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Evidence for distinct conformations of the two α_1 subunits in diazepam-bound GABA_A receptors

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Abstract

Benzodiazepines allosterically modulate GABA_A receptors to increase currents induced by submaximal GABA concentrations. Benzodiazepine-induced conformational changes in the transmembrane domain increase the reactivity of cysteines substituted for a subset of residues in the α_1 subunit M3 membrane-spanning segment. With the cysteine-substitution mutant α_1 F296C $\beta_1\gamma_2$ we previously noted that p-chloromercuribenzenesulfonate (pCMBS⁻) modification in the presence of diazepam potentiated subsequent GABA-induced currents. In contrast, pCMBS⁻ modification in the presence of GABA caused inhibition of subsequent responses. We now show that in the presence of diazepam, pCMBS⁻ only reacts with the engineered cysteine in one of the two α subunits; whereas, in the presence of GABA, pCMBS⁻ reacts with the cysteine in the other α subunit, or with both cysteines. This implies that the two α subunits have distinct conformations in the diazepam-bound state. Based on analysis of single channel kinetic data, others have hypothesized that diazepam only alters the GABA affinity of one of the two GABA binding sites. The results presented here provide structural evidence to support the hypothesis that diazepam binding only alters the conformation of one of the two α subunits in a GABA_A receptor and provides new insights into the mechanism of allosteric potentiation by benzodiazepines. © 2001 Elsevier Science Ltd. All rights reserved.

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GABA_A receptors form ligand-gated Cl⁻ channels that are the major inhibitory neurotransmitter receptors in the central nervous system (Macdonald and Olsen, 1994; Sieghart et al., 1999). The receptors are formed by the assembly of five homologous subunits around the central channel axis. Multiple subunits have been cloned and subdivided into families based on the extent of sequence similarity. In heterologous expression systems, and probably in vivo also, the most common subunit stoichiometry is 2 α , 2 β and 1 γ subunits (Chang et al., 1996; Tretter et al., 1997; Farrar et al., 1999; Sieghart et al., 1999). The order of the subunits around the pore is presumably β - α - β - α - γ . Thus, the conformations of the two α subunits are not necessarily identical because one α subunit has two β subunits as neighbors and the other α subunit has a β and a γ . Each subunit has an ~200

amino acid extracellular N-terminal domain and a similarly sized C-terminal domain with four membrane-spanning segments (M1, M2, M3, M4) that forms the channel (Macdonald and Olsen, 1994; Karlin and Akabas, 1995). The two GABA binding sites are formed in the extracellular domain at the α - β subunit interfaces (Sigel et al., 1992; Amin and Weiss, 1993; Smith and Olsen, 1994; Boileau et al., 1999).

The GABA_A receptors are subject to allosteric modulation by multiple classes of clinically used drugs including benzodiazepines, barbiturates, ethanol and intravenous and volatile anesthetics. Extensive work has focused on identifying the binding sites and elucidating the molecular mechanisms of action for these drugs (Macdonald and Olsen, 1994; Krasowski and Harrison, 1999). For benzodiazepines, the high affinity binding site is located in the extracellular domain at the interface of the α and γ subunits. Many residues in and surrounding the high affinity benzodiazepine binding site have been identified and are aligned with residues in the β and α subunits that form the GABA binding sites (Wieland et

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al., 1992; Buhr et al., 1996; Duncalfe et al., 1996; Amin et al., 1997; Wingrove et al., 1997; Boileau et al., 1998; Kucken et al., 2000). Recent work has also suggested that a second, low affinity binding site is located in the transmembrane domain (Walters et al., 2000).

Benzodiazepine binding potentiates the currents elicited by submaximal GABA concentrations but does not directly activate the receptor by itself (Macdonald and Olsen, 1994; Rabow et al., 1995). Diazepam, a benzodiazepine, increases the single channel opening frequency but does not alter the open channel or burst duration histograms (Study and Barker, 1981; Rogers et al., 1994). Thus, based on a kinetic model for the GABA_A receptor it was inferred that diazepam increases the affinity for GABA at only one of the two GABA binding sites by increasing the GABA association rate (Rogers et al., 1994; Lavoie and Twyman, 1996).

