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Benzodiazepines Induce a Conformational Change in the Region of the γ-Aminobutyric Acid Type A Receptor α_1-Subunit M3 Membrane-Spanning Segment

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ABSTRACT

Benzodiazepine binding to γ-aminobutyric acid type A ($GABA_A$) receptors allosterically modulates GABA binding and increases the currents induced by submaximal GABA concentrations. Benzodiazepines induce conformational changes in the GABA-binding site in the extracellular domain, but it is uncertain whether these conformational changes extend into the membrane-spanning domain where the channel gate is located. Alone, benzodiazepines do not open the channel. We used the substituted-cysteine-accessibility method to investigate diazepam-induced conformational changes in the region of the α_1-subunit M3 membrane-spanning segment. In the absence of diazepam or GABA, $pCMBS^-$ did not react at a measurable rate with cysteine-substitution mutants between α_1Phe296 and α_1Glu303. In the presence of 100 nM diazepam, $pCMBS^-$ reacted with α_1F296C, α_1F298C, and α_1L301C but not with the other cysteine mutants between α_1Phe296 and α_1Glu303. These three mutants are a subset of the five residues that we previously showed reacted with $pCMBS^-$ applied in the presence of GABA. The $pCMBS^-$ reaction rates with these three cysteine mutants were similar in the presence of diazepam and GABA. Thus, diazepam, which binds to the extracellular domain, induces a conformational change in the membrane-spanning domain that is similar to a portion of the change induced by GABA. Because diazepam does not open the channel, these results provide structural evidence that the diazepam-bound state represents an intermediate conformation distinct from the open and resting/closed states of the receptor. The diazepam-induced conformational change in the M3 segment vicinity may be related to the mechanism of allosteric potentiation.

Benzodiazepines are used to treat anxiety disorders and epilepsy as well as in the induction of general anesthesia. The γ-aminobutyric acid (GABA) type A ($GABA_A$) receptors are the molecular targets for these clinical effects. $GABA_A$ receptors are the major inhibitory neurotransmitter receptors in the central nervous system. Five homologous subunits arranged pseudosymmetrically around a central pore form the receptor-channel complex (Macdonald and Olsen, 1994; Nayeem et al., 1994; Karlin and Akabas, 1995). Each subunit has an ~200-amino acid extracellular N-terminal domain, four membrane-spanning segments (M1, M2, M3, and M4), a large intracellular loop between M3 and M4, and a short extracellular C terminus (Schofield et al., 1987; Macdonald and Olsen, 1994). Each subunit contributes to the channel lining that is largely formed by residues from the M2, and possibly from the M3, membrane-spanning segments (Xu and Akabas, 1996; Williams and Akabas, 1999). The subunit stoichiometry varies in different brain regions, but a common stoichiometry is two α-, two β-, and one γ-subunits (Chang et al., 1996; McKernan and Whiting, 1996; Tretter et al., 1997; Farrar et al., 1999).

Benzodiazepine binding allosterically potentiates GABA binding. This increases the currents induced by submaximal GABA concentrations (Macdonald and Olsen, 1994; Rabow et al., 1995; Sieghart, 1995; Hevers and Luddens, 1998). At the single channel level, benzodiazepines increase channel-opening rates (Study and Barker, 1981), whereas closing and desensitization rates are not altered (Rogers et al., 1994; Ghansah and Weiss, 1999). In hippocampal cells, the $GABA_A$ receptor single channel conductance was reportedly increased by benzodiazepines (Eghbali et al., 1997), but in other cells this has not been observed (Study and Barker, 1981; Rogers et al., 1994).

The γ-subunit is critical for benzodiazepine potentiation of GABA-induced currents (Pritchett et al., 1989; Gunther et al., 1995). The main site of photoaffinity labeling by flunitrazepam, however, is in the α-subunit at position α_1His101

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ABBREVIATIONS: GABA, γ-aminobutyric acid; $GABA_A$, GABA type A receptor; Cys, cysteine; $pCMBS^-$, *p*-chloromercuribenzenesulfonate; β-CCE, ethyl-β-carboline-3-carboxylate.

(Stephenson et al., 1990; Duncalfe et al., 1996). This and other evidence suggests that the benzodiazepine-binding site is located in the extracellular domain at the interface between the α- and γ-subunits (Duncalfe et al., 1996; Amin et al., 1997; Sigel and Buhr, 1997; Boileau et al., 1998). The GABA-binding sites are located in similar regions of the β- and α-subunit interface (Amin and Weiss, 1993; Smith and Olsen, 1995; Boileau et al., 1999).

