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ACCELERATED COMMUNICATION

Benzodiazepines Affect Channel Opening of $GABA_A$ Receptors Induced by Either Agonist Binding Site

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ABSTRACT

Benzodiazepines are widely used as anxiolytics, sedatives, muscle relaxants, and anticonvulsants. They allosterically modulate GABA type A ($GABA_A$) receptors by increasing the apparent affinity of the agonist GABA to elicit chloride currents. Such an increase in apparent affinity of channel gating could either be caused by an increase in affinity for GABA or by a facilitation of channel opening. In the first case, conformation of the affected sites would have to be altered. In the second case, the affected sites are not necessarily altered, because diazepam could facilitate conformational changes leading to the open channel. It is controversial as to whether benzodiazepines affect only channel opening induced by the occupation of one of the two agonist binding sites or by both. We used receptors formed by concatenated subunits to selectively destroy one of the two agonist sites by point mutation. Both of the receptors harboring only one active agonist site could be stimulated by diazepam. We therefore present evidence that binding of diazepam can affect channel opening induced by either agonist binding site.

Benzodiazepines are mainly used for their sedative/hypnotic and anxiolytic effects. They bind to high-affinity binding sites on $GABA_A$ receptors (Sigel, 2002). Initial purification of a $GABA_A$ receptor (Sigel et al., 1983) using a benzodiazepine affinity chromatography resulted in isolation of two subunits termed α and β. A variety of subunit isoforms have been cloned since then: α_{1-6}, β_{1-3}, γ_{1-3}, δ, ϵ, π, and θ (Schofield et al., 1987; Macdonald and Olsen, 1994; Davies et al., 1997; Whiting et al., 1999; Sieghart and Sperk, 2002). The $GABA_A$ receptors consists of five subunits arranged pseudosymmetrically around a Cl^- ion selective-channel pore. The stoichiometry of the most abundant adult receptors is probably two α, two β, and one γ subunits (Chang et al., 1996; Tretter et al., 1997; Farrar et al., 1999; Baumann et al., 2001). The subunit arrangement around the channel pore is $\gamma\beta\alpha\beta\alpha$ counterclockwise as viewed from synaptic cleft (Baumann et al., 2002). The large extracellular N-terminal part is implicated in the formation of binding sites for agonists and benzodiazepines.

Agonist binding sites (Sigel et al., 1992; Amin and Weiss, 1993; Smith and Olsen, 1994; Westh-Hansen et al., 1997; Boileau et al., 1999; Wagner and Czajkowski, 2001) are homologous (Sigel and Buhr, 1997; Sigel, 2002) to the binding site for allosteric modulators of the benzodiazepine type (Wieland et al., 1992; Amin et al., 1997; Buhr et al., 1997a,b; Buhr and Sigel, 1997; Teissere and Czajkowski, 2001). Binding of GABA to agonist sites is coupled to the opening of the channel pore, which then allows the flow of Cl^- ions down their electrochemical gradient.

Benzodiazepines allosterically modulate activation of $GABA_A$ receptors by GABA (Choi et al., 1977; Macdonald and Barker, 1978). Binding of benzodiazepines to their binding site increases the apparent affinity of at least one agonist site (Gallager and Tallman, 1983; Serfozo and Cash, 1992; Lavoie and Twyman, 1996).

We sought to determine here whether occupancy of the benzodiazepine binding site facilitates channel opening by occupancy of only one or both agonist binding sites. For this purpose, we used concatenated $GABA_A$ receptors in which the function of one of the agonist sites was selectively disrupted by the β_2Y205S mutation (Amin and Weiss, 1993).

Materials and Methods

Construction of Receptor Subunits. The cDNAs coding for the α_1, β_2, and γ_2S subunits of the rat $GABA_A$ receptor channel have

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been described earlier (Lolait et al., 1989; Malherbe et al., 1990a,b). For cell transfection, the cDNAs were subcloned into the polylinker of pBC/CMV (Bertocci et al., 1991). This expression vector allows high-level expression of a foreign gene under control of the cytomegalovirus promoter. Construction of the tandem subunit construct γ_2-β_2 and the triple subunit construct α_1-β_2-α_1 has been described previously (Baumann et al., 2002). The introduction of the mutation β_2Y205S into multisubunit constructs has been described elsewhere (Baumann et al., 2003).