Diazepam binding in the extracellular domain induces a conformational change in the transmembrane domain that increases the reactivity of cysteines substituted for α_1 F296, α_1 F298 and α_1 L301, residues in the α_1 subunit M3 membrane-spanning segment, with pCMBS⁻ added extracellularly (Williams and Akabas, 2000). The M3 segment engineered cysteine residues that reacted with pCMBS⁻ in the presence of diazepam are a subset of the residues that react with pCMBS⁻ in the presence of GABA (α_1 F296C, α_1 F298C, α_1 A300C, α_1 L301C and α_1 E303C) (Williams and Akabas, 1999). Thus, we previously concluded that the diazepam-bound state represents a structural state of the receptor that is distinct from the resting and GABA-activated states (Williams and Akabas, 2000). During the course of those experiments we observed that at position α_1 F296C the subsequent GABA-induced currents were potentiated following pCMBS⁻ modification in the presence of diazepam but the subsequent currents were inhibited when pCMBS⁻ modification occurred in the presence of GABA. This is the only M3 cysteine-substitution mutant where the effects of pCMBS⁻ modification in the presence of GABA and diazepam were qualitatively different, i.e. inhibition vs. potentiation. We now show that this differential effect of modification in the presence of diazepam and GABA occurs because, at least in the presence of diazepam, the conformation of the two α subunits within a functional receptor are different. Thus, in the presence of diazepam the engineered cysteine in only one α subunit is accessible to react with pCMBS⁻, the cysteine in the other α subunit remains buried. In contrast, in the presence of GABA, modification of the engineered cysteine in the other α subunit, or of both cysteines, has a dominant, inhibitory effect on the subsequent GABA-induced currents.

1. Methods

The rat GABA_A α_1 F296C mutant was generated, characterized and mRNA made by in vitro transcription as previously described (Williams and Akabas, 1999). *Xenopus laevis* oocytes were prepared and injected (50 nl) with mRNA (200 pg/nl) encoding wild type β_1 and γ_{2s} subunits and the mutant α_1 subunit in a 1:1:1 ratio as was described previously (Williams and Akabas 1999, 2000). Currents were recorded from two electrode voltage clamped oocytes continuously perfused (5 ml/min) with 115 mM NaCl, 2.5 mM KCl, 1.8 mM MgCl₂, 10 mM HEPES, pH 7.5 with NaOH at room temperature using a TEV-200 amplifier (Dagan, MN) and a TL1 analog/digital interface (Axon Instruments, CA). Chamber volume was about 200 μ l. The holding potential was -80 mV.

pCMBS⁻ (Aldrich, St. Louis, MO) reacts with cysteines to add a mercuribenzenesulfonate group onto the free sulfhydryl. It reacts 10³ times faster with the ionized thiolate anion (S⁻) than with the unionized thiol (SH) and only cysteines on the water-accessible surface will ionize to any significant extent (Hasinoff et al., 1971). If the average of the two GABA-induced currents 5–10 min after application of pCMBS⁻ are significantly different from the response to GABA before the application of pCMBS⁻ we infer that the currents have been irreversibly changed. We infer that an irreversible change in the GABA-induced current following pCMBS⁻ application is due to the modification of an engineered cysteine. The effects of covalent modification were assayed with test pulses of GABA at an EC₅₀ concentration. Data are expressed as the percent effect $[(I_{\text{final}}/I_{\text{initial}})-1]\times 100$ where I_{final} is the magnitude of the GABA-induced currents after application of pCMBS⁻ and I_{initial} is the magnitude of the initial GABA currents. Mean \pm SEM are given. Significant differences were determined by Student's *t*-test.

The sequence of reagent additions is indicated by the bars above the current traces in the figures. The current traces are separated by wash periods. Following applications of EC₅₀ concentration GABA (1–2 μ M) oocytes were washed for 3 min with buffer to allow complete recovery from desensitization. Following applications of saturating GABA (100 μ M) the wash period was extended to 7 min to allow complete recovery from desensitization.

2. Results

We showed previously that for wild-type $\alpha_1\beta_1\gamma_2$ GABA_A receptors expressed in *Xenopus* oocytes, a 1-min application of 0.5 mM pCMBS⁻ in the absence or in the presence of either 100 μ M GABA or 100 nM diazepam had no effect on the subsequent GABA-

induced currents (Williams and Akabas 1999, 2000). Thus, we infer that the endogenous cysteine residues in the wild type subunits are not accessible for reaction with pCMBS⁻ applied extracellularly or, alternatively but less likely, reaction with these cysteines has no functional effect.

