM for GABA, GABA plus 1 μM DZP, and GABA plus 1 μM DMCM.

4 DZP- and DMCM-mediated modulations were examined in $GABA_A$ receptors in which the α-subunit carries the L263S mutation. This mutation also produced spontaneous opening and a leftward shift of the GABA dose–response relation, but to a lesser extent than that of βL259S. In this case, the leftward and rightward shifts for DZP and DMCM were still present with EC_{50}'s $=0.24\pm0.03$, 0.14 ± 0.02, and 1.2 ± 0.04 μM for GABA, GABA plus 1 μM DZP, and GABA plus 1 μM DMCM, respectively.

5 Oocytes expressing ultrahigh levels of wild-type $GABA_A$ receptors exhibited currents in response to 1 μM DZP alone, whereas DMCM decreased the baseline current. The DZP-mediated activation currents were determined in wild-type receptors as well as receptors in which the GABA binding site was mutated (β2Y205S). The EC_{50}'s for DZP-mediated activation were 72.0 ± 2.0 and 115 ± 6.2 nM, respectively, similar to the EC_{50} for DZP-mediated enhancement of the wild-type GABA-activated current (64.8 ± 3.7 nM).

6 Our results support a mechanism in which DZP increases the apparent affinity of the receptor, not by altering the affinity of the closed state, but rather by shifting the equilibrium towards the high-affinity open state.

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Abbreviations: BZD, benzodiazepine; *c*, ratio of K_{R*}/K_R in Scheme I and K_{B*}/K_B in Scheme II; *d*, ratio of K_{B*}/K_B; DEPC, diethyl pyrocarbonate; DMCM, methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate; DZP, diazepam; EC_{50}, an effective concentration inducing 50% of maximal response; GABA, γ-aminobutyric acid; ID, inner diameter; K_B, diazepam dissociation constant of the receptors in closed states; K_{B*}, diazepam dissociation constant of the receptors in open states; K_G, GABA dissociation constant of the receptors in closed states; K_{G*}, GABA dissociation constant of the receptors in open states; *L*, equilibrium gating constant for unliganded receptor; MWC model, Monod–Wyman–Changeux allosteric model; OD, outer diameter; OR2, oocyte Ringer's solution; R, unliganded receptor; RB, receptor with one diazepam molecule bound; RG, receptor with one GABA molecule bound; RG_2, receptor with two GABA molecules bound; RGB, receptor with one GABA molecule and one diazepam molecule bound; RG_2B, receptor with two GABA molecules and one diazepam molecule bound; s.e.m., standard error of the mean

Introduction

That the $GABA_A$ receptor is the main target for the central actions of benzodiazepines has been known for several decades (Costa *et al*., 1975; Haefely *et al*., 1975). The mechanism by which benzodiazepines, such as diazepam (DZP), enhance GABA receptor function has been termed allosteric. Allosteric, in this sense, refers to DZP binding at a site distinct from the agonist (GABA) binding site. Structure–function studies have indeed verified that the DZP binding site is distinct from the GABA binding site (Wieland *et al*., 1992; Amin *et al*., 1997; Chang & Weiss, 2000). The central question to be answered, however, is how specifically does DZP alter receptor function.

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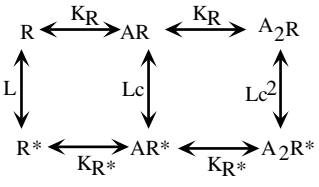
Note: We use B (instead of D) for diazepam to avoid confusion with the general meaning of K_D.

Early on, and still a popular notion is that DZP can enhance the affinity of GABA for its binding site. Single channel studies have proposed a more specific mechanism whereby DZP increases the rate at which mono-liganded receptors open, although typically two GABA molecules must bind to gate the pore (Twyman *et al*., 1989; Rogers *et al*., 1994). Even if this mechanism were true, it is not clear how the binding of DZP mechanistically achieves this particular action. For example, are the DZP and GABA sites coupled such that DZP alters the affinity of one of the two GABA binding sites? Or, alternatively, does DZP exert its actions at some step subsequent to the binding of GABA. It should be mentioned that, irrespective of actions on receptor kinetics, it has been documented that DZP can increase the conductance of individual $GABA_A$ receptors (Eghbali *et al*., 1997). Suffice it to say, the molecular mechanism of DZP is still unresolved.

In a previous study (Chang & Weiss, 1999), we provided strong support for the following activation mechanism of the $GABA_A$ receptor:



Scheme 1

In this scenario, the probabilities of channel opening from the nonliganded (R), mono-liganded (AR), and di-liganded (A_2R) states were 9.9×10^{-6}, 0.007, and 0.84, respectively. What this implies is that an overwhelming majority of the receptors, in the absence of agonist, are in the R state. A pulse of GABA at the synaptic cleft would then drive the receptors through the AR and A_2R states to the open A_2R^* state. For this reason, a majority of kinetic studies (ours included) have typically (and safely) ignored openings of the unliganded and mono-liganded states. At the end of the manuscript where we proposed this activation mechanism, we concurred that while normal activation can be adequately described by this submechanism, the more comprehensive model might be necessary to account for the actions of selective GABA receptor modulators (Chang & Weiss, 1999).