Expression in *Xenopus laevis* Oocytes. Capped cRNAs were synthesized (Ambion, Austin, TX) from the linearized pCMV vectors containing the different subunits, respectively. A poly(A) tail of approximately 400 residues was added to each transcript using yeast poly(A) polymerase (USB, Cleveland, OH). The concentration of the cRNA was quantified on a formaldehyde gel using Radiant Red stain (Bio-Rad, Hercules, CA) for visualization of the RNA and known concentrations of RNA ladder (Invitrogen, Carlsbad, CA) as standard on the same gel. cRNA combinations were stored in water at −80°C. Oocytes were injected with 50 nl of the cRNA solution containing cRNA coding for the dual and triple constructs at 10 nM/10 nM concentration. The injected oocytes were incubated in modified Barth's solution [10 mM HEPES, pH 7.5, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.34 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 100 U/ml penicillin, and 100 μg/ml streptomycin] at 18°C for at least 24 h before the measurements. *X. laevis* oocytes were prepared, injected, and defolliculated as described previously (Sigel, 1987; Sigel et al., 1990).

Two-Electrode Voltage Clamp. Electrophysiological experiments were performed by the two-electrode voltage-clamp method at a holding potential of −80 mV. The perfusion medium contained 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM sodium HEPES, pH 7.4. Relative stimulation by diazepam of currents elicited by GABA in concatenated α_1-β_2-α_1/γ_2-β_2, α_1-β_2Y205S-α_1/γ_2-β_2, and α_1-β_2-α_1/γ_2-β_2Y205S receptors was performed at a concentration of GABA eliciting $\leq EC_8$. The stimulation by diazepam was calculated as Stimulation = $((I_{\text{after diazepam}}/I_{\text{before diazepam}}) - 1) \times 100\%$. Between two experiments, the perfusion system was washed with 100% dimethyl sulfoxide to avoid contamination.

Results

Diazepam Potentiation of Nonmutated $GABA_A$ Receptors. It has been proposed that an allosteric effect by diazepam is induced at only one of two α-subunits of the $GABA_A$ receptor (Williams and Akabas, 2001). Furthermore, it has been suggested that diazepam facilitates the opening of the singly ligated state of the channel (Serfozo and Cash, 1992; Lavoie and Twyman, 1996), leaving it open whether or not the same site always is occupied first. To further investigate these suggestions, we used wild-type and mutated receptors in which subunit composition and stoichiometry are predefined by using subunit concatenation (Baumann et al., 2001). Here, mutations may be placed into only one of the two identical subunits occurring in the pentamer (Minier and Sigel, 2004). A nonmutated receptor composed of a triple α_1-β_2-α_1 and a dual γ_2-β_2 subunit construct was characterized by an EC_{50} value for GABA of approximately 120 μM (Baumann et al., 2002). Currents elicited by 20 μM GABA in the nonmutated receptor were stimulated by 1 μM diazepam (Fig. 2). Stimulation amounted to 333 ± 50% (mean ± S.D., $n = 7$). This value is slightly higher than the value of approximately 270% found previously for γ_2-β_2-α_1/β_2-α_1 and higher than the value of approximately 170% found for nonlinked $\alpha_1\beta_2\gamma_2$ receptors (Baumann et al., 2002). The difference between linked and nonlinked receptors might be explained by the formation of diazepam-insensitive $\alpha_1\beta_2$ receptors in *X. laevis* oocytes in the case of nonlinked receptors (Boileau et al., 2002).