Several classes of drugs interact with the benzodiazepine site (Macdonald and Olsen, 1994; Rabow et al., 1995; Sieghart, 1995; Hevers and Luddens, 1998). At low concentrations, flumazenil is a benzodiazepine antagonist and β-carbolines are inverse agonists (Chan and Farb, 1985; Sigel and Baur, 1988). At higher concentrations, however, flumazenil and β-carbolines potentiate GABA-induced currents. This potentiation presumably involves interactions at other sites on the $GABA_A$ receptors (Chan and Farb, 1985; Sigel and Baur, 1988; Mehta and Ticku, 1989; Yakushiji et al., 1989; Stevenson et al., 1995). In some mutant $GABA_A$ receptors, low concentrations of flumazenil and/or β-carbolines cause benzodiazepine-like potentiation (Dunn et al., 1999).

In contrast to GABA and many general anesthetics, benzodiazepines do not directly open the channel gate (Macdonald and Olsen, 1994; Rabow et al., 1995), which is located in the membrane-spanning domain (Xu and Akabas, 1996). It is not known whether the benzodiazepine-induced conformational changes are confined to the extracellular domain or whether they extend into the membrane-spanning, channel-forming domains. Mutation of three residues in the extracellular loop connecting the M2 and M3 segments altered allosteric modulation by benzodiazepines but did not affect their binding (Boileau and Czajkowski, 1999). This and other evidence suggest that the transduction of benzodiazepine binding may involve conformational changes in the membrane-spanning domain.

Using the substituted-cysteine (Cys)-accessibility method, we previously showed that the ability of *p*-chloromercuribenzenesulfonate ($pCMBS^-$) to react with the α_1 M3 segment Cys-substitution mutants α_1F298C, α_1A300C, α_1L301C, and α_1E303C was state dependent, i.e., the residues were accessible only in the presence of GABA (Williams and Akabas, 1999). At a fifth position, α_1F296C, the effect of $pCMBS^-$ applied in the presence of GABA was equivocal (as discussed below in the statistics section of *Materials and Methods*). In addition, $pCMBS^-$ reacted with α_1A291C and α_1Y294C when applied in the presence or in the absence of GABA. We inferred that the GABA-induced conformational change creates a water-filled crevice(s) that extends from the extracellular surface into the interior of the membrane-spanning segments. This crevice allows extracellularly applied sulfhydryl reagents to gain access to these residues. We used the state-dependent accessibility of the M3 substituted Cys to probe for conformational changes induced by benzodiazepine binding. We show that benzodiazepine binding alters the accessibility of a subset of the residues that were reactive only in the presence of GABA.

Materials and Methods

Mutants and Expression. The rat α_1M3 segment Cys-substitution mutants were generated and characterized previously (Williams and Akabas, 1999). In vitro mRNA transcription and *Xenopus* oocyte preparation and injection were as described previously (Xu and Akabas, 1993; Williams and Akabas, 1999). Oocytes were injected with 50 nl of a 200 pg/nl solution of subunit mRNA in a 1:1:1 ratio of α_1:β_1:γ_2.

Electrophysiology. Two-electrode voltage clamp recording from *Xenopus* oocytes and data acquisition and analysis were performed as described previously (Williams and Akabas, 1999). Oocytes were continuously perfused at 5 ml/min with calcium-free-frog Ringers (CFFR; 115 mM NaCl, 2.5 mM KCl, 1.8 mM $MgCl_2$, 10 mM HEPES, pH 7.5 with NaOH) at room temperature. The holding potential was −80 mV.

Experimental Protocols. The sulfhydryl-specific reagent used in these experiments was $pCMBS^-$; Sigma, St. Louis, MO). After reaction with a Cys $pCMBS^-$ adds $-HgC_6H_4SO_3^-$ onto the reactive sulfhydryl.