A salient feature of this type of mechanism originally proposed some 50 years ago (Del Castillo & Katz, 1957) is that the agonist affinity is higher for the open states (A*, AR*, and A_2R^*) compared to the closed states (R, AR, and A_2R). In our particular case ($\alpha1\beta2\gamma2$ $GABA_A$ receptors), the affinity of the closed state for GABA was $\approx$650-fold less than the affinity of the open state (Chang & Weiss, 1999). Based on our proposed activation mechanism (Chang & Weiss, 1999), we reasoned that DZP could alter the sensitivity of the receptor by shifting the equilibrium between R and R* such that relatively more receptors reside in the unliganded open state. As more receptors are in the high-affinity open state, the sensitivity to GABA-mediated activation would be increased. Here, we show this is indeed what happens and this concept can account for the actions of DZP on $GABA_A$ receptors. It also provides a simple approachable notion of allosteric regulation; simply put, weak agonism at a site distinct from that of agonist.

Methods

Site-directed mutagenesis and in vitro *transcription*

Unless otherwise noted, all $GABA_A$ receptor isoforms used in this study were rat $\alpha1$, $\beta2$, and $\gamma2$. The rat $\alpha1$-, $\beta2$-, and $\gamma2$L-subunits were obtained by polymerase chain reaction from a rat brain cDNA library (Amin *et al*., 1994). The three subunits were cloned into pALTER-1 (Promega, Madison, WI, U.S.A.) and pGEMHE (Liman *et al*., 1992) vectors. The $\alpha1$- and $\gamma2$-subunits were both cloned in pALTER-1 and pGEMHE between *Hin*dIII and *Xba*I, whereas $\beta2$ in pALTER-1 was cloned between *Sal*I and *Bam*HI, and in pGEMHE, $\beta2$ was cloned at the *Hin*dIII site. All mutations were confirmed by cDNA sequencing.

The wild-type and mutant cDNAs of the $\alpha1$-, $\beta2$-, $\gamma2$L-subunits in pALTER-1 were linearized by *Ssp*I and those in pGEMHE were linearized by *Nhe*I. This linearization process leaves a tail of several hundred base pairs for RNA stability. Capped cRNAs from the pALTER-1 and pGEMHE vectors were transcribed using SP6 or T7 RNA polymerase (Ambion, Austin, TX, U.S.A.), respectively, using the RNeasy Mini Kit (Qiagen, Valencia, CA, U.S.A.). After degradation of the DNA template by RNase-free DNase I, the cRNAs were purified and suspended in diethyl pyrocarbonate (DEPC)-treated water. The cRNA yield and integrity were examined on a 1% agarose gel.

Oocyte isolation and cRNA injection

Female *Xenopus laevis* (*Xenopus* I, Ann Arbor MI, U.S.A.) were anesthetized with 0.2% MS-222 and the ovarian lobes were surgically removed and placed in a Ca^{2+}-free oocyte Ringer's solution (OR2) consisting of (in mM) 92.5 NaCl, 5 HEPES, 2.5 KCl, and 1 $MgCl_2$ (pH 7.5). The lobes were cut into small pieces and digested with 0.2% collagenase A (Roche Diagnostics, Indianapolis, IN, U.S.A.) in the above solution at room temperature with continuous stirring until the oocytes were dispersed (1–2 h). The oocytes were then thoroughly rinsed with ND-96 incubation solution consisting of (in mM) 96 NaCl, 5 HEPES, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 2.5 $NaCH_3COCO_2$, 5% horse serum, 0.05 mg ml^{-1} gentamycin, and 10 U ml^{-1} penicillin/streptomycin (pH 7.5). Stage VI oocytes were selected and incubated at 14°C.

A P87 horizontal puller (Sutter Instrument Co., Novato, CA, U.S.A.) was used to make micropipettes from borosilicate glass (Drummond Scientific, Broomall, PA, U.S.A.) for cRNA injection. The micropipette tips were cut with microscissors to $\sim$40 μm OD. The cRNA for $\alpha:\beta:\gamma$-subunits were mixed in a 1 : 1 :2 ratio and diluted 45- to 100-fold with DEPC-treated water. No dilution was employed for the high expression experiments. The cRNA was injected into the oocytes with a Nanoject microinjection system (Drummond Scientific, Broomall, PA, U.S.A.). The volume of the microinjection into each oocyte was varied from 27 to 84 nl to provide a range of expression levels. Typically, a total of 0.1–1 ng of cRNA was injected into each individual oocyte.