GABA_A receptors formed by the coexpression of the M3 segment cysteine-substitution mutant α_1 F296C with wild type β_1 and γ_2 subunits have a GABA EC₅₀ of 1.4±0.4 μ M (Williams and Akabas, 1999). To ensure that the cell surface population of GABA_A receptors was mainly formed as α_1 F296C $\beta_1\gamma_2$ and did not include a significant proportion of α_1 F296C β_1 receptors we tested the effect of coapplication of Zn²⁺ on the GABA-induced currents. Coapplication of 100 μ M ZnCl₂ with 100 μ M GABA caused -1±4% (*n*=3) inhibition of the GABA-induced currents (data not shown). As we previously showed the Zn²⁺ IC₅₀ for $\alpha_1\beta_1$ receptors is 0.54 μ M whereas $\alpha_1\beta_1\gamma_2$ receptors are not inhibited by 1 mM Zn²⁺ (Horenstein and Akabas, 1998), thus we concluded that there was not a significant population of α_1 F296C β_1 receptors on the oocyte surface.

For α_1 F296C $\beta_1\gamma_2$ receptors the currents elicited by an EC₅₀ GABA concentration were potentiated by 100 nM diazepam and these effects washed out completely following removal of the diazepam (Fig. 1C). Furthermore, by itself 100 nM diazepam did not elicit a current (Fig. 1C).

As we observed previously, a 1-min application of 0.5 mM pCMBS⁻ by itself did not alter the subsequent GABA-induced currents (+3±5%; *n*=3) in oocytes expressing α_1 F296C $\beta_1\gamma_2$ (Fig. 1A) (Williams and Akabas, 1999). We inferred that in the resting state the engineered cysteine residues were inaccessible to react with pCMBS⁻ added extracellularly. A 1-min application of 0.5 mM pCMBS⁻ in the presence of 100 μ M GABA, however, resulted in -23±5% (*n*=10) inhibition of subsequent GABA-induced currents (Fig. 1B). In contrast, a 1-min application of 0.5 mM pCMBS⁻+100 nM diazepam resulted in +24±2% (*n*=8) potentiation of subsequent GABA-induced currents (Fig. 1D) (Williams and Akabas 1999, 2000). From the irreversible change in current following the application of the sulfhydryl reactive reagent, pCMBS⁻, we inferred that in the presence of GABA or diazepam one or both of the engineered cysteine residues in the two α_1 F296C subunits were becoming accessible to react with pCMBS⁻.

Following pCMBS⁻ modification of α_1 F296C $\beta_1\gamma_2$ receptors in the presence of either GABA or diazepam the currents elicited by EC₅₀ GABA were still potentiated by 100 nM diazepam. Before pCMBS⁻ modification, 100 nM diazepam potentiated the EC₅₀ GABA current by 23±4% (*n*=7). After modification by pCMBS⁻ in the presence of GABA, 100 nM diazepam potentiated EC₅₀ GABA currents by 14±3% (*n*=4) and after modification by pCMBS⁻ in the presence of

diazepam EC₅₀ GABA currents were potentiated by 18±9% (*n*=3). Thus, diazepam still potentiated submaximal GABA-induced currents after pCMBS⁻ modification of α_1 F296C either in the presence of GABA or diazepam.

Because there are two α subunits in each functional receptor, and thus two engineered cysteines, this differential effect, of inhibition following modification in the presence of GABA and potentiation following modification in the presence of diazepam, could arise from modification of the cysteine in one but not the other subunit. Alternatively, the position of the cysteines may be different in the presence of GABA and of diazepam. In this scenario, pCMBS⁻ modification would trap the cysteine in different positions depending on whether modification took place in the presence of GABA or diazepam. To distinguish between these possibilities we applied pCMBS⁻ sequentially with diazepam and then with GABA. As seen in Fig. 2A, coapplication of pCMBS⁻+diazepam results in +25±4% potentiation of the subsequent GABA-induced test current. A subsequent application of pCMBS⁻+GABA, however, causes -17±5% (*n*=3) inhibition of the subsequent GABA test current relative to the initial GABA test current. This is similar to the extent of inhibition seen without the prior pCMBS⁻+diazepam application. The ability of the second pCMBS⁻ application, in the presence of GABA, to inhibit the GABA test currents indicated that one of the two engineered cysteines was not modified by the first application of pCMBS⁻ in the presence of diazepam.