Diazepam Potentiation of Mutated $GABA_A$ Receptors. To engineer two mutant receptors in each of which one of the two agonist binding sites was destroyed, the β_2Y205S point mutation was introduced site-specifically. This mutation has been shown to disrupt affinity of agonist binding sites for GABA in $\alpha_1\beta_2\gamma_2$ receptors (Amin and Weiss, 1993). The α_1-β_2Y205S-α_1 or γ_2-β_2Y205S constructs were coexpressed with corresponding nonmutated dual or triple subunit construct (α_1-β_2Y205S-α_1/γ_2-β_2 and α_1-β_2-α_1/γ_2-β_2Y205S). Wild-type concatenated receptors α_1-β_2-α_1/γ_2-β_2 were also expressed. Figure 1 illustrates the receptor types. The two agonist binding sites are located at β/α subunit interfaces. We call the one located next to the benzodiazepine binding site at the α/γ subunit interface site 2 and the second site 1. In α_1-β_2Y205S-α_1/γ_2-β_2 receptors, site 2 is strongly affected, and in α_1-β_2-α_1/γ_2-β_2Y205S receptors, site 1 is strongly affected. All concatenated receptors were able to form fully functional receptors when expressed in *X. laevis* oocytes. Their functional properties have been described elsewhere (Baumann et al., 2002, 2003). Both mutated receptors displayed two-phasic GABA concentration-response curves. The respective EC_{50} values for GABA were approximately 900 μM for site 1 and 10 M for site 2 on α_1-β_2Y205S-α_1/γ_2-β_2 receptors and approximately 0.8 M for site 1 and 400 μM for site 2 on α_1-β_2-α_1/γ_2-β_2Y205S mutant receptors (Baumann et al., 2003). In the case of α_1-β_2Y205S-α_1/γ_2-β_2 and α_1-β_2-α_1/γ_2-β_2Y205S mutant receptors, 30 μM GABA was applied in the absence or presence of 1 μM diazepam (Fig. 2). These GABA concentrations were all $< EC_8$ (Baumann et al., 2003)

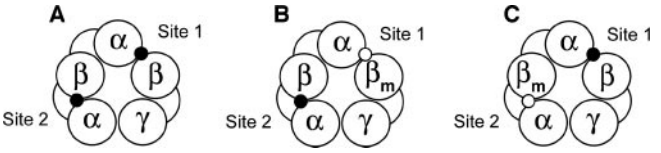

Fig. 1. Concatenated receptors and location of the mutations. ●, intact site; ○, mutated site. A, α_1-β_2-α_1/γ_2-β_2 receptor. B, α_1-β_2-α_1/γ_2-β_2Y205S receptor. C, α_1-β_2Y205S-α_1/γ_2-β_2 receptor.

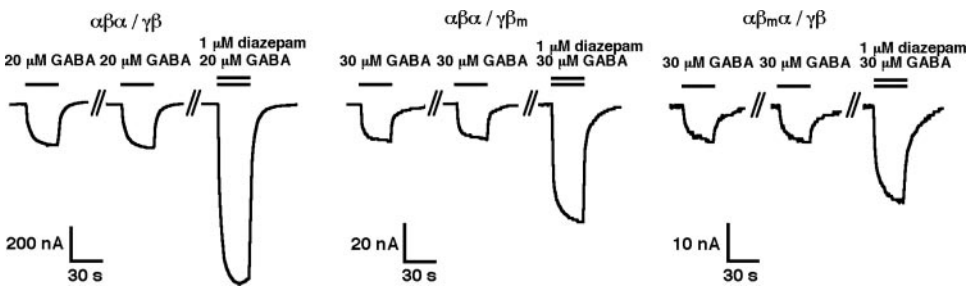

Fig. 2. Stimulation by 1 μM diazepam of currents elicited by GABA in concatenated receptors α_1-β_2-α_1/γ_2-β_2 ($\alpha\beta\alpha/\gamma\beta$), α_1-β_2-α_1/γ_2-β_2Y205S ($\alpha\beta\alpha/\gamma\beta_2$), and α_1-β_2Y205S-α_1/γ_2-β_2 ($\alpha\beta_2\alpha/\gamma\beta$).

for the nonmutated sites in the case of the mutant channels. Stimulation by diazepam was 261 ± 39% (mean ± S.D., n = 6) and 182 ± 27% (mean ± S.D., n = 5) in α_1-β_2-α_1/γ_2-β_2Y205S and α_1-β_2Y205S-α_1/γ_2-β_2 receptors, respectively. This corresponds to approximately 78 and 55% of the stimulation observed in nonmutated receptors, respectively. These preliminary experiments demonstrated that both receptors, in each of which one agonist site was destroyed, were still modulated by diazepam.