Recording from oocytes

At 1–3 days after cRNA injection, oocytes were placed in a small volume chamber ($<$100 μl) with a 300-μm nylon mesh

support. The oocyte was continuously perfused at a rate of 150–200 $\mu l\,s^{-1}$ with the oocyte Ringer's solution (OR2), consisting of (in mM) 92.5 NaCl, 2.5 KCl, 5 HEPES, 1 $CaCl_2$, 1 $MgCl_2$ (pH 7.5), and briefly switched to OR2 plus drug (e.g., GABA, DZP, DMCM, etc.). GABA, picrotoxin, DZP, and DMCM were obtained from Sigma Chemicals (St Louis, MO, U.S.A.). GABA and picrotoxin were prepared daily from powder; however, DZP and DMCM were prepared from stock solution that was made with PEG-300 and ethanol, respectively. Stock solutions of DZP were kept at −20°C and those of DMCM were stored at room temperature.

Microelectrodes were made from filamented borosilicate glass (OD = 1.0 mm and ID = 0.75 mm) using the P87 horizontal puller. The electrodes were filled with 3 M KCl and had resistances of 1–3 MΩ. The perfusion chamber was grounded with a KCl agar bridge. The standard two-electrode voltage-clamp technique was carried out using the GeneClamp 500 voltage-clamp amplifier (Axon Instruments, Foster City, CA, U.S.A.). The current signal was low-pass filtered at 10 Hz and digitized at 50 Hz with 16-bit resolution. Data were analyzed using Igor software (Wavemetrics, Lake Oswego, OR, U.S.A.).

Data analysis

Dose–response relationships were fit with the following form of the Hill equation using a nonlinear least-squares method

$$I = \frac{I_{max}}{1 + (EC_{50}/[A])^n} \quad (1)$$

where I is the peak current response at a given concentration of agonist (A), I_{max} is the maximum current response, EC_{50} is the concentration of the agonist yielding half-maximal activation, and n is the Hill coefficient. Data were compared statistically by a Student's t-test. Statistical significance was determined at the 5% level. All results are presented as the mean ± s.e.m.

Results

Figure 1a shows GABA-activated currents from oocytes expressing recombinant $\alpha1\beta2\gamma2$ $GABA_A$ receptors. The top row of traces are currents in response to a range of GABA concentrations and the bottom row of traces are currents in response to the same concentrations of GABA, but in the presence of the benzodiazepine agonist DZP (1 μM). The dose–response relationships for GABA, GABA plus 1 μM DZP, and GABA plus 1 μM DMCM (an inverse benzodiazepine agonist) are plotted in Figure 1b. Fitting Equation (1) (see Methods) to the dose–response relationships yielded EC_{50}'s (concentration of GABA required for half-maximal activation) of 41.0 ± 3.0, 21.7 ± 2.7, and 118.3 ± 6.8 μM for GABA only, GABA plus DZP, and GABA plus DMCM, respectively (Table 1). Thus, DZP and DMCM have opposing actions on GABA sensitivity.

Prediction 1. Lack of a DZP-mediated shift in a spontaneously opening mutant

In Scheme 1, L is equal to [R]/[R*], or the ratio of the number of receptors in the unbound closed and unbound open conformations. As L decreases (increasing population of the R* state), the receptors become more sensitive to GABA (EC_{50} decreases) owing to the higher affinity of the open state (R*)