To determine the irreversible effects of $pCMBS^-$ on the GABA-induced currents, the following series of reagents were applied to two-electrode voltage clamped oocytes: 100 μM GABA, 20 s; 100 μM GABA, 20 s; EC_{50} GABA, 20 s; drug alone, 20 s; drug plus EC_{50} GABA, 20 s; EC_{50} GABA, 20 s; 0.5 mM $pCMBS^-$ with or without drug, 1 min; 100 μM GABA, 20 s; 100 μM GABA, 20 s; EC_{50} GABA, 20 s; EC_{50} GABA, 20 s (see Fig. 1 for example). The applications of GABA and reagents were separated by 3- to 5-min washes with CFFR to allow recovery from desensitization. The drugs coapplied with $pCMBS^-$ were 100 nM diazepam (RBI, Natick, MA), 10 or 100 nM flumazenil (gift of Hoffman-LaRoche, Nutley, NJ), and 10 or 100 nM ethyl-β-carboline-3-carboxylate (β-CCE) (RBI). Stock solutions of the three drugs were dissolved in dimethyl sulfoxide and diluted in CFFR immediately before application. The percentage of dimethyl sulfoxide was never greater than 0.02% and had no effect on GABA-induced current.

We infer that an irreversible change in the GABA-induced currents after $pCMBS^-$ application is due to the covalent modification of a Cys by $pCMBS^-$. The percentage effect of $pCMBS^- = \{(I_{GABA,\ after}/I_{GABA,\ before}) - 1\} \times 100$. $I_{GABA,\ after}$ is the average peak current of the two GABA test pulses after the application of $pCMBS^-$, and $I_{GABA,\ before}$ is the average of the peak current of the initial two GABA applications. Test pulses of GABA were applied at two concentrations, EC_{50} and more than 5 times EC_{50} (near-saturating GABA, generally 100 μM). Changes in the peak current induced by the EC_{50} GABA test pulses are more sensitive to effects of $pCMBS^-$ modification on gating kinetics, whereas changes in the peak current induced by the near-saturating GABA test pulses are more sensitive to effects of modification on conductance (Williams and Akabas, 1999).

For screening experiments, $pCMBS^-$ was applied for 1 min at 0.5 mM. This combination of time and concentration were chosen because they were the maximal concentration and duration that caused no significant increase in the leak conductance of uninjected *Xenopus* oocytes. This limits our ability to detect reactive residues. As discussed below, for a given mutant, given the variability of responses, application of a reagent must cause a net change in current greater than ~30% to be statistically significantly different from wild type by a one-way ANOVA (for n between 4 and 6). Given this threshold and the reaction conditions that we used for $pCMBS^-$, 0.5 mM applied for 1 min, if complete reaction caused 100% inhibition of the GABA-induced current, we would detect as reactive positions with a second order reaction rate >12 l/mol-s.

Measurement of Reaction Rates. Rates of reaction of $pCMBS^-$ with the engineered Cys mutants were determined by the effect of sequential brief applications of $pCMBS^-$. A test pulse of GABA was applied to measure the GABA-induced current. GABA plus $pCMBS^-$ (0.2–0.5 mM) was applied for 15 to 60 s. After washout of $pCMBS^-$, a test pulse of GABA was applied, and the GABA-induced current was measured. The effect of five to eight brief, sequential applications of $pCMBS^-$ was determined. The magnitudes of the GABA test currents were normalized relative to the initial test pulse. The normalized current was plotted as a function of the cumulative duration

of $pCMBS^-$ treatment and fitted with a single exponential function using Prism2 software (GraphPad, San Diego, CA). The second order rate constant was calculated by dividing the pseudo-first order rate constant obtained from the exponential fit by the $pCMBS^-$ concentration.

Statistics and Curve Fitting. Data are expressed as the percentage changes of current after modification ± S.E.M. The significance of differences between each mutant and wild type was determined by one-way ANOVA using the Student-Newman-Kuels post hoc test (SPSS for Windows, SPSS, Inc., Chicago, IL). Dose-response curves were fit using Prism2 software.

It is important to recognize that $pCMBS^-$-reactive residues are identified based on the functional effect of modification. Functional effects are determined by the statistical significance of the effect on a mutant relative to the effect on wild type. For mutants for which the average effect after application of $pCMBS^-$ is small, whether the effect is judged to be significant depends, in part, on the stringency of the one-way ANOVA post hoc test used. In our previous work on the α_1 M3 segment, using the Student-Newman-Kuels post hoc test to determine significance of effects, we found that the effect of $pCMBS^-$ applied in the presence of GABA was significant at six residues, α_1A291C, α_1Y294C, α_1F298C, α_1A300C, α_1L301C, and α_1E303C. With the less stringent Duncan post hoc test, an additional residue α_1F296C would be judged to be reactive with $pCMBS^-$ applied in the presence of GABA (Williams and Akabas, 1999). The choice of post hoc test is, unfortunately, somewhat arbitrary. Thus, for Cys mutants for which the effects of complete reaction are small, it may be difficult to determine functionally whether reaction has occurred. In the presence of GABA, α_1F296C is such a case for which determining whether it is reactive is difficult because reaction may cause only a small effect on subsequent GABA-induced currents.