Concentration-Dependence of the Stimulation by Diazepam. Fig. 3 shows the concentration-dependence on diazepam of the stimulation of the currents elicited by GABA. Table 1 summarizes the results. Wild-type α_1-β_2-α_1/γ_2-β_2 and mutant α_1-β_2-α_1/γ_2-β_2Y205S and α_1-β_2-α_1/γ_2-β_2Y205S receptors showed similar values for half-maximal stimulation between 47 and 87 nM. Thus, the concentration-dependence on diazepam was not significantly affected by mutation within either agonist site. Maximal stimulation was observed at 1 μM diazepam. Values were somewhat smaller than indicated above, but the relative extents were similar. The reasons for this discrepancy are not known. Experiments were carried out at GABA concentrations of 5 μM for wild-type receptors and 15 and 30 μM for mutant receptors α_1-β_2-α_1/γ_2-β_2Y205S and at 15, 30, and 60 μM for mutant receptors α_1-β_2Y205S-α_1/γ_2-β_2. In the case of α_1-β_2-α_1/γ_2-β_2Y205S receptors, the average stimulation was nearly constant, whereas maximal stimulation at α_1-β_2Y205S-α_1/γ_2-β_2 receptors significantly decreased with increasing concentrations of GABA, at least at 60 μM GABA.

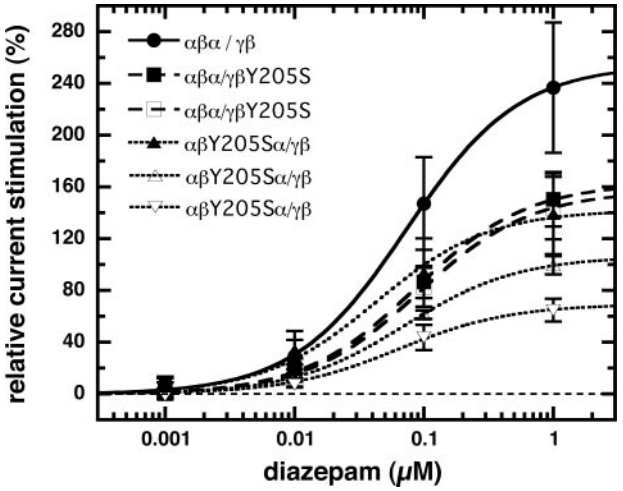

Fig. 3. Concentration-dependence of the stimulation by diazepam of currents elicited by 5 μM GABA in concatenated wild-type receptors α_1-β_2-α_1/γ_2-β_2 (●); 15 μM GABA (■) and 30 μM GABA (□) in concatenated mutant receptors α_1-β_2-α_1/γ_2-β_2Y205S; and 15 μM GABA (▲), 30 μM GABA (△) and 60 μM GABA (▽) in concatenated mutant receptors α_1-β_2Y205S-α_1/γ_2-β_2. Data are given as mean ± S.D. (n = 4).

TABLE 1

Dose-response curves for current stimulation by diazepam

Cumulative concentration-response curves were carried out at different GABA concentrations. The diazepam concentrations were 1, 10, 100, and 1000 nM. Stimulation went through an optimum. At 10,000 nM, stimulation was less than at 1000 nM. Data are given as means ± S.D. from four experiments each.

Subunit Combination	GABA	Diazepam K_a	Diazepam E_{max}
	μM	nM	%
α_1-β_2-α_1/γ_2-β_2	5	75 ± 15	255 ± 51
α_1-β_2-α_1/γ_2-β_2Y205S	15	87 ± 20	163 ± 22
α_1-β_2-α_1/γ_2-β_2Y205S	30	87 ± 23	157 ± 24
α_1-β_2Y205S-α_1/γ_2-β_2	15	47 ± 14	142 ± 28
α_1-β_2Y205S-α_1/γ_2-β_2	30	72 ± 10	106 ± 13
α_1-β_2Y205S-α_1/γ_2-β_2	60	68 ± 19	70 ± 9