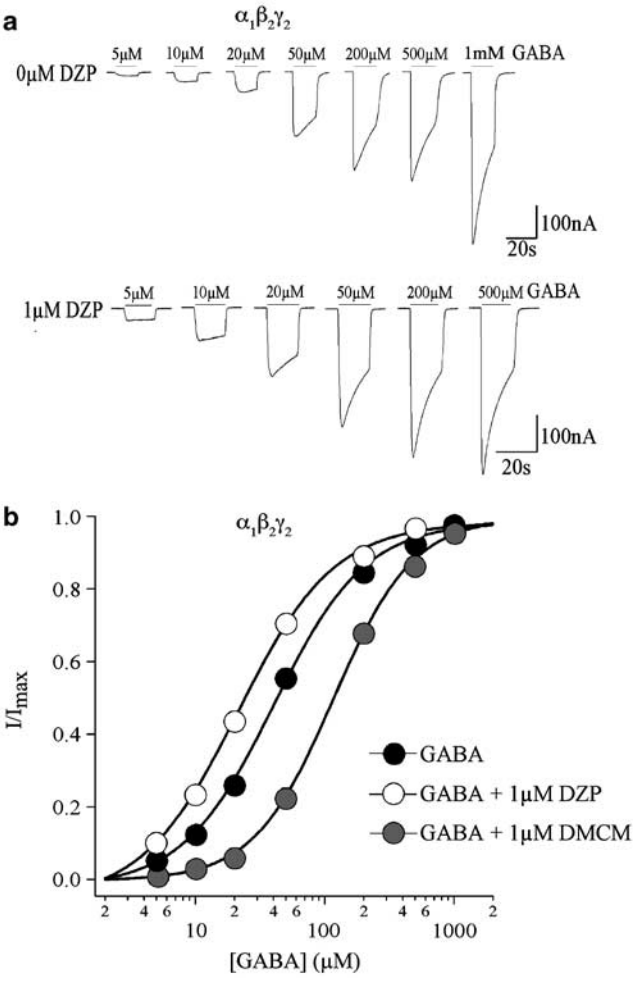


Figure 1 DZP and DMCM induce opposing actions on the GABA dose–response relationship. (a) Oocytes expressing recombinant $GABA_A$ receptors were exposed to increasing concentrations of GABA. The top row of traces are currents in response to GABA, but in the absence of DZP. The bottom row of traces are currents from the same occyte, but with coapplication of 1 μM DZP. (b) The maximum GABA-activated currents are plotted for GABA alone (filled circles), GABA plus 1 μM DZP (open circles), and GABA plus DMCM (shaded circles). The data were fitted with Equation (1) and the EC_{50}'s were 41.0 ± 3.0 (N = 6), 21.7 ± 2.7 (N = 6), and 118.3 ± 6.8 μM (N = 5), respectively.

compared to that of the closed state (R). The continuous line in the inset in Figure 2a shows the theoretical relationship between L and EC_{50} based on our working hypothesis for the activation mechanism (Chang & Weiss, 1999). In a previous study, we demonstrated that mutation of a highly conserved residue in the second membrane-spanning domain (TM2) of the β2-subunit (L259S) stabilized the open state of the receptor and produced an EC_{50} of 0.052 ± 0.005 μM, very close to the theoretical limit for the EC_{50} of 0.05 μM (Chang & Weiss, 1999). This theoretical limit is related to the affinity of the open state. The position of $\alpha1\beta2$L259Sγ in terms of L is indicated by the leftmost vertical line in the inset of Figure 2a. In that study, using a simultaneous mutation in the GABA binding site (βY157S), we also demonstrated that the shift in EC_{50} induced by the L259S mutation was independent of any effects on agonist binding. Stated more simply, the affinity of the closed state appeared unaltered. If indeed the mechanism of the βL259S-induced mutation was a maximal stabilization

Table 1 EC_{50} and Hill coefficients for the wild-type and mutant $GABA_A$ receptors

Combination	*EC_{50}* (μM)	*Hill*	N
GABA-mediated currents			
$\alpha_1\beta_2\gamma_2$			
GABA	41.0±3.0	1.23±0.12	6
GABA + 1 μM DZP	21.7±2.7	1.17±0.16	6
GABA + 1 μM DMCM	118.3±6.8	1.41±0.10	5
$\alpha_1 L263S\beta_2\gamma_2$			
GABA	0.24±0.03	1.02±0.12	5
GABA + 1 μM DZP	0.14±0.02	0.96±0.09	5
GABA + 1 μM DMCM	1.2±0.04	1.41±0.09	3
$\alpha_1\beta_2 L259S\ \gamma_2$			
GABA	0.078±0.005	0.91±0.05	8
GABA + 1 μM DZP	0.12±0.03	1.13±0.31	5
GABA + 1 μM DMCM	0.14±0.004	1.04±0.02	7
Combination	*EC_{50}* (nM)	*Hill*	N
DZP-mediated currents			
$\alpha_1\beta_2\gamma_2$	72.0±2.0	1.58±0.07	7
$\alpha_1\beta_2$Y205Sγ_2	115.0±6.2	1.14±0.08	3

of the open state, then one would predict that DZP would not further increase the sensitivity of the L259S mutant. Figure 2a shows dose–response relationships from GABA-mediated currents in the absence (filled circles) and presence (open circles) of DZP for αβL259Sγ. The EC_{50}'s were 0.078±0.005 and 0.12±0.03 μM, respectively (Table 1). These two values are statistically indistinguishable ($P>0.05$). This supports the notion that DZP and the βL259S mutation converge mechanistically.

The homologous mutation in the α-subunit (αL263S) also increased the sensitivity to GABA, although to a lesser extent than βL259S with an $EC_{50}=0.24\pm0.03$ μM for αL263S as compared to 0.078±0.005 μM for βL259S (Chang & Weiss, 1999). The position of α1L263Sβ2γ in terms of L is indicated by the rightmost vertical line in the inset of Figure 2a. In this case, we would predict that DZP, in contrast to βL259S where the sensitivity was maximally shifted, could further increase the sensitivity to GABA. In fact, we observed an increase in GABA-mediated sensitivity for αL263S ($EC_{50}=0.14\pm0.02$ μM) and this increase in sensitivity was approximately twofold as was the DZP-mediated shift for the wild-type receptor (compare Figures 1b and 2b and see Table 1).

