Benzodiazepines act on $GABA_A$ receptors via two distinct and separable mechanisms

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Benzodiazepines (BZs) act on γ-aminobutyric acid type A ($GABA_A$) receptors such as $\alpha_1\beta_2\gamma_2$ through key residues within the N-terminal region of α subunits, to render their sedative and anxiolytic actions. However, the molecular mechanisms underlying the BZs' other clinical actions are not known. Here we show that, with low concentrations of GABA, diazepam produces a biphasic potentiation for the $\alpha_1\beta_2\gamma_2$-receptor channel, with distinct components in the nanomolar and micromolar concentration ranges. Mutations at equivalent residues within the second transmembrane domains (TM_2) of α, β and γ subunits, proven important for the action of other anesthetics, abolish the micromolar, but not the nanomolar component. Converse mutation of the corresponding TM_2 residue and a TM_3 residue within ρ_1 subunits confers diazepam sensitivity on homo-oligomeric ρ_1-receptor channels that are otherwise insensitive to BZs. Thus, specific and distinct residues contribute to a previously unresolved component (micromolar) of diazepam action, indicating that diazepam can modulate the $GABA_A$-receptor channel through two separable mechanisms.

Sedative-hypnotic drugs such as diazepam (a benzodiazepine) can impose graded effects on the function of the central nervous system (CNS), resulting in a spectrum of clinical actions. This range of therapeutic actions progresses from sedation at low doses, to induction of anesthesia at significantly higher doses. BZs act at nanomolar concentrations to modulate chloride ion flux through $GABA_A$-receptor channels, thereby affecting synaptic transmission[1–3]. The crucial amino acids responsible for this potent action of BZs in part have been determined, and are located within the N-terminal domains of α and γ subunits[1–16]. The importance of two α subunit isoforms (α_1 or α_2) in mediating sedative or anxiolytic action of diazepam, respectively, has been demonstrated using transgenic animals[17–20].

Certain BZs such as diazepam, midazolam and lorazepam are also known for their anesthetic properties[21] and are routinely used in surgical procedures. However, in comparison to the extensive studies on the high-affinity action of BZs, little is known about the molecular determinants of the anesthetic action of BZs.

In this study, we have demonstrated that diazepam, in the presence of low GABA concentrations, produces two distinct components of potentiation within the nanomolar (nM) and micromolar (μM) concentration ranges for the $\alpha_1\beta_2\gamma_2$-receptor channel. In contrast to the nM component, the μM component of diazepam action is independent of the γ_2 subunit and is insensitive to the selective BZ antagonist flumazenil. Mutations at equivalent residues within the TM_2 of α, β and γ subunits, proven important for the action of other anesthetics, nullify the μM component without significantly affecting the nM component of response to diazepam. Furthermore, the μM action of diazepam can also be conferred upon the BZ-insensitive ρ_1-receptor channel by converse mutation of the corresponding TM_2 residue and a TM_3 residue. The physiological significance of the μM action of diazepam is discussed in the light of the dynamics of benzodiazepine concentrations, the persistence of low concentrations of GABA and the tonic level of $GABA_A$ receptor activity within the CNS.

Results

Biphasic potentiation of $\alpha_1\beta_2\gamma_2$ by diazepam

Complementary RNA (cRNA) from rat wild-type α_1, β_2 and γ_2 (γ_{2s})[22] subunits were co-injected into *Xenopus laevis* oocytes. Three days after injection, GABA-elicited currents were recorded in the absence and presence of a broad range of diazepam concentrations (2 to 150,000 nM) using the two-electrode voltage-clamp technique. Figure 1a and b depicts current traces and the concentration–response relationship for diazepam at EC_3, EC_8 and EC_{16} (effective concentration giving rise to 3%, 8% and 16% of the maximal GABA current, respectively) GABA for the $\alpha_1\beta_2\gamma_2$-receptor channel. These data reveal two different patterns of response to diazepam, which are dependent on the GABA concentration. At all three GABA concentrations tested, diazepam produced a two- to three-fold potentiation of the GABA-evoked currents, with EC_{50} values between 32 and 54 nM, and a Hill coefficient approaching unity (nM component, **Table 1**). On the other hand, at EC_3 and EC_8 GABA, μM concentrations of diazepam (20 μM and above) evoked a second component of potentiation, further increasing GABA-elicited currents at EC_3 from three-fold (the nM component) to approximately eight-fold. At EC_3 GABA, the μM component of diazepam had an apparent EC_{50} of 63 ± 8.5 μM and a notably greater Hill coefficient (3.04 ± 0.37) than that of the nM component (**Table 1**). The extent of the μM diazepam-induced potentiation was markedly dependent upon the GABA concentration;

Table 1. Parameters obtained from fitting the Hill equation to GABA, diazepam and flurazepam data.

Subunit	Fractional potentiation	EC_{50} (μM)	Hill number	*n**
GABA-dependent activation				
$\alpha_1\beta_2\gamma_2$	–	43.9 ± 2.37	1.35 ± 0.07	12
$\alpha_1\beta_2$	3.52 ± 0.72	1.24 ± 0.15	6	
$\alpha_{S267I}\beta_{N265I}\gamma_{S280I}$	–	1.13 ± 0.17	1.57 ± 0.27	19
ρ_1	1.0 ± 0.15	2.02 ± 0.41	21	
ρ_{W328M}†	–	1.31 ± 0.30	2.39 ± 0.15	6
ρ_{I307S}	–	0.257 ± 0.006	3.32 ± 0.10	6
$\rho_{I307S/W328M}$	–	0.095 ± 0.003	3.45 ± 0.08	4
Diazepam potentiation of GABA receptor current				
$\alpha_1\beta_2\gamma_2$ GABA EC_3				
nM component	3.30 ± 0.22	0.032 ± 0.008	1.26 ± 0.32	4
μM component	4.88 ± 0.18	63 ± 8.5	3.04 ± 0.37	4
$\alpha_1\beta_2\gamma_2$ GABA EC_8				
nM component	1.99 ± 0.28	0.042 ± 0.003	1.38 ± 0.19	6
μM component	1.33 ± 0.15	51 ± 3.9	‡	6
$\alpha_1\beta_2\gamma_2$ GABA EC_{16}	1.91 ± 0.22	0.054 ± 0.021	1.00 ± 0.15	5
$\alpha_1\beta_2$ GABA EC_3	14 ± 1.65	46.2 ± 2.5	2.05 ± 0.10	5
$\alpha_1\beta_2\gamma_2$ GABA EC_3 + 10 μM Zn^{+2}				
nM component	3.44 ± 0.26	0.048 ± 0.003	1.23 ± 0.05	4
μM component	4.34 ± 0.26	60.5 ± 4.7	2.97 ± 0.25	4
$\alpha_1\beta_2\gamma_2$ GABA EC_3 +20 μM flumazenil	3.31 ± 0.59	81.2 ± 0.51	2.43 ± 0.24	4
$\alpha_{S267I}\beta_{N265I}\gamma_{S280I}$ GABA EC_3§	2.28 ± 0.35	0.032 ± 0.004	0.81 ± 0.04	6
$\alpha_{S267I}\beta_{N265I}\gamma_{S280I}$ GABA EC_8§	1.39 ± 0.035	0.041 ± 0.005	1.00 ± 0.07	3
$\alpha_{S267I}\beta_{N265I}\gamma_{S280I}$ GABA EC_{16}§	1.10 ± 0.083	0.055 ± 0.005	1.08 ± 0.08	5
Diazepam direct activation				
$\rho_{I307S/W328M}$	–	93.0 ± 1.9	3.3 ± 0.18	4
Flurazepam potentiation				
$\alpha_1\beta_2\gamma_2$ GABA EC_3#	2.65 ± 0.10	0.50 ± 0.10	0.89 ± 0.06	4