Discussion

We investigated two types of receptors with predefined subunit arrangement, in each of which one of the two GABA binding was destroyed by the point mutation β_2Y205S (Amin and Weiss, 1993). Diazepam was able to potentiate GABA-elicited currents in both receptor types with only one functional agonist site. This indicates that conformational changes induced by occupancy of the benzodiazepine binding site are transduced either to both agonist sites or to domains in the channel involved in channel gating by the two individual agonist sites. Stimulation at all receptors α_1-β_2-α_1/γ_2-β_2, α_1-β_2Y205S-α_1/γ_2-β_2, and α_1-β_2-α_1/γ_2-β_2Y205S was tested at $EC_{<8}$ for the nonmutated site in the case of the mutant channels. At this concentration of GABA, a near-maximal stimulation by diazepam may be measured, assuming a classic channel model (Sigel, 2002). Measurement in mutant channels was confined to a narrow concentration range of the agonist. At lower concentrations of GABA, elicited current amplitudes were too small. This small current amplitude is not caused by a lack of expression but rather by a consequence of exclusive occupation of one agonist site. The probability to open is low for channels occupied by a single agonist molecule. Larger current amplitudes were observed at higher concentrations of GABA because the second binding site became occupied (Baumann et al., 2003). Although stimulation by diazepam of α_1-β_2-α_1/γ_2-β_2Y205S receptors (site 2 intact) was only slightly larger at 15 than at 30 μM GABA; maximal stimulation of α_1-β_2Y205S-α_1/γ_2-β_2 receptors (site 1 intact) seemed to decrease with increasing concentrations of GABA in the range of 15 to 60 μM GABA. Assuming a classic channel model, these observations would argue for higher apparent agonist affinity at site 1 than at site 2 (Sigel, 2002), in contrast to previous observations (Baumann et al., 2003). If the model that was able to successfully simulate data obtained for concatenated $\alpha_1\beta_2\gamma_2$ receptors (Baumann et al., 2003) and receptors containing α_1 and α_6 subunits (Minier and Sigel, 2004) is accepted, this would indicate that the two agonist sites respond subtly differently to diazepam. In α_1-β_2-α_1/γ_2-β_2Y205S receptors in which site 2 is not mutated, diazepam would lead to a slight increase in the agonist affinity and stimulate channel gating of the singly ligated state approximately 2-fold; however, in α_1-β_2Y205S-α_1/γ_2-β_2 receptors in which site 1 is not mutated, diazepam would increase the agonist affinity approximately 4-fold and slightly decrease channel gating of the singly ligated state. Agonist site 2 is located at the subunit interface neighboring the interface α_1/γ_2 with the benzodiazepine binding pocket, whereas agonist site 1 is located one subunit interface farther away. Note that it is assumed here that the mutations themselves do not interfere with the stimulation by diazepam.

Our observations are relevant for the molecular mechanism of action of benzodiazepines. Because we describe that either the conformation of the agonist sites or their associated domains responsible for channel opening are affected by benzodiazepines, our conclusions are in apparent or real contrast to several previous studies. On the basis of observations

of diazepam effects on single channel kinetic properties of $GABA_A$ channels, Rogers et al. (1994) hypothesized that exclusively "the rate of entry into the singly bound closed state" was increased by diazepam. However, the authors did not specify that such an increase in rate would only occur at one of the two agonist sites, but they excluded an increase in the rate of entry in the doubly bound state. Lavoie and Twyman (1996) hypothesized "that diazepam likely enhances GABA receptor currents primarily by accelerating GABA association to its receptor at the first agonist binding site". Again, the authors did not specify that such an increase in rate of GABA association would exclusively occur at one of the two agonist sites. Williams and Akabas (2001) mutated amino acid residues in M3 of the two α subunits to cysteine and treated mutated $\alpha\beta\gamma$ receptors with a cysteine-reactive reagent. Modification in the presence of GABA strongly decreased, and modification in the presence of diazepam strongly increased subsequent current responses to GABA. Receptor premodified in the presence of GABA could not be modified in the presence of diazepam, whereas the opposite was still possible. The authors hypothesized that in the presence of diazepam, only one mutated residue is accessible to modification, whereas in the presence of GABA, the second or both residues are accessible. Thus, diazepam would lead to different conformations in the M3 portions of the two α subunits. In agreement with this, we postulate differential effects by diazepam on the channel gating mediated by the two agonist sites.

In summary, our observations argue against the hypothesis that one agonist site exclusively or its associated domain responsible for channel opening is selectively affected in response to occupation of the benzodiazepine binding site. Although diazepam could still affect channel opening induced by the singly ligated state, this does not seem limited to one of the two agonist binding sites.

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