We next examined the actions of DMCM on the GABA dose–response relationships of the two mutants. In the case of αβL259Sγ, the EC_{50}'s were 0.078±0.005 and 0.14±0.004 μM for GABA alone and GABA plus DMCM, respectively. These values were not statistically different. While the absence of a leftward shift with DZP was predicted, at face value it seemed counter-intuitive that DMCM would not shift the dose–response relationship back to the right. However, examination of the inset in Figure 2a provides a rational explanation. Owing to the plateau of the relationship between L and EC_{50} at lower values of L, modest shifts of L in either direction would not produce a detectable change in the EC_{50}. We did observe a significant rightward shift imparted by DMCM for αL263S as would be predicted by the position of this mutant along the L–EC_{50} relationship. DMCM increased the EC_{50} for GABA from 0.24±0.03 to 1.2±0.04 μM (Table 1) and this difference was statistically significant ($P<0.05$). The data in Figure 2 support the conclusion that the changes in GABA sensitivity imparted by the TM2 mutation and DZP involve a common mechanism.

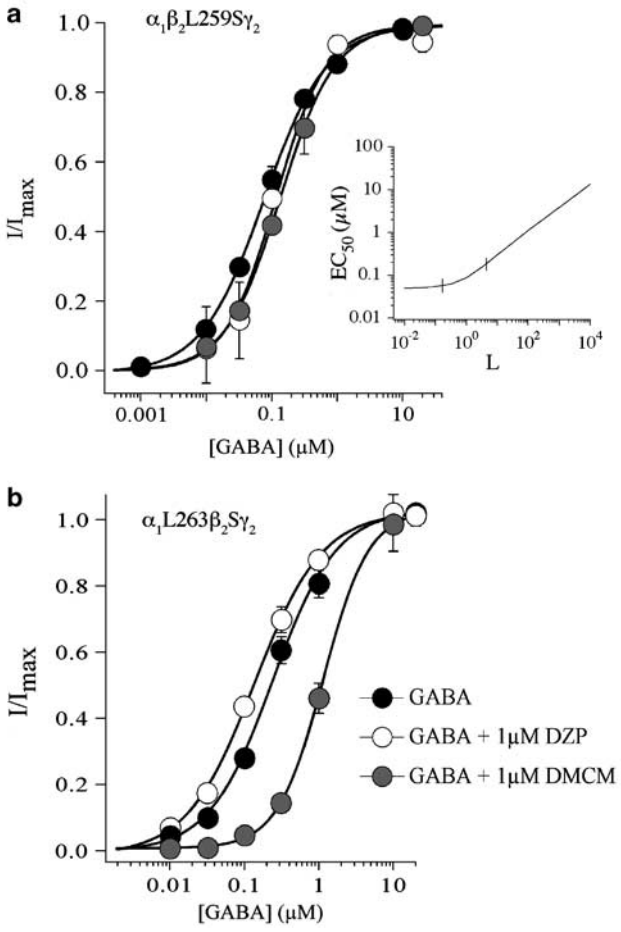


Figure 2 Effects of DZP on spontaneously opening mutant $GABA_A$ receptors. (a) Dose–response relationships for αβL259Sγ $GABA_A$ receptors in the presence of GABA alone, GABA plus 1 μM DZP, and GABA plus 1 μM DMCM. The EC_{50}'s were 0.078±0.005 ($N=8$), 0.12±0.03 ($N=5$), and 0.14±0.004 μM ($N=7$), respectively, and were statistically indistinguishable. The inset shows the predicted relationship between L and EC_{50} for the allosteric activation mechanism. The leftmost vertical line is the position of the αβL259Sγ in terms of L and the rightmost vertical line is the position of the αL263Sβγ in terms of L. This plot provides an explanation as to why neither DZP or DMCM altered the EC_{50} of the β mutant. (b) Dose–response relationships for αL263Sβγ $GABA_A$ receptors in the absence or presence of 1 μM DZP or 1 μM DMCM. The EC_{50}'s were 0.24±0.03 ($N=5$), 0.14±0.02 ($N=5$), and 1.2±0.04 μM ($N=3$), respectively. Both the increase and decrease in sensitivity with DZP and DMCM were statistically significant when compared to GABA alone.

Prediction 2. Direct activation of the GABA receptor by DZP

If DZP shifts receptors from R to R*, then one would predict a DZP-mediated current. In our hands, a typical oocyte with exogenously expressed recombinant $GABA_A$ receptors exhibits a maximum GABA-activated current in the range of 1–5 μA. If DZP were to activate the receptors to a degree that is 1/27,000 that of GABA as predicted from our value of L (Chang & Weiss, 1999), the DZP-activated current would be in the range of 0.04–0.19 nA, well below our level of resolution of approximately 2 nA. Using a high-expression vector and concentrated cRNA, we have been able to increase substantially expression levels of the GABA receptor. In fact, the expression is too high to measure reliably the maximum