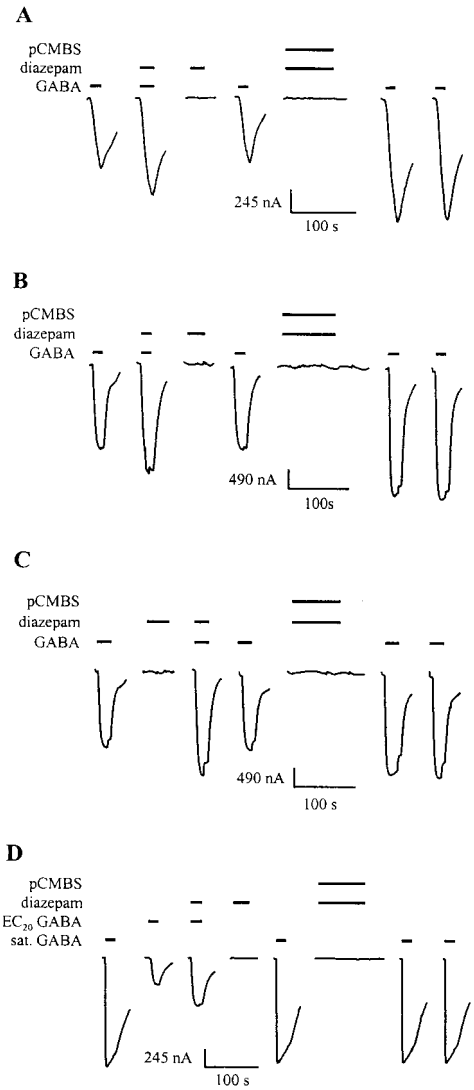

Fig. 1. Effect of a 1-min coapplication of 0.5 mM $pCMBS^-$ and 100 nM diazepam on M3 segment Cys-substitution mutants. Currents were recorded from individual oocytes expressing α_1F296C (A), α_1F298C (B), α_1L301C (C), and α_1E303C (D). The initial four traces for each mutant demonstrate that 100 nM diazepam potentiates GABA-induced currents and that diazepam alone does not induce a current. The final four traces for each mutant show the effect of a 1-min application of $pCMBS^-$ and diazepam on the subsequent GABA-induced currents. For the mutants α_1F296C (A), α_1F298C (B), and α_1L301C (C), subsequent currents were potentiated. For α_1E303C (D), the subsequent currents were unchanged. The concentration of the GABA test pulses was 1 μM (A), 30 μM (B), and 30 μM (C). In D, the EC_{20} GABA concentration was 1 μM and the saturating GABA concentration was 100 μM. Bars above current traces indicate the period of application of the reagent indicated to the left. Traces are separated by 3- to 5-min washes with CFFR buffer to allow recovery from desensitization.

Results

Reactions with $pCMBS^-$ Applied in the Presence of Diazepam. The EC_{50} for GABA activation of wild-type $\alpha_1\beta_1\gamma_2$ receptors was 2.1 ± 1.3 μM (n = 6) and for the Cys-substitution mutants ranged between 0.5 ± 0.1 μM for α_1A291C and α_1Y294C and 48 ± 15 μM for α_1L301C as reported previously (Williams and Akabas, 1999). For wild-type receptors, 100 nM diazepam potentiated the currents induced by an EC_{30} GABA concentration by 26 ± 3% (n = 3). For the Cys-substitution mutants, potentiation by 100 nM diazepam ranged between 21 ± 3% (n = 4) for α_1V297C and 73 ± 24% (n = 4) for α_1Y294C (Table 1). Application of 100 nM diazepam alone did not elicit a current in wild type or any of the Cys-substitution mutants.

For wild-type, a 1-min application of 0.5 mM $pCMBS^-$ in the presence or in the absence of 100 nM diazepam had no effect on subsequent currents induced by either near-saturating or EC_{50} GABA concentrations (Table 2). As we reported previously, for the Cys-substitution mutants between α_1Ala291 and α_1Val307, a 1-min application of 0.5 mM $pCMBS^-$ in the absence of GABA reacted with only α_1A291C and α_1Y294C (Williams and Akabas, 1999). These two residues were accessible both in the absence and in the presence of GABA (Williams and Akabas, 1999). At the other M3 Cys-substitution mutants, $pCMBS^-$, applied in the absence of GABA, did not effect subsequent GABA-induced currents.