* Number of individual oocytes tested. †Data obtained from ref. 28. ‡Within the μM concentration range, more data points needed to obtain a reliable Hill coefficient. §Hill equation was fit to the data points up to the 5 μM concentration. #Hill equation was fit to the data points up to the 20 μM concentration.

potentiation was diminished significantly at EC_8 GABA, and eventually abolished at EC_{16} GABA.

Monophasic potentiation of $\alpha_1\beta_2$ by diazepam

Co-expression of cRNA for α_1 and β_2 subunits also yielded GABA-gated receptor channels. The $\alpha_1\beta_2$-receptor channel can be distinguished from the $\alpha_1\beta_2\gamma_2$-receptor channel due to $\alpha_1\beta_2$'s unique pharmacological and kinetic characteristics. For example, the $\alpha_1\beta_2$-receptor channel is more sensitive to GABA in comparison to the $\alpha_1\beta_2\gamma_2$-receptor channel (**Table 1**). Figure 1c and d shows the diazepam concentration–response relationship (10 to 150,000 nM) at EC_3 GABA for the $\alpha_1\beta_2$-receptor channel. Diazepam elicited only a single component of potentiation within the μM concentration range for the $\alpha_1\beta_2$-receptor channel, increasing the control EC_3 GABA currents by more than twelve-fold (**Fig. 1c** and **d**). Furthermore, the EC_{50} and the Hill coefficient values for the μM component of diazepam action were similar for both $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_2$-receptor channels (at EC_3 GABA, **Table 1**). The lack of $\alpha_1\beta_2$-receptor channel sensitivity to diazepam within the nM concentration range has been documented previously, as the presence of the γ subunit is essential in conferring diazepam's nM action[11].

Zn^{2+} does not block the μM action of diazepam

Zinc inhibits the GABA-evoked currents from $\alpha_1\beta_2$- but not from $\alpha_1\beta_2\gamma_2$-receptor channels[23,24] (**Fig. 2**). Zinc exhibited properties of an open channel blocker for $\alpha_1\beta_2$, inhibiting the GABA-evoked currents back to the baseline. In comparison, the GABA currents for $\alpha_1\beta_2\gamma_2$-receptor channels were reduced to 64 ± 4% of the control in the presence of 10 μM Zn^{2+}, suggesting that a fraction of the total GABA current may have arisen from $\alpha_1\beta_2$-type-receptor channels. To confirm that the μM potentiation of $\alpha_1\beta_2\gamma_2$ at EC_3 GABA was not due to the presence of $\alpha_1\beta_2$-receptor channels (see above), the $\alpha_1\beta_2\gamma_2$ diazepam concentration–response relationship at EC_3 GABA was determined in the presence of 10 μM Zn^{2+} (**Fig. 2b**). In these experiments, the characteristics of $\alpha_1\beta_2\gamma_2$'s biphasic potentiation by diazepam were unaltered in the presence of Zn^{2+} (**Table 1**). For the nM and the μM components, the EC_{50}/Hill coefficient values obtained in the presence of Zn^{2+} were 0.048 ± 0.003/1.23 ± 0.05 and 60.5 ± 4.7/2.97 ± 0.25, respectively. Moreover, the absolute magnitudes of potentiation for the two components were 3.44 ± 0.26 for the nM and 4.34 ± 0.26 for the μM component of diazepam action. These values are similar to the values determined for the $\alpha_1\beta_2\gamma_2$-receptor channel in the absence of Zn^{2+} (**Table 1**). These data indicate that the significant potentiation observed at μM concentration of diazepam for $\alpha_1\beta_2\gamma_2$ (at EC_3 GABA) does not arise from the concomitant presence of $\alpha_1\beta_2$-receptor channels.