GABA-activated current. Nevertheless, under these conditions, we can measure a DZP-mediated current. Figure 3 shows recordings from oocytes expressing high levels of wild-type $\alpha1\beta2\gamma2$ $GABA_A$ receptors. As evident in Figure 3a, 1 μM GABA induced a current of 3.4 μA. Figure 3a also shows the current in response to 1 μM DZP alone. As will be shown subsequently, this is a maximal concentration of DZP. We also were able to observe a DZP-mediated current in oocytes expressing $\alpha1\beta2Y205S\gamma$ receptors (data not shown). This mutation in the GABA binding site imparts a 950-fold increase in the EC_{50}, thus negating the possibility that residual GABA might be assisting the DZP in activating the receptor. In addition, Figure 3a shows a response to 500 μM picrotoxin, an antagonist of the GABA receptor. Note the decrease in holding current indicating spontaneous GABA receptor activity. In fact, Scheme 1 predicts (*via* R*) some level of spontaneous wild-type GABA receptor activity ($P_{open} = 9.9 \times 10^{-6}$) and such a block of spontaneous GABA receptor activity has been documented previously.

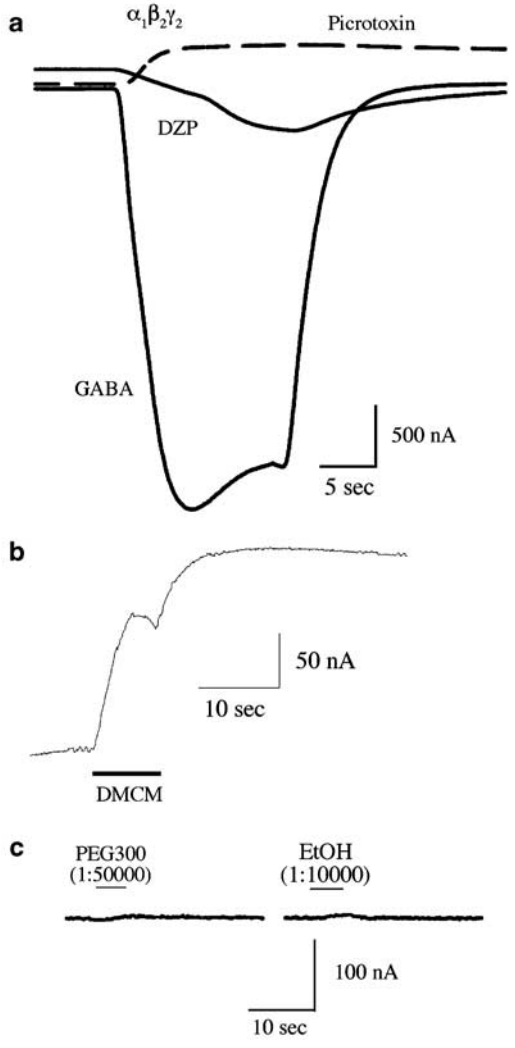


Figure 3 DZP directly activates $GABA_A$ receptors. (a) The current in response 1 μM GABA and 1 μM DZP in an oocyte expressing high levels of $GABA_A$ receptors are shown. With this high level of expression, we were unable to measure the maximum GABA-activated current. The dashed line is in response to 500 μM picrotoxin and indicates the population of spontaneously opening wild-type $\alpha\beta\gamma$ receptors predicted by Scheme 1 (Chang & Weiss, 1999). Similar results were obtained in 16 experiments. (b) DMCM (1 μM) reduced the baseline current consistent with a decrease in spontaneous opening. The notch on the falling phase of the current trace represents a modest activation that also occurs with this concentration of DMCM. Note the slow return toward baseline after DMCM removal, suggesting a high affinity for DMCM. (c) The traces at the bottom show the negative vehicle controls for DZP (PEG-300) and DMCM (ethanol). These dilutions would correspond to concentrations of 1 μM for DZP and DMCM.

Figure 3b shows wild-type $\alpha1\beta2\beta2$ GABA receptors exposed to 1 μM DMCM alone. In this case, the baseline current is decreased, consistent with a diminution in the spontaneous opening rate. Finally, Figure 3c shows the vehicle controls for DZP and DMCM. Taken together, the data thus far support opposing actions of DZP and DMCM at the benzodiazepine binding.