TABLE 1

Percentage potentiation by 100 nM diazepam of currents induced by EC_{50} GABA concentration for wild type and the Cys-substitution mutants

Mutant	Diazepam-induced potentiation	GABA EC_{50}[a]
	%	μM
Wild type	26 ± 3	2.1 ± 1.3
Y294C	73 ± 24	4.9 ± 1.6
F296C	54 ± 16	1.4 ± 0.4
V297C	21 ± 3	2.4 ± 0.6
F298C	38 ± 18	28 ± 4.0
S299C	22 ± 2	4.5 ± 0.2
A300C	39 ± 1	3.5 ± 0.5
L301C	52 ± 31	48 ± 15
I302C	27 ± 15	1.9 ± 0.8
E303C	37 ± 17	1.4 ± 0.3

[a] Data from Williams and Akabas (1999).

In contrast, a 1-min application of 0.5 mM $pCMBS^-$ and 100 nM diazepam caused significant potentiation of the subsequent currents induced by EC_{50} concentration GABA test pulses for the mutants α_1F296C, α_1F298C, and α_1L301C (Fig. 1 and Table 2). At α_1F298C and α_1L301C, the extent of potentiation by a 1-min application of 0.5 mM $pCMBS^-$ was similar in the presence of diazepam and in the presence of GABA (Table 2). In contrast, at α_1F296C, the effect of $pCMBS^-$ modification in the presence of diazepam had the opposite effect on the subsequent GABA-induced currents as modification in the presence of GABA. The subsequent GABA-induced currents were potentiated by 24 ± 2% after application of 0.5 mM $pCMBS^-$ in the presence of diazepam but were inhibited by −23 ± 5% after application of 0.5 mM $pCMBS^-$ in the presence of GABA (Table 2; Williams and Akabas, 1999). This suggests that the position of the Cys substituted for α_1Phe296 is different in the presence of diazepam and GABA. Thus, modification by $pCMBS^-$ has a different effect on subsequent GABA-induced currents.

At the other two positions that reacted with $pCMBS^-$ in the presence of GABA, α_1A300C and α_1E303C, a 1-min application of 0.5 mM $pCMBS^-$ in the presence of 100 nM diazepam had no effect on the subsequent GABA-induced currents (Fig. 1D and Table 2). Thus, of the five positions that were accessible only in the presence of GABA, three were also accessible in the presence of diazepam, although at one of these positions the effect of modification was opposite when modified in the presence of diazepam compared with the presence of GABA.

At the intervening positions in the α_1 M3 segment, α_1V297C, α_1S299C, and α_1I302C, that were not accessible either in the presence or in the absence of GABA, there was no effect of a 1-min application of 0.5 mM $pCMBS^-$ plus 100 nM diazepam on subsequent GABA-induced currents (data not shown). We infer that $pCMBS^-$ did not react with these residues in the presence of diazepam, but negative results must be interpreted with caution. An alternative explanation that cannot be excluded is that $pCMBS^-$ reacted but had no functional effect on the receptor.

We infer that engineered Cys residues that react with $pCMBS^-$ are on the water-accessible surface of the protein, at least part of the time, because $pCMBS^-$ reacts 1000 times faster with ionized thiolates compared with un-ionized thiols (Hasinoff et al., 1971), and only thiols on the water-accessible protein surface will ionize to a significant extent. In addition, $pCMBS^-$ is membrane impermeant, and thus, when applied extracellularly, it will have access only to residues that are on the extracellular, water-accessible surface of the protein (VanSteveninck et al., 1965; Olami et al., 1997). Furthermore, reactivity of substituted Cys was shown to be well correlated with calculated solvent accessibility in the aspartate chemotaxis receptor (Danielson et al., 1997). Thus, we infer that diazepam induced a conformational change that moves α_1Phe296, α_1Phe298, and α_1Leu301 onto the extracellular, water-accessible surface of the receptor.

Reaction of $pCMBS^-$ Applied in the Presence of the Inverse Agonist β-CCE. Application of 100 nM β-CCE alone did not elicit a current in wild type or any of the Cys-substitution mutants; nor did it have an effect on subsequent GABA-induced currents. Coapplication of 100 nM β-CCE had no significant effects on the current induced by an EC_{30} GABA concentration in oocytes expressing either wild type or the Cys-substitution mutants (data not shown).