The μM action of diazepam is insensitive to flumazenil

Flumazenil is a selective antagonist for the high-affinity (nM) com-

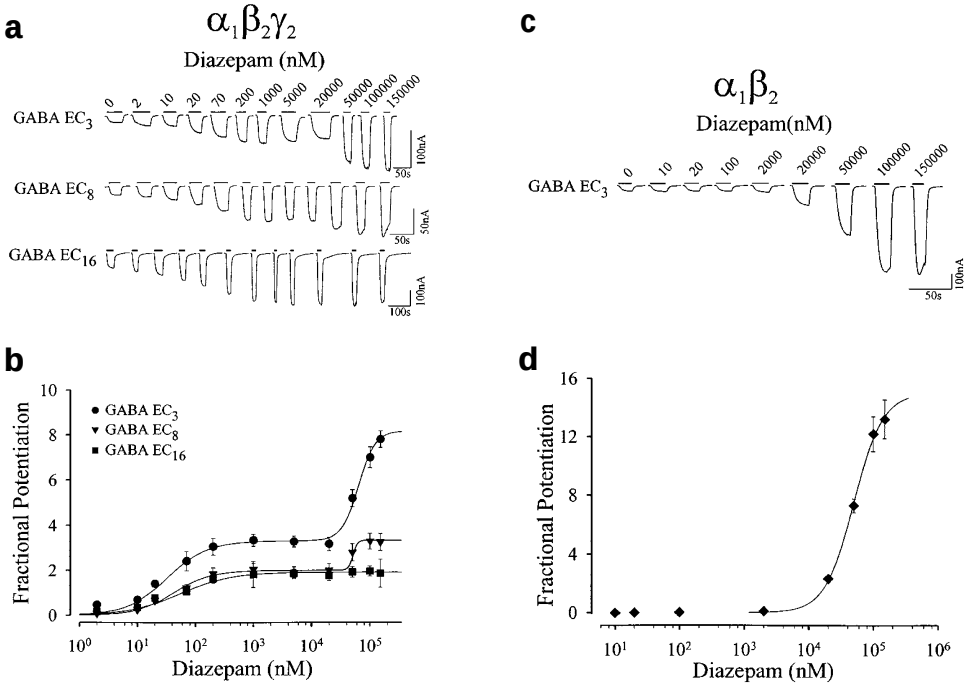
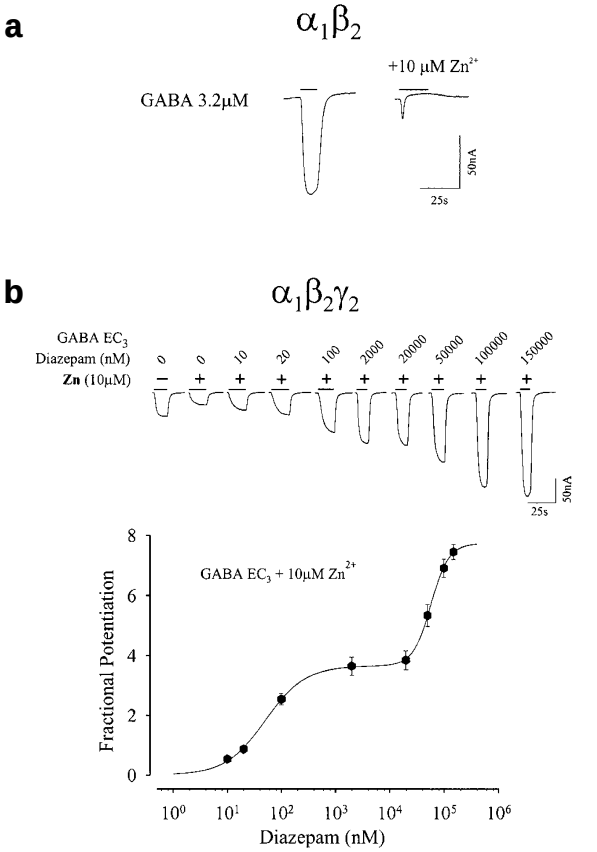


Fig. 1. Diazepam modulation of wild type $\alpha_1\beta_2\gamma_2$- or $\alpha_1\beta_2$-receptor channels. (**a**, **b**) The current traces and concentration–response relationship for diazepam at EC_3 (3.2 μM), EC_8 (4.6 μM) and EC_{16} (7.75 μM) GABA for the $\alpha_1\beta_2\gamma_2$-receptor channel. The presence of the diazepam μM component of potentiation is highly dependent on GABA concentration. (**c, d**) The diazepam concentration–response relationship (10 to 150,000 nM) and current traces at EC_3 GABA (0.2 μM) for the $\alpha_1\beta_2$-receptor channel. Diazepam at μM concentrations (but not at nM concentrations) elicited a significant potentiation for the $\alpha_1\beta_2$-receptor channel.

ponent of BZ action upon the $GABA_A$-receptor channel[25]. For the $\alpha_1\beta_2\gamma_2$-receptor channel at EC_3 GABA, flumazenil at concentrations of 20 μM (or 10 μM, data not shown) abolished the nM, but not the μM, component of diazepam action (**Fig. 3**). The overall potentiation at the μM component was reduced from approximately 8 to 3.31 ± 0.59. However, the EC_{50} and the Hill coefficient values derived for the diazepam μM component in the presence and absence of flumazenil were similar for the $\alpha_1\beta_2\gamma_2$-receptor channel at EC_3 GABA (**Table 1**). Concentrations of flumazenil up to 100 μM were co-applied with 100 μM diazepam (at EC_3 GABA) to test the effect of flumazenil at equivalent concentrations on μM potentiation by diazepam. In the presence of such high concentrations of flumazenil, 100 μM diazepam also produced a significant potentiation (data not shown). These experiments suggest that the nM and μM actions of diazepam are mediated through distinct mechanisms.



TM_2 mutations abolished the μM action of diazepam

The diazepam concentration–response relationships for $\alpha_{S267I}\beta_{N265I}\gamma_{S280I}$ at EC_3, EC_8 and EC_{16} GABA were next determined. At all three GABA concentrations tested, diazepam produced only a single component of potentiation within the nM concentration range (**Fig. 4b**). The EC_{50} and Hill coefficient values derived for $\alpha_{S267I}\beta_{N265I}\gamma_{S280I}$ (~32 to 55 nM and 1.1 to 0.8 nM, respectively; **Table 1**) were similar to the values determined for the nM component of wild-type $\alpha_1\beta_2\gamma_2$ (**Table 1**). The $\alpha_{S267I}\beta_{N265I}\gamma_{S280I}$-receptor channel experiments further demonstrate that the nM and the μM actions of diazepam are distinct and separable.

In comparison to the $\alpha_1\beta_2\gamma_2$,-receptor channel, the $\alpha_{S267I}\beta_{N265I}\gamma_{S280I}$-receptor channel exhibited an approximately 40-fold higher sensitivity to GABA (**Table 1**). This difference in

Fig. 2. Diazepam biphasic potentiation of the $\alpha_1\beta_2\gamma_2$-receptor channel is unaltered in the presence of zinc. (**a**, **b**) Differential effect of Zn^{2+} (10 μM) on the $\alpha_1\beta_2$- versus $\alpha_1\beta_2\gamma_2$-receptor channel. (**a**) The GABA-evoked currents (3.2 μM) for the $\alpha_1\beta_2$-receptor channel are completely blocked by 10 μM Zn^{2+}. (**b**) The current traces for the diazepam concentration–response relationship for the $\alpha_1\beta_2\gamma_2$-receptor channel in the presence of Zn^{2+} (10 μM). Although Zn^{2+} partially blocks the GABA-evoked currents for the $\alpha_1\beta_2\gamma_2$-receptor channel (see current traces in **b**), it does not significantly affect the biphasic potentiation of the $\alpha_1\beta_2\gamma_2$-receptor channel by diazepam at EC_3 GABA.