Prediction 3. Similar EC_{50}'s for potentiation and activation

If the modulation and direct activation by DZP were the same, as opposed to different mechanisms acting *via* different binding sites, then the EC_{50}'s for activation and modulation should be similar. To test this possibility, we compared the sensitivities of activation and modulation by DZP. The top row of current traces in Figure 4a are direct activation by various concentrations of DZP in wild-type $\alpha\beta\gamma$ receptors (ultrahigh expression). The bottom row of traces in Figure 4a are currents from DZP-mediated activation of $\alpha\beta Y205S\gamma$. This mutation is in the GABA binding site and results in a reduced sensitivity such that no GABA-mediated current can be detected at GABA concentrations as high as 20 mM (Amin & Weiss, 1993). In this case, however, we can still detect DZP-mediated currents with high expression. The resulting dose–response relationships for $\alpha\beta\gamma$ (filled circles) and $\alpha\beta Y205S\gamma$ (open circles) are plotted in Figure 4b. The EC_{50}'s for DZP-mediated activation were 72.0 ± 2.0 and 115.0 ± 6.2 nM for $\alpha\beta\gamma$ and $\alpha\beta Y205S\gamma$, respectively (Table 1). The shaded line in Figure 4b is the dose–response relationship for wild-type $\alpha\beta\gamma$ in the presence of 3 μM GABA and increasing concentrations of DZP taken from a previous study (Amin *et al*., 1997). In this case, the EC_{50} for modulation was 64.6 ± 3.7 nM, very close to that for direct activation in $\alpha\beta\gamma$ and $\alpha\beta Y205S\gamma$. These data support the hypothesis that DZP-mediated activation and modulation are through the same DZP binding site.

Discussion

The classic notion of how benzodiazepines, such as DZP, modulate GABA receptor function is *via* an allosteric mechanism (Del Castillo & Katz, 1957; Study & Barker, 1981; Twyman *et al*., 1989). In such a mechanism, DZP binds to a site distinct from that of GABA and enhances receptor sensitivity. Single channel studies extended this mechanism and proposed that DZP increases the opening rate of mono-liganded (GABA) receptors (Vicini *et al*., 1987; Twyman *et al*., 1989; Rogers *et al*., 1994; Lavoie & Twyman, 1996). The specifics of how DZP binding is coupled to the increase in GABA sensitivity have been unclear.

Since these earlier studies, structure–function studies of the GABA receptor have revealed domains and residues involved in ligand binding (Chang & Weiss, 2000). We, and others, initially identified several amino acids on the α1-subunit

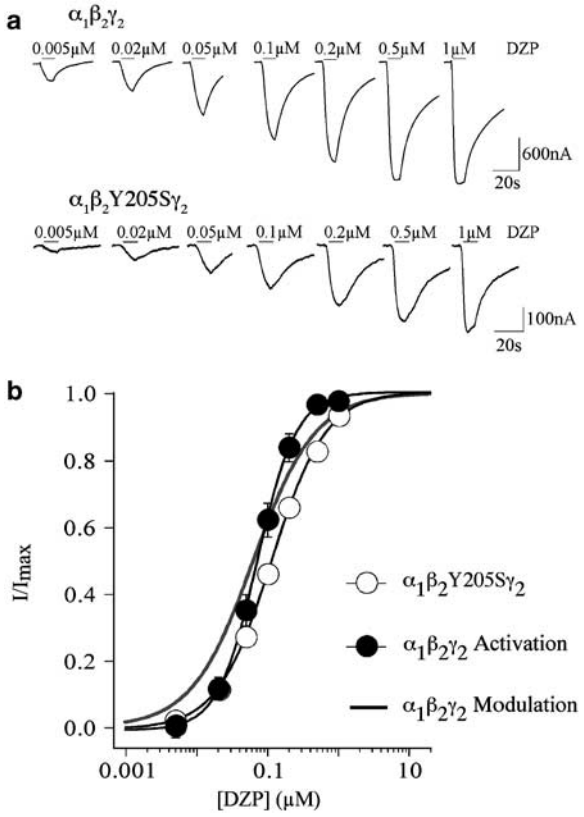
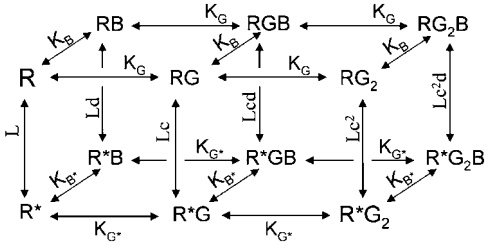


Figure 4 Comparison of the EC_{50} for the activation and modulation of wild-type and $\alpha\beta$Y205Sγ $GABA_A$ receptors. (a) The top row of traces are currents in response to increasing concentrations of DZP in oocytes expressing high levels of wild-type $\alpha\beta\gamma$ $GABA_A$ receptors. The bottom row of traces are currents in response to increasing concentrations of DZP for $\alpha\beta$Y205Sγ $GABA_A$ receptors. (b) The dose–response relationships were plotted for direct activation of $\alpha\beta\gamma$ and $\alpha\beta$Y205Sγ by DZP. The continuous lines are fits of Equation (1) and yielded similar EC_{50}'s of 72.0 ± 2.0 ($N=7$) and 115.0 ± 6.2 nM ($N=3$) for $\alpha\beta\gamma$ and $\alpha\beta$Y205Sγ, respectively. The gray continuous line plots the increase in GABA-mediated current for $\alpha\beta\gamma$ $GABA_A$ receptors in the presence of 3 μM GABA. In this case, the EC_{50} for DZP was 62.0 ± 3.4 nM ($N=24$), again, similar to that for direct activation by DZP.

extracellular domain that contribute to the DZP binding pocket (Pritchett & Seeburg, 1991; Amin *et al*., 1997; Boileau *et al*., 1998; Teissere & Czajkowski, 2001). Interesting to us at the time was the observation that these residues aligned with amino acids on the β2-subunit that contribute to GABA binding (Amin *et al*., 1997). A picture has emerged wherein the DZP binding site, presumably located at the α–γ interface, may be structurally homologous to the two GABA binding sites located at the two α–β interfaces (Smith & Olsen, 1995; Sigel & Buhr, 1997; Tretter *et al*., 1997; Jones-Davis *et al*., 2005).