A 1-min application of 0.5 mM $pCMBS^-$ in the presence of 100 nM β-CCE had no effect on the subsequent GABA-induced currents of wild type or of any of the Cys-substitution mutants except α_1F298C (Fig. 2A and Table 2). For α_1F298C, a 1-min application of 100 nM β-CCE and 0.5 mM $pCMBS^-$ caused 45 ± 11% (n = 3) potentiation of the subsequent currents elicited by GABA applied at an EC_{50} concentration. Therefore, for α_1F298C, we also tested the effect of a lower concentration of β-CCE, 10 nM. A 1-min application of 10 nM β-CCE and 0.5 mM $pCMBS^-$ altered subsequent GABA-induced currents by 9 ± 3% (n = 3); an amount not significantly different from wild type.

Reaction of $pCMBS^-$ Applied in the Presence of Flumazenil. At concentrations below 10 nM, flumazenil behaves as a benzodiazepine antagonist; however, at concentrations greater than 1 μM, it potentiates GABA-induced currents (Chan and Farb, 1985; Sigel and Baur, 1988; Mehta and Ticku, 1989; Yakushiji et al., 1989; Stevenson et al., 1995). For wild-type and the Cys-substitution mutants, application of 100 nM flumazenil in the absence of GABA did not elicit any current. After washout of flumazenil, there were no effects on subsequent GABA-induced currents (data not shown). Furthermore, coapplication of 100 nM flumazenil with submaximal GABA concentrations did not significantly alter the magnitude of the GABA-induced currents (data not shown).

Surprisingly, a 1-min application of 0.5 mM $pCMBS^-$ in the presence of 100 nM flumazenil irreversibly potentiated the subsequent GABA-induced currents at α_1F298C and

TABLE 2

Percentage change in the subsequent GABA-induced currents due to a 1-min application of 0.5 mM $pCMBS^-$ in the presence of the indicated reagent[a]

Data given as means ± S.E.M. (n).

	Saturating GABA[a]	100 nM Diazepam	100 nM β-CCE	100 nM Flumazenil
WT[b]	−12 ± 3 (8)	5 ± 5 (4)	5 ± 3 (3)	4 ± 4 (3)
F296C[b]	−23 ± 5* (10)	24 ± 2* (8)	−5 ± 8 (3)	5 ± 2 (3)
F298C[b]	30 ± 6* (9)	36 ± 12* (6)	45 ± 11[c]* (3)	20 ± 6* (6)
L301C[b]	37 ± 9* (8)	40 ± 10* (5)	3 ± 3 (3)	52 ± 13* (5)
WT[d]	−4 ± 2 (8)	−2 ± 3 (3)	3 ± 6 (3)	1 ± 4 (3)
A300C[d]	45 ± 6* (11)	4 ± 8 (3)	4 ± 9 (3)	10 ± 6 (3)
E303C[d]	−32 ± 4* (20)	5 ± 9 (4)	12 ± 3 (3)	3 ± 1 (3)

[a] Results in the presence of saturating GABA taken from Williams and Akabas (1999).
[b] Effect on EC_{50} GABA test pulses.
[c] For F298C, the effect was 9 ± 3% in the presence of 10 nM β-CCE.
[d] Effect on near-saturating GABA test pulses.
* Statistically different than corresponding wild type by one-way ANOVA.

α_1L301C by 20 ± 6% (n = 6) and 52 ± 13% (n = 5), respectively (Fig. 2, B and C, and Table 2). We also tested the reactivity of these two mutants in the presence of 10 nM flumazenil. At these two positions, we also tested the effect of a 1-min application of 0.5 mM pCMBS$^-$ in the presence of 10 nM flumazenil. For α_1F298C, the subsequent GABA-induced currents were potentiated by 9 ± 7% (n = 3), which is not significantly different from wild type. For α_1L301C, the subsequent currents were potentiated still by 52 ± 9% (n = 5). Thus, we infer that flumazenil at both 10 and 100 nM concentrations increased the reactivity of the Cys at position α_1Leu301. None of the other M3 Cys-substitution mutants between α_1Tyr294 and α_1Glu303 reacted with pCMBS$^-$ applied in the presence of 100 nM flumazenil.