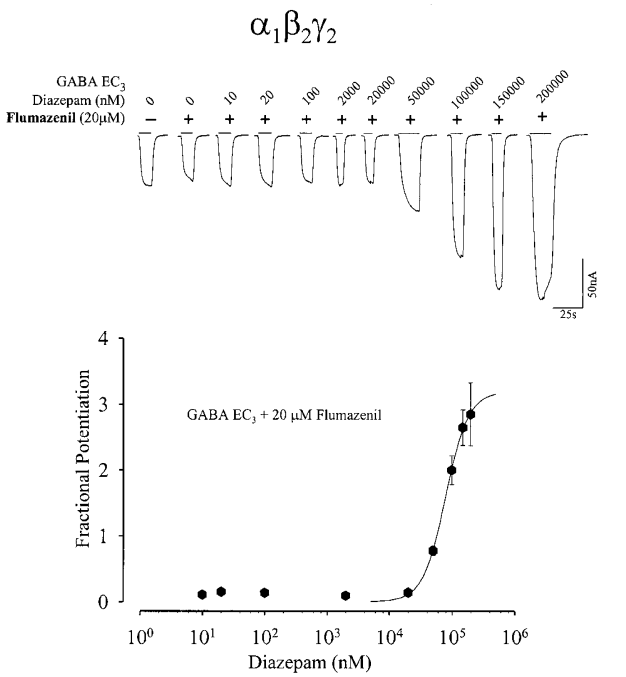


Fig. 3. Flumazenil abolishes the nM but not the μM component of diazepam action upon the $\alpha_1\beta_2\gamma_2$-receptor channel. Top, current traces for diazepam modulation of GABA currents in the presence of 20 μM flumazenil. The nM but not the μM component of diazepam action is eliminated by the co-application of flumazenil (bottom).

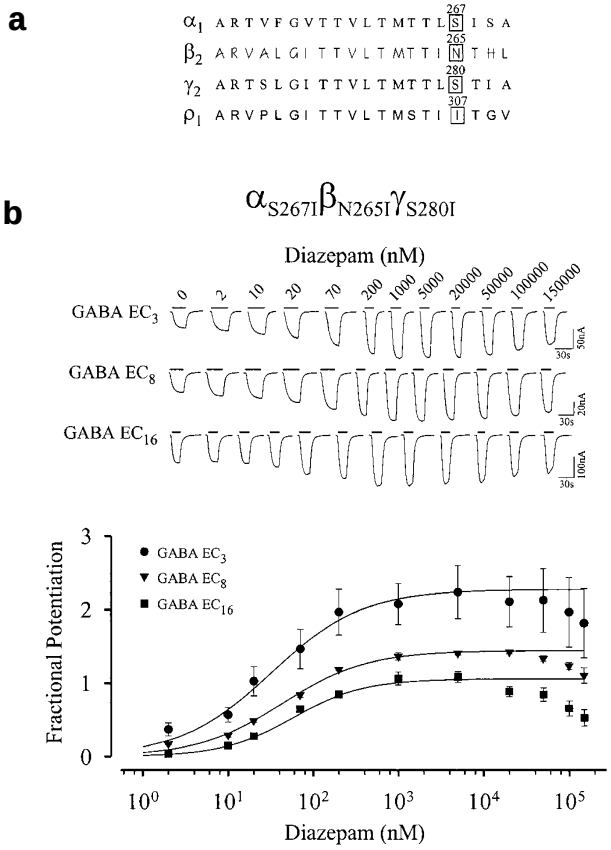


Fig. 4. Diazepam modulation of the $\alpha_{S267I}\beta_{N265I}\gamma_{S280I}$-receptor channel. (**a**) Amino-acid sequence comparison for the TM_2 domains of α_1, β_2, γ_2 and ρ_1 subunits. The mutated residues are shown in boxes. (**b**) The current traces and the concentration–response relationship for diazepam, for the $\alpha_{S267I}\beta_{N265I}\gamma_{S280I}$-receptor channel at EC_3 (0.09 μM), EC_8 (0.17 μM) and EC_{16} (0.35 μM) GABA. At all three GABA concentrations examined, diazepam produced a single component of potentiation occurring within the nM concentration range. The line is derived from the fit of the Hill equation to the data up to 5 μM diazepam.

sensitivity indicates the importance of these TM_2 residues in the GABA-dependent modulation of the $\alpha_1\beta_2\gamma_2$-receptor channel.

Imparting diazepam sensitivity to ρ_1-receptor channels

ρ_1-receptor channels are insensitive to BZs and other intravenous anesthetics[26] (excepting neuroactive steroids[27]). This property of the homo-oligomeric ρ_1-receptor channel has been used to study the molecular basis of anesthetic action by conferring anesthetic sensitivity upon ρ_1. For example, the specific mutation of residue 307 within TM_2 or residue 328 within TM_3 of the ρ_1 subunit (**Fig. 5a**) can impart pentobarbital sensitivity upon the ρ_1-receptor channel[28,29]. Within the ρ_1 subunit, residues 307 and 328 were substituted, either singly or in combination, to their amino acid counterparts present within the α_1 (Ser, TM_2) and β_2 (Met, TM_3) subunits. Diazepam did not modulate the GABA-elicited currents at EC_4 in wild-type ρ_1-, ρ_{I307S}- and ρ_{W328M}- receptor channels (**Fig. 5b**). In contrast, doubly mutated ρ_1 became highly sensitive to diazepam (**Fig. 5c** and **d**). The EC_4 GABA currents for $\rho_{I307S/W328M}$ were markedly potentiated in the presence of diazepam, resulting in an increase of GABA currents up to 9-fold (20 μM diazepam). Figure 5e and f show the potentiation of GABA currents by diazepam (10 μM) at EC_1, EC_4, EC_{50} and EC_{99} GABA for ρ_1, ρ_{I307S}, ρ_{W328M} and $\rho_{I307S/W328M}$ mutants. For $\rho_{I307S/W328M}$, diazepam potentiation was most prominent at GABA concentrations below the EC_{50} value. The fractional potentiation by diazepam (10 μM) was greatest at low GABA concentrations (EC_1 5.40 ± 0.50 and EC_4 3.20 ± 0.50), whereas at higher GABA concentrations (EC_{99}), little potentiation was observed. In comparison, ρ_1, ρ_{W328M} and ρ_{I307S} did not respond to diazepam at any of the GABA concentrations tested.