In order to determine whether the order of application affected the result, we first applied pCMBS⁻+GABA. This resulted in -20±10% inhibition of the subsequent GABA test current. A subsequent application of pCMBS⁻+diazepam, however, had no effect (-3±1%; *n*=3) on the subsequent GABA test current (Fig. 2B). This indicates that in the presence of GABA either pCMBS⁻ reacted with both engineered cysteines or the reaction of pCMBS⁻ in the presence of GABA with one of the cysteines causes a dominant inhibitory effect. We cannot distinguish these possibilities. Application of pCMBS⁻ in the presence of both GABA and diazepam resulted in -18±6% (*n*=3) inhibition of the subsequent GABA test currents (Fig. 2C). This is consistent with the dominant effect of modification in the presence of GABA.

3. Discussion

Substitution of cysteine for α_1 F296 places one engineered cysteine in the α subunit but because there are two α subunits in a functional receptor there are two engineered cysteines per receptor. The results presented above indicate that in the presence of diazepam, pCMBS⁻ only

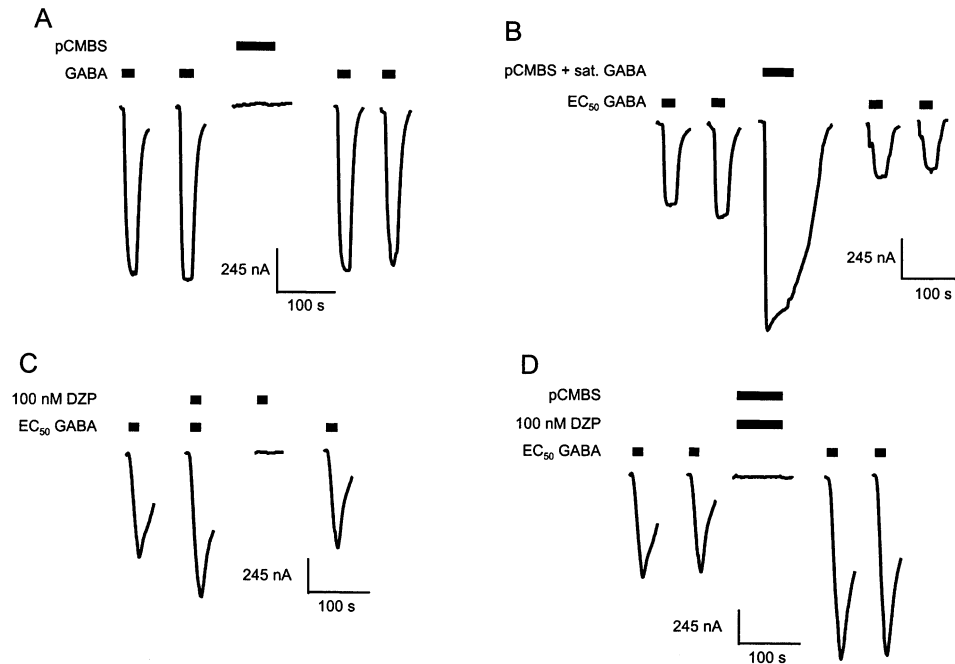


Fig. 1. Effect of a 1-min application of 0.5 mM pCMBS⁻ applied in the absence or in the presence of GABA or diazepam on subsequent GABA-induced currents in oocytes expressing $\alpha_1F296C\beta_1\gamma_2$ mutant receptors. (A) Applied alone pCMBS⁻ has no effect on subsequent GABA-induced currents indicating that it has not reacted with the engineered cysteine residues. (B) Currents elicited by two 1 μ M GABA test pulses after application of pCMBS⁻ in the presence of a near saturating concentration of GABA (100 μ M) are reduced compared to the initial GABA responses. This indicates that pCMBS⁻ has covalently modified one or both of the engineered cysteine residues. (C) Demonstrates that 100 nM diazepam potentiates EC₅₀ GABA-induced currents, that by itself 100 nM diazepam does not induce currents and that the diazepam effects washout. (D) Currents elicited by two 1 μ M GABA test pulses (EC₅₀) after application of pCMBS⁻ in the presence of 100 nM diazepam are potentiated compared to the initial GABA responses. This indicates that pCMBS⁻ has covalently modified one or both of the engineered cysteine residues. Holding potential is -80 mV. In this and the other figures, the time between sequential current traces is 3 min after EC₅₀ GABA applications and 7 min after 100 μ M GABA applications to allow complete recovery from desensitization.

reacted with one of the two engineered cysteine residues per receptor. We infer this because following the potentiation induced by application of pCMBS⁻ with diazepam, the subsequent application of pCMBS⁻ with GABA inhibited the subsequent GABA test currents. This could only occur if one of the two engineered cysteines was not modified by the initial application of pCMBS⁻ with diazepam. The fact that pCMBS⁻ when coapplied with diazepam only reacted with one of the two engineered cysteines implies that the conformations of the two α subunits are different in the presence of diazepam. Thus, the diazepam-induced conformational change only increased the accessibility of the engineered cysteine in one of the two α subunits for reaction with pCMBS⁻.