Reaction Rates of pCMBS$^-$ with Accessible Cys-Substitution Mutants. The reaction rates of sulfhydryl reagents with substituted Cys residues depend on multiple factors, including time-averaged water surface accessibility, local steric factors, and local reagent concentration. In the aspartate chemotaxis receptor, reactivity of substituted Cys was well correlated with solvent accessibility calculated from the crystal structure (Danielson et al., 1997). Thus, the pCMBS$^-$ reaction rates with the reactive Cys-substitution mutants were measured in the presence of GABA, diazepam, flumazenil, and β-CCE.

For the mutants α_1F298C and α_1L301C, the pCMBS$^-$ reaction rates in the presence of 100 nM diazepam and flumazenil were of similar magnitude to the reaction rates in the presence of GABA (Fig. 3 and Table 3). This suggests that these reagents cause a similar increase in the accessibility of these substituted Cys residues as the GABA-induced conformational change. These mutants did not react with pCMBS$^-$ in the absence of GABA.

The residue with the fastest rate of reaction in the presence of GABA, α_1E303C, did not react in the presence of diazepam (Table 3). This suggests that the conformation induced by diazepam is not merely a short-lived version of a GABA-induced conformation because in that case one would

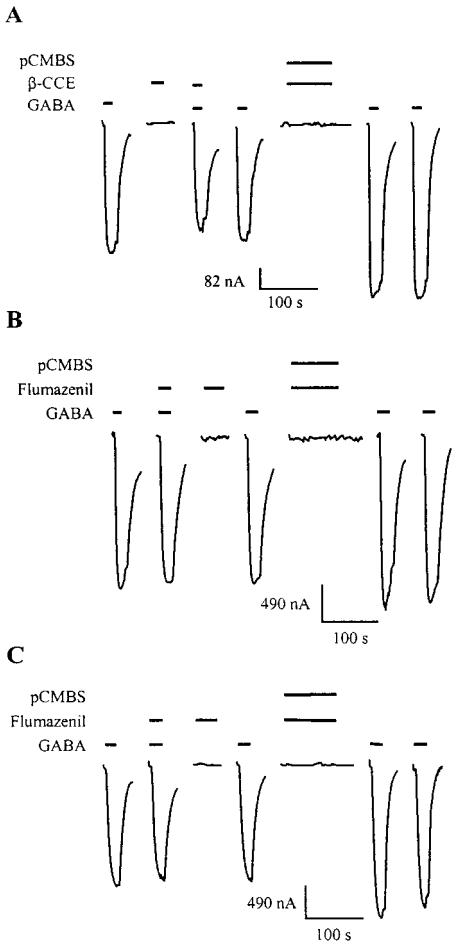

Fig. 2. Effect of a 1-min application of 0.5 mM pCMBS$^-$ applied in the presence of β-CCE or flumazenil on the subsequent GABA-induced currents for the mutants α_1F298C and α_1L301C. Currents were recorded from oocytes expressing α_1F298C (A and B) and α_1L301C (C). The initial four traces for each mutant demonstrate that 100 nM β-CCE (A) or 100 nM flumazenil (B and C) did not significantly effect the GABA-induced currents and that the drugs alone did not induce a current. The final four traces for each mutant show the effect of a 1-min application of pCMBS$^-$ and drug on the subsequent GABA-induced currents. In all three cases the subsequent currents were potentiated. The GABA concentration was 30 μM. Bars above current traces indicate the period of application of the reagent indicated to the left. Traces are separated by 3- to 5-min washes with CFFR buffer to allow recovery from desensitization.