The co-application of 20 μM flumazenil and 20 μM diazepam at EC_4 GABA did not significantly alter the diazepam-induced potentiation of the GABA currents elicited from $\rho_{I307S/W328M}$ (data not shown). These data indicate that the μM action of diazepam on $\rho_{I307S/W328M}$ and its nM action on the $\alpha_1\beta_2\gamma_2$-receptor channel are intrinsically different.

Diazepam at higher concentrations is an agonist for $\rho_{I307S/W328M}$ (**Fig. 6**). The EC_{50} value for the direct action of diazepam was 93 ± 1.9 μM associated with a high Hill coefficient (3.3 ± 0.18, **Table 1**). Diazepam was highly efficacious relative to GABA, eliciting a relative maximum current of 0.8. In comparison, wild-type ρ_1 and singly mutated ρ_1 were not directly activated by diazepam at concentrations up to 300 μM.

Differential sensitivity of $\rho_{I307S/W328M}$ to different BZs

The structure–function relationships of different BZs were assessed using the $\rho_{I307S/W328M}$ model. Figure 7 shows the current traces and the relative efficacies of diazepam, midazolam, flunitrazepam and flurazepam in potentiating GABA responses (EC_4) of the $\rho_{I307S/W328M}$-receptor channel. Diazepam was the most efficacious among the BZs tested. Nevertheless, midazolam and flunitrazepam also elicited a marked potentiation, increas-

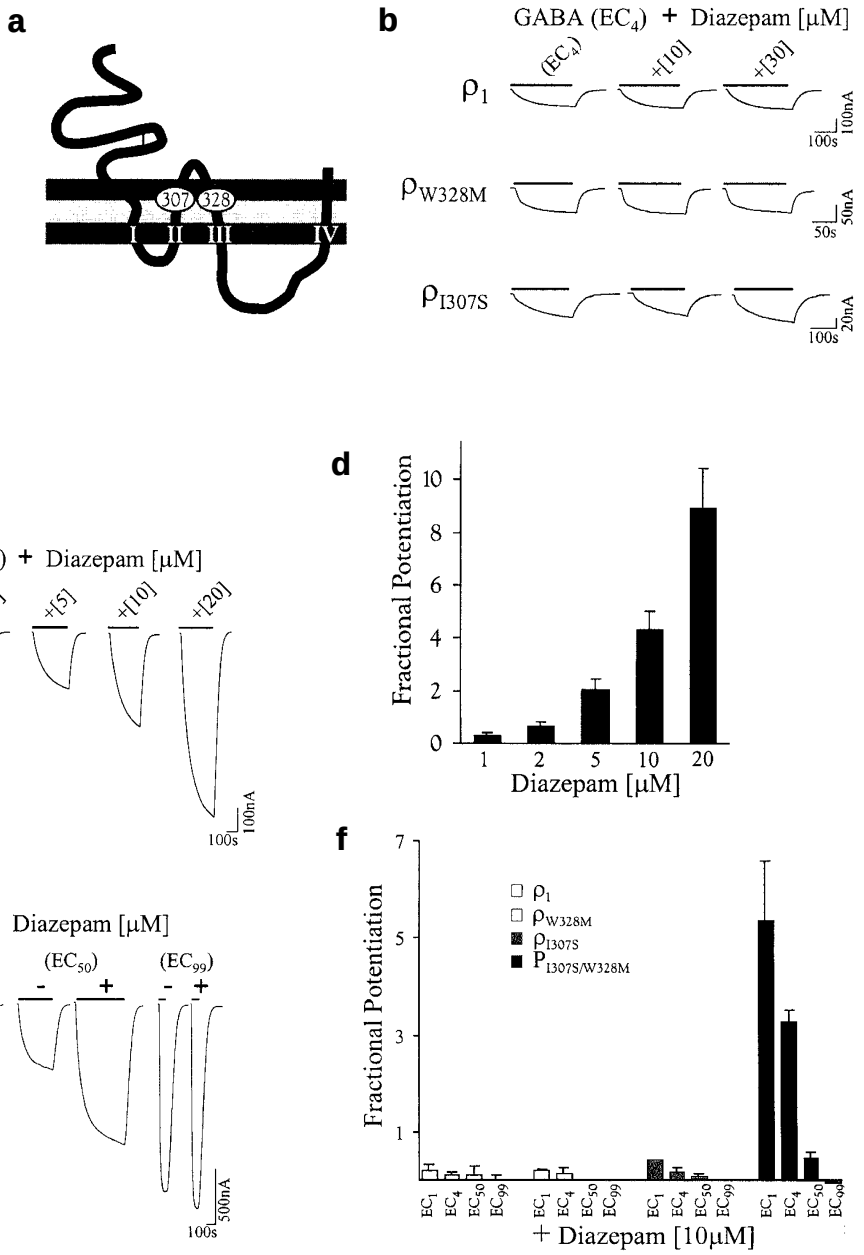


Fig. 5. Imparting diazepam sensitivity to the ρ_1-receptor channel by double mutation. (**a**) The position of the crucial amino acids within the putative second and third transmembrane domains. (**b**) GABA-evoked currents at EC_4 from ρ_1 (0.27 μM GABA), ρ_{W328M} (0.35 μM GABA) or ρ_{I307S} (0.11 μM GABA) are insensitive to diazepam concentrations up to 30 μM. (**c**, **d**) GABA currents (EC_4 = 0.04 μM) from the $\rho_{I307S/W328M}$ are markedly potentiated by μM concentrations of diazepam. (**e**, **f**) Potentiation of GABA currents by diazepam (10 μM) at EC_1, EC_4, EC_{50} and EC_{99} GABA for ρ_1, ρ_{I307S}, ρ_{W328M} and $\rho_{I307S/W328M}$ mutants. The potentiation of the $\rho_{I307S/W328M}$-receptor channel is dependent on the GABA concentrations. ρ_1, ρ_{W328M} and ρ_{I307S} do not respond to diazepam across the range of GABA concentrations tested (relative to their EC_{50} values).

ing GABA currents by approximately 0.5-fold and 3-fold at 5 and 20 μM concentrations, respectively. In contrast, flurazepam at concentrations up to 20 μM did not significantly alter the GABA-evoked currents from the $\rho_{I307S/W328M}$-receptor channel.