We do not know, whether in the presence of GABA, pCMBS⁻ reacted only with the engineered cysteine in the other α subunit or, more likely, with both engineered cysteines to inhibit the subsequent GABA-induced currents. Because the effect of pCMBS⁻ modification in the presence of GABA was dominant over the potentiation following modification in the presence of diazepam we cannot distinguish these possibilities. The molecular

mechanism by which reaction of pCMBS⁻ with the cysteine in one α subunit causes potentiation of GABA-induced currents whereas modification of the other cysteine or both cysteines causes inhibition is uncertain. Nevertheless, it provides a tool to distinguish the effects of modification in the presence of diazepam and GABA.

We hypothesize that the α subunit that is modified in the presence of diazepam is the α subunit that is adjacent to the γ subunit (Fig. 3). This α subunit contributes to the formation of the diazepam binding site at the α - γ interface in the extracellular domain (Fig. 3). At present, we have no way to identify which of the two α subunits is covalently modified. Nonetheless, given the position of the diazepam binding site it seems more likely that a conformational change induced by diazepam binding will propagate into the α subunit forming the binding site rather than into the non-adjacent α subunit.

These results have important implications for the molecular mechanism of action of diazepam and other benzodiazepines. If diazepam binding only induces a conformational change in the membrane-spanning domain of one of the two α subunits, the question arises as to whether the effect of diazepam binding in the extra-

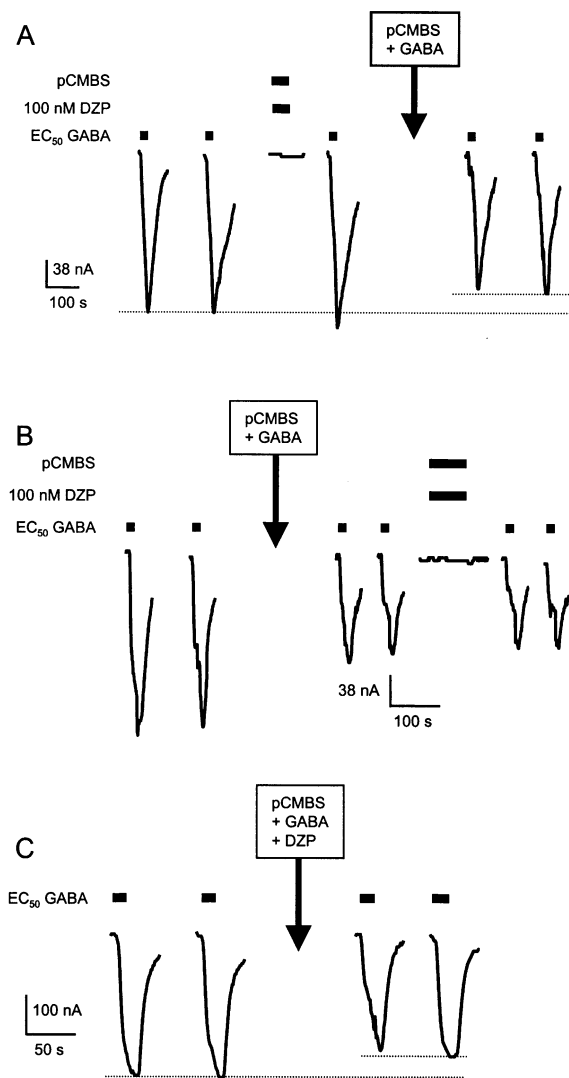


Fig. 2. Effects of sequential applications of 0.5 mM pCMBS⁻ with diazepam and GABA on GABA-induced currents in oocytes expressing α_1 F296C $\beta_1\gamma_2$ mutant receptors. (A) The GABA currents are potentiated following application of pCMBS⁻+100 nM diazepam. A subsequent 1-min application of pCMBS⁻+100 μ M GABA, at the arrow, results in inhibition of the currents elicited by the GABA test pulses. This indicates that one of the engineered cysteine residues was not modified during the initial application of pCMBS⁻ with diazepam and was therefore available to be modified during the subsequent application of pCMBS⁻ with GABA. Currents during co-application of pCMBS⁻ and GABA are not shown for aesthetic reasons because they were much larger due to the use of near-saturating concentrations of GABA. (B) A 1-min application of pCMBS⁻+100 μ M GABA, at the arrow, results in inhibition of the subsequent currents elicited by the GABA test pulses. Subsequent application of pCMBS⁻+100 nM diazepam does not further alter the currents induced by the subsequent GABA test pulses. (C) Simultaneous application of pCMBS⁻ with 100 μ M GABA and 100 nM diazepam results in inhibition of subsequent GABA-induced currents.