An earlier study revealed that benzodiazepines have a dual effect on $\alpha1\beta2\gamma2$ $GABA_A$ receptors (Walters *et al*., 2000). One component of modulation was in the nanomolar range and required the presence of the γ-subunit. The second component was in the micromolar range and did not require the presence of the γ-subunit. The actions of benzodiazepines being investigated in the present study represent the presumed high-affinity component. Thus, 1 μM DZP (the highest concentration used here) is saturating for the high-affinity component, yet below the concentration necessary for the low-affinity component.

Structural evidence from the homologous muscle nACh receptor, as well as structure–function studies of the GABA receptor, indicate that agonist binding at subunit interfaces induces helix rotation (perhaps TM2) that leads to pore opening (Horenstein *et al*., 2001; Unwin *et al*., 2002; Miyazawa *et al*., 2003). In this model, two GABA molecules bind and impart a structural perturbation that is transferred to the other subunits or subunit interfaces. The structural perturbation imparted by DZP in this case, however, is much less efficient at opening the pore. Based on this presumed homology, we reasoned that DZP binding at its subunit interface may be acting in a similar mechanistic manner as GABA. Based on the results from this study, we would have to extend the gating mechanism for the $GABA_A$ receptor (Scheme 1 in the Introduction) to incorporate binding of both GABA and benzodiazepines as shown in Scheme 2 below.



Scheme 2

In this mechanism, K_G represents GABA binding affinities, whereas K_B represents benzodiazepine binding affinities. All other abbreviations are similar as that in Scheme 1 and described in our previous study of allosteric gating of the $GABA_A$ receptor (Chang & Weiss, 1999). While we do not have sufficient data to fully resolve the transition and binding rates in this scheme, we can make some useful generalizations related to the actions of GABA and DZP. According to our allosteric model (Chang & Weiss, 1999), a GABA receptor bound by a single GABA molecule has a fractional open time of 0.007, which is about 700-fold greater than the spontaneous opening rate. The binding of a second GABA molecule increases the fractional open time another 120-fold compared to the single-bound receptor. DZP, on the other hand, is 1/27,000 as efficacious as GABA. In this case, a single molecule of GABA is 225-fold more efficacious than a single molecule of DZP. Further support of this mechanism comes from cysteine scanning studies that suggest (1), structural rearrangements are induced by DZP binding alone and (2), these structural rearrangements share features with rearrangements imparted by the binding of GABA (Williams & Akabas, 2000). In addition, the Gibbs lab has published a model for BZDs based upon our previous allosteric activation mechanism (Chang & Weiss, 1999) and supported by direct activation of TM2 mutant $GABA_A$ receptors by bezodiazepines (Downing *et al*., 2005). However, the present study is the first to show direct activation of wild-type GABA receptor by benzodiazepines.

Our data do not allow us to distinguish between a mechanism where subunits are activated (gated) individually *versus* a mechanism where there is a concerted gating transition subsequent to agonist binding. Our working hypothesis, however, is the conceptually simpler concerted model. In this scenario, ligand binding (GABA and/or DZP) imparts a structural rearrangement at the agonist binding site that is then

transduced to the gating domain. The concerted opening transition occurs when there is a sufficient perturbation at the gate to overcome the energy barrier for channel opening.

In summary, we have postulated a mechanism for allosteric potentiation that is actually conceptually simpler than classic models that relied upon a coupling between the activation and modulation pathways leading to alterations in receptor affinity. In the present model, the increased sensitivity imparted by DZP comes about from a modest de-stabilization of the closed state of the receptor. In this scenario, DZP acts as a very weak partial agonist for the GABA receptor and acts at a site that is structurally comparable, yet physically distinct, from that of the GABA binding site. In addition to being a common mechanism for allosteric modulation of other receptors, these findings support the working hypothesis of an allosteric GABA-mediated activation mechanism. Classic linear activation mechanisms in which DZP enhances receptor affinity or stabilizes specific open states, could not account for spontaneous activity or DZP-mediated activation. Furthermore, that GABA receptors can sample the conformational space in the absence of agonist may have analogies in enzyme catalysis where it has recently been documented that cyclophillin A can undergo similar motions, and at similar rates, in either the presence or absence of substrate (Eisenmesser *et al*., 2005). Thus, as is true for the GABA receptor, the conformational changes necessary for function are an intrinsic property of the protein.

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