At EC_3 GABA, the flurazepam concentration–response relationship was determined for the wild-type $\alpha_1\beta_2\gamma_2$-receptor channel to examine whether flurazepam could also produce a biphasic potentiation of GABA currents similar to that observed for diazepam (**Fig. 8**). In contrast to diazepam, flurazepam elicited only a single component of potentiation with an EC_{50} of 501 ± 96 nM and a Hill coefficient of 0.89 ± 0.06 (**Table 1**). Thus, at μM concentration, flurazepam neither produced a μM component of potentiation for $\alpha_1\beta_2\gamma_2$, nor augmented the GABA-evoked current from the $\rho_{I307S/W328M}$-receptor channel. Diazepam and midazolam, which possess a μM action upon $\rho_{I307S/W328M}$, are used routinely to induce and maintain anesthesia on their own, but flurazepam is not[21].

However, at higher concentrations (20 μM and above), flurazepam produced a significant inhibition of the current (**Fig. 8**) similar to the inhibitory effect observed at equivalent concentrations of diazepam for $\alpha_{S267I}\beta_{N265I}\gamma_{S280I}$ (**Fig. 4**) and wild-type $\alpha_1\beta_2\gamma_2$ (between 5 and 20 μM, **Fig. 1**). Previous studies have shown that impairing the high-affinity (nM) component of diazepam action within the $\alpha_1\beta_2\gamma_2$ by mutation of a single residue within the N-terminal domain of the α subunit causes the μM component to become more prominent[5]. On the other hand, eliminating the diazepam μM component (or its absence in the case of flurazepam) seems to reveal an inhibitory phase (which is otherwise masked by μM concentrations of diazepam; **Figs. 4** and **8**, respectively). The co-existence of an inhibitory phase associated with the nM component, as well as the apparent absence of an inhibitory phase following the abolition of the nM component[5], suggests a close association between the diazepam inhibitory action and the nM component. BZs can

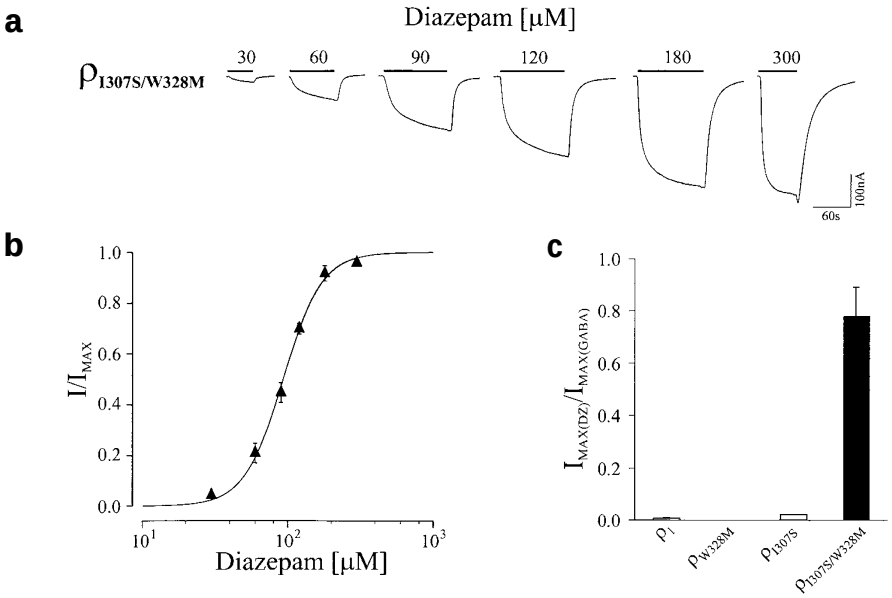


Fig. 6. The diazepam concentration–response relationship, and diazepam's relative efficacy with respect to GABA in its direct activation of $\rho_{I307S/W328M}$. Diazepam is an agonist for the $\rho_{I307S/W328M}$-receptor channel. (**a**) Current traces showing the direct activation of $\rho_{I307S/W328M}$ by 30 to 300 μM diazepam. (**b**) Concentration–response relationship for diazepam. Diazepam activation of the $\rho_{I307S/W328M}$-receptor channel appears to be highly cooperative (high Hill coefficient, **Table 1**). (**c**) Diazepam was highly effective relative to GABA (current magnitude at 300 μM diazepam divided by current magnitude at 10 μM GABA; EC_{50} GABA for $\rho_{I307S/W328M}$, 0.095 ± 0.003 μM) for direct activation of the $\rho_{I307S/W328M}$-receptor channel. In comparison, diazepam alone, at concentrations up to 300 μM, did not evoke a significant response in either wild-type or singly mutated ρ_I.

enhance the desensitization of GABA currents[30,31]. It is thus possible that the inhibitory action observed here is a reflection of this induced desensitization upon the GABA current.