cellular domain is similarly limited to a conformational change in only one of the two GABA binding sites. Analysis of the effects of diazepam on single channel kinetics showed that diazepam increases the opening frequency and the burst frequency without significantly alt-

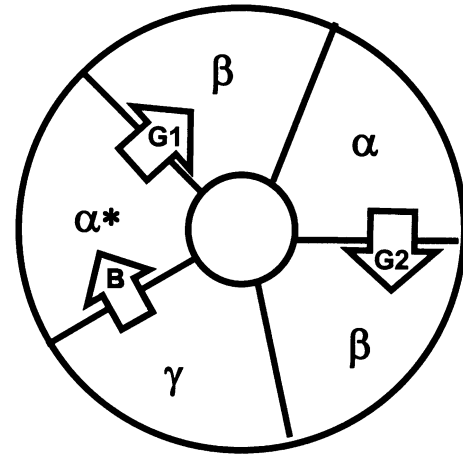


Fig. 3. Cartoon illustrating a top view of a GABA_A receptor to show the likely position of the subunits around the central channel axis. The location of the benzodiazepine binding site at the γ - α interface is indicated by the arrow containing the letter B. The location of the two GABA binding sites at the α - β subunit interfaces is indicated by the arrows containing the letter G. The α subunit involved in the formation of both the benzodiazepine and GABA binding sites is labeled α^* . We hypothesize that it is the engineered cysteine residue in this α subunit that is being modified by pCMBS⁻ in the presence of diazepam. Furthermore, we suggest that it is the affinity of the GABA binding site labeled G1, involving the α^* subunit, that changes following benzodiazepine binding.

tering burst duration or mean open duration or the relative frequency of occurrence of openings of the three open states (Study and Barker, 1981; Vicini et al., 1987; Rogers et al., 1994). Based on a kinetic model of the GABA_A receptor it was hypothesized that diazepam increased the rate of entry into the mono-liganded closed state by increasing the GABA affinity of the first binding step (Rogers et al., 1994). This appears to occur due to an increase in the GABA association rate at one of the two binding sites (Lavoie and Twyman, 1996). Thus, the kinetic data suggests that diazepam only alters the structure of one of the two GABA binding sites. Furthermore, in order to explain the GABA concentration dependence of Cl⁻ efflux from synaptosomes in the presence of benzodiazepine it was hypothesized that with benzodiazepine bound, channels opened following the binding of only one GABA molecule (Serfozo and Cash, 1992). This may be due to an increase in mono-liganded channel openings in the presence of benzodiazepines at low GABA concentrations. These results suggest that diazepam binding only alters the structure of one of the two GABA binding sites. Our data provide structural evidence to support the hypothesis that diazepam only alters the structure of one of the two α subunits.

It should be noted that in the presence of diazepam, pCMBS⁻ also reacted with cysteines substituted for α_1 F298 and α_1 L301. Because the effect of modification of these two cysteine-substitution mutants was the same

in the presence of GABA and diazepam we cannot use them to determine whether in the presence of diazepam only one of the two engineered cysteines are being modified at those positions.

In summary, diazepam binding to the extracellular domain induces a conformational change in the α_1 subunit membrane-spanning domain. This conformational change induces the formation of a receptor state whose structure is distinct from the closed and GABA-activated states. In the membrane-spanning domain the conformational change appears to occur in only one of the two α subunits. Consistent with this finding, single channel kinetic data indicates that benzodiazepines only increase the affinity for binding the first GABA molecule. It is likely that the α subunit involved is the one adjacent to the γ subunit. Thus, the diazepam induced conformational change propagates from the γ - α subunit interface, through the α subunit to the GABA-binding site at the α - β subunit interface. The conformational change does not seem to extend to the other α subunit, which may explain why benzodiazepines are unable to open GABA_A receptors by themselves. Thus, our results provide new structural insights into the molecular mechanism of action of benzodiazepines.

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