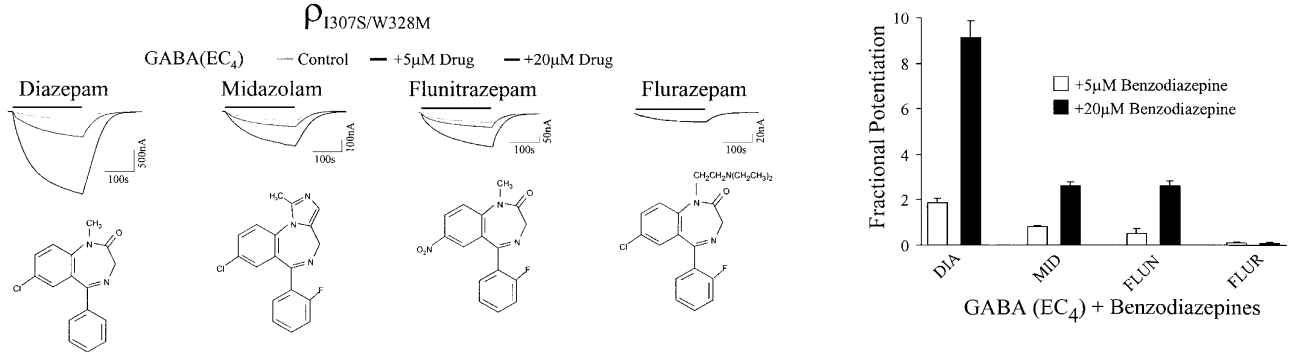
Discussion

The nM and the μM components of diazepam action within the $\alpha_1\beta_2\gamma_2$-receptor channel have intrinsic differences. First, the nM component of diazepam action depends on the presence of the γ subunit. However, both $\alpha_1\beta_2$- and $\alpha_1\beta_2\gamma_2$-receptor channels are sensitive to μM concentrations of diazepam. Second, for the $\alpha_1\beta_2\gamma_2$-receptor channel, flumazenil inhibits the nM but not the μM component of potentiation by diazepam. Third, there is a difference of three orders of magnitude in potency between the two components. Fourth, mutation of specific TM_2 residues within the α_1, β_2 and γ_2 subunits abolishes the μM but not the nM effect of diazepam. Fifth, the action of diazepam in eliciting the μM component of both $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2$ receptors appears highly cooperative as inferred from the Hill coefficient. Diazepam's direct action upon the $\rho_{I307S/W328M}$-receptor channel and pentobarbital's direct action on ρ_{W328M} (ref. 28) also have a high Hill coefficient (**Table 1**), whereas the nM component persistently displays a low cooperativity (Hill coefficient ~1). Sixth, there is a correlation between lipid solubility and the extent of the μM potentiation for the BZs tested. For example, diazepam, which has the highest octanol:buffer partition coefficient[32] among the BZs tested, has the greatest efficacy for the $\rho_{I307S/W328M}$-receptor channel. In comparison, no such relationship has been shown for the nM component. These differences provide strong evidence that the nM and the μM component of diazepam action are mediated via distinct and separable mechanisms.

The aforementioned transmembrane residues (α_{S267} and β_{M286} and the equivalent residues within ρ_1) are centrally important for a number of anesthetic compounds[28,29,33–37]. Also, mutation of the equivalent residue (β_{N265}) within human β_2 or β_3 subunits (to a Ser) of $\alpha\beta\gamma$ is sufficient to impair the action of loreclezole, a broad-spectrum anticonvulsant that acts at μM concentrations[38]. It is intriguing that so many structurally diverse and hydrophobic compounds mediate their action through these common residues.

The prevalent activation scheme for $GABA_A$ receptors postulates two open states[39]. In this scheme, one open state is derived from receptor channels occupied by a single GABA, whereas the other open state arises from doubly bound receptor channel.

Fig. 7. The relative effectiveness of diazepam, midazolam, flunitrazepam and flurazepam in potentiating the GABA responses (EC_4) of the $\rho_{I307S/W328M}$-receptor channel. Diazepam is the most effective among the benzodiazepines tested. In comparison, flurazepam up to 20 μM concentration did not potentiate the GABA-evoked currents for the $\rho_{I307S/W328M}$-receptor channel.



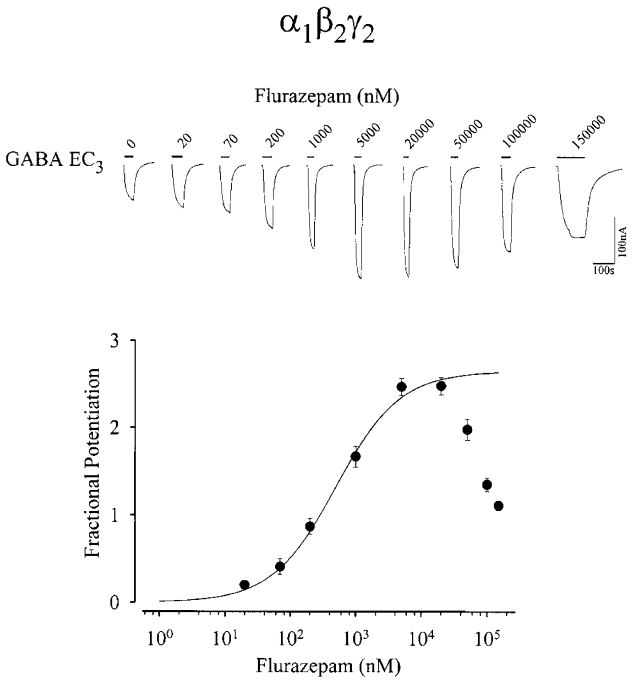


Fig. 8. Flurazepam elicits only a single component of potentiation within the nM concentration range. Note that the flurazepam induces inhibition at μM concentrations. The line is derived from the fit of the Hill equation to the data up to 20 μM flurazepam.

From the simple law of mass action, the proportion of the singly-bound form of $\alpha_1\beta_2\gamma_2$-receptor channels may predominate at EC_3 and EC_8 GABA. Thus, the μM component of potentiation by diazepam could be the result of selective enhancement of currents from $\alpha_1\beta_2\gamma_2$-receptor channels, which are present in a mono-liganded state.

It has been suggested that the incremental CNS effects of benzodiazepines, from sedation to the induction of anesthesia, are all consequences of the relative occupancy of the benzodiazepine nM receptor. We postulate that the more marked actions of benzodiazepine upon the CNS, such as induction of anesthesia, could be the result of its μM action upon $GABA_A$-receptor channels. Three observations suggest this view. First, the nM action of a benzodiazepine such as diazepam is dependent upon the isoform of the α subunit expressed as well as the presence of the γ subunit, and therefore, its nM activity is limited to only certain subtypes of $GABA_A$ receptors. On the other hand, diazepam's μM action seems less specific (since both $\alpha_1\beta_2$- and $\alpha_1\beta_2\gamma_2$-receptor channels are sensitive to μM concentrations of diazepam) and thus may occur at all $GABA_A$ receptors. Second, diazepam produces the second component of potentiation only at low concentrations of GABA. Within the CNS, a low extracellular concentration of GABA has been documented[40,41]. In addition, a tonic level of functional $GABA_A$ channel activity in a number of CNS regions has been shown[42,43]. Third, concentrations of benzodiazepines can reach μM levels within the plasma[44], and given their high lipid solubility, their concentrations can reach even higher levels within the CNS[45]. Finally, the additional chloride flux due to the μM effect upon $GABA_A$-receptor channels can be predicted to dramatically increase diazepam's overall CNS depressant action. Therefore, under circumstances in which a large dose of diazepam is administered, the marked and ubiquitous action of diazepam at μM concentrations may be central in producing a state of general CNS depression, such as the induction of anesthesia.

Methods

Oligonucleotide-mediated site-directed mutagenesis was done according to the manufacturer's protocol (Altered Sites, Promega, Madison, Wisconsin). Successful mutagenesis was verified by DNA sequencing. The cDNAs were linearized with *Hpa*I (for ρ_1) or *Ssp*I (for α_1,β_2, or γ_2 subunits) leaving a 3′ tail several hundred base pairs long. These additional 3′ sequences may increase cRNA stability within the oocyte. The cRNA was transcribed from the linearized cDNAs by standard *in vitro* transcription procedures (Megascript, Ambion, Austin, Texas).

Xenopus laevis (Xenopus I, Ann Arbor, Michigan) were anesthetized by hypothermia, and oocytes were surgically removed and placed in oocyte Ringer (OR_2) that consisted of 82.5 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 50 U/ml penicillin, 50 μg/ml streptomycin, pH 7.5. Oocytes were dispersed and then incubated in OR_2 without Ca^{2+} plus 0.3% collagenase A (Boehringer Mannheim, Indianapolis, Indiana) for approximately 1 h. After isolation, the oocytes were thoroughly rinsed with OR_2, and stage VI oocytes were separated and maintained at 18°C.

Micropipettes for injecting cRNA were made on a Sutter P87 puller (Sutter Instrument Company, Novato, California), and the tips were cut with microscissors. The cRNA in diethylpyrocarbonate-treated water was drawn up into the micropipette with negative pressure and then injected into the oocytes by applying positive pressure using a Picospritzer II (General Valve Corporation, Fairfield, New Jersey). The oocytes were incubated in OR_2 at 18°C for three days before the experiment. To ensure quality and to estimate the quantity of the cRNA, set dilutions of cRNA from mutants were electrophoresed on a 1% formaldehyde-containing agarose gel. The amount of cRNA was judged and matched by interpolation of lanes containing different dilutions of the corresponding cRNA. In addition, for nearly all mutants, two independent isolates were characterized and tested. The α_1, β_2 and γ_2 subunits were co-injected into *Xenopus laevis* oocytes in the ratio of 1:1:1.8 to ensure the incorporation of γ_2 subunits.

Three days after injection, oocytes were placed on a mesh machined in a small perfusing volume chamber (~75 μl), with a $t_{1/2}$ and clearance time of approximately 3 and 10 seconds, respectively. The chamber had an inlet in the top and an outlet in the bottom, which allowed continuous and rapid perfusion. Twenty separate reservoirs (100-ml glass containers) were connected to four six-way valves and the outlet of each of these six-way valves (the sixth position was continuous with the reservoir containing the control solution) was connected to one four-way valve. The outlet of the four-way valve led to the chamber. In this way, up to 20 different solutions could be bath-applied to an individual oocyte. Switching between the different solutions was controlled manually. The oocyte was continuously perfused with recording OR_2 (without antibiotics and the 1 mM Na_2HPO_4, which was replaced with 1 mM NaCl) and switched to the test solution containing drug.

Recording microelectrodes were made with a Narishige PP-83 puller (Tokyo, Japan) and filled with 3 M KCl. Electrodes with resistances of 0.6–1.6 MΩ were used. A two-electrode voltage-clamp amplifier (Turbo TEC-05 npi, Adams and List, Westbury, New York) was used to record currents in response to the application of drugs. In all cases, membrane potential was clamped to –70 mV. Data were visualized on a Gould TA240 chart recorder (Valley View, Ohio) during the experiment and stored online using Pulse Fit or on a VCR using Instrutech 10b (Port Washington, New York) for subsequent off-line analysis.

The EC_{50} and Hill coefficients were estimated by fitting the concentration–response relationships to the Hill equation according to the following formula (Sigma plot, or software provided by D.S. Weiss).

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

Alternatively, the data containing two components of potentiation were fitted with a sum of two Hill equations.

$$I = I_{maxa}/(1+ [EC_{50a}/(A)]^{n}_{a}) + I_{maxb}/(1+ [EC_{50b}/(A)]^{n}_{b})$$

Here, I is the peak current at a given concentration of agonist A, I_{max} is the maximum current, EC_{50} is the concentration of agonist yielding a half-maximal current, and *n* is the Hill coefficient.

The fractional potentiation was calculated using the following equation:

fractional potentiation = $(I_{BZ} - I_{GABA})/I_{GABA}$

Here, I_{BZ} is the measurement of GABA current in the presence of a given concentration of BZ, and the I_{GABA} is the amplitude of the GABA current alone. In addition, a concentration of 70 nM BZ was bath-applied to the oocytes before collection of BZ data points.

The GABA concentration–response relationships were shifted to lower concentrations (~two-fold) in oocytes that had high levels of expression. For this reason, oocytes used to collect the data were mostly limited to those that had less than 1 μA in maximal response to GABA. The EC values (for example, EC_4) were first determined from the averaged concentration–response relationship. The obtained values were then empirically tested for each experiment by comparing the elicited GABA response at this concentration and the maximal response evoked by a GABA concentration equivalent to $20 \times EC_{50}$. The values were then adjusted and retested accordingly until a true EC value was determined. The low EC values (for example, EC_1) often had a standard error of 30% relative to expected values.

All BZs were purchased from Sigma, except for diazepam, which was purchased from Sigma or BIOMOL (Plymouth Meeting, Pennsylvania). Stock solutions of BZs at 100 mM were made in DMSO. Test solutions containing drugs were made by adding the BZ stock solutions to rapidly stirred recording OR_2 or OR_2 containing GABA. The presence of the vehicle solution DMSO at the maximum tested concentration (0.2%) did not alter the GABA-induced current from ρ_1, $\alpha_1\beta_2\gamma_2$ or $\alpha_{S267I}\beta_{N265I}\gamma_{S280I}$ receptor channels.

All data presented are the mean ± standard error (s.e.m.).

Acknowledgements

We thank H. Schwiger for insight concerning benzodiazepine use in anesthesia and E. Bennett, M. Pacheco, B. Pattnaik, L. Wecker. B. Lindsey and P. Coppock for reading this manuscript. This work was supported by a grant from the NEI (EY11531).

Received 19 September; Accepted 19 October 2000

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