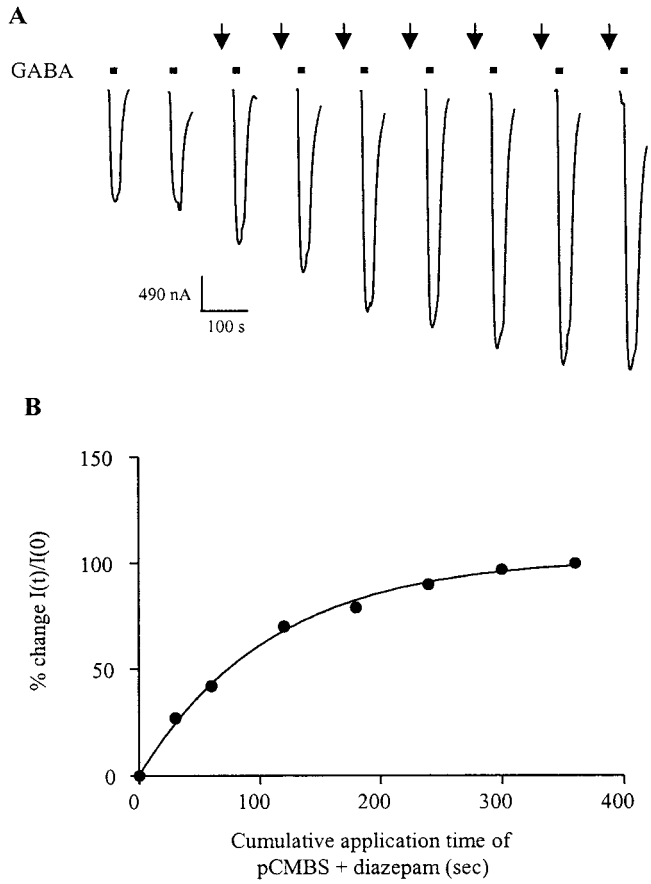

Fig. 3. Measurement of rate of reaction of pCMBS$^-$ applied in the presence of 100 nM diazepam. A, currents recorded from an oocyte expressing α_1F298C. Bars indicate period of application of GABA test pulses; arrows indicate application of 0.5 mM pCMBS$^-$ plus 100 nM diazepam. The first two applications were for 30 s, and the subsequent five applications were for 60 s each. Current traces during application of pCMBS$^-$ and diazepam are not shown. Each application of GABA and drugs were separated by 3-min washes with CFFR. B, plots of the percentage change in the GABA test peak currents relative to the initial peak currents as a function of the cumulative duration of application of pCMBS$^-$ plus diazepam. The smooth line is a single exponential fit of the data.

have expected reaction with α_1E303C as well as at the other positions.

Two Cys mutants, α_1A291C and α_1Y294C, near the extracellular end of the M3 segment, reacted with pCMBS$^-$ both in the absence and in the presence of GABA (Williams and Akabas, 1999). At α_1A291C, the pCMBS$^-$ reaction rate was similar in the absence and in the presence of GABA (Table 3). Thus, we could not distinguish whether diazepam altered the conformation in the region of this residues. At α_1Tyr294 the pCMBS$^-$ reaction rate was 2 times faster in the presence of GABA than in the absence of GABA: in the presence of diazepam, the rate of reaction was similar to the rate in the absence of GABA (Table 3). This implies that diazepam does not alter the reactivity of α_1Y294C, and therefore, we infer that it does not alter the conformation of α_1Y294C in a manner similar to GABA.

Discussion

In the presence of diazepam, pCMBS$^-$ reacted with Cys substituted for three residues in the M3 membrane-spanning segment, α_1Phe296, α_1Phe298, and α_1Leu301. In the absence of diazepam or GABA, pCMBS$^-$ did not react at a measurable rate with any of these Cys-substitution mutants. This implies that diazepam induced a conformational change in the $GABA_A$ receptor α_1-subunit membrane-spanning domain to increase the reactivity of the engineered Cys at these positions. The residues accessible in the presence of diazepam are a subset of the M3 segment residues that became accessible in the presence of GABA (Table 4). In the presence of GABA, pCMBS$^-$ reacted with the three M3 segment, diazepam-accessible residues plus two others, α_1Ala300 and α_1Glu303 (Williams and Akabas, 1999).

Several pieces of evidence suggest that pCMBS$^-$ reaction in the diazepam-bound conformation is not occurring because of a diazepam-induced increase in the spontaneous open probability of the receptor. First, at the three reactive residues the reaction rates were similar in the presence of diazepam and GABA. This suggests that the reactive engineered Cys residues were spending a similar percentage of time on the water-accessible surface with diazepam and GABA. Second, the residue that reacted fastest in the presence of GABA, α_1E303C, did not react in the presence of diazepam. If reaction were occurring in a short-lived state structurally similar to those occurring in the presence of GABA this residue should have reacted. Third, the effect of pCMBS$^-$ modification of α_1F296C was different in the presence of GABA and diazepam. In the presence of GABA, covalent modification of α_1F296C caused inhibition of subsequent GABA-induced currents, whereas in the presence of diazepam, modification caused potentiation of subsequent currents. Thus, pCMBS$^-$ modification appeared to trap the receptor in distinct conformations depending on whether pCMBS$^-$ was applied in the presence of diazepam or GABA. We infer that the position of α_1F296C relative to the rest of the protein is different in the presence of the two reagents. A similar phenomenon, opposite effects on subsequent currents, was observed when the nicotinic acetylcholine receptor mutant αS252C was covalently modified in the presence and in the absence of acetylcholine (Akabas et al., 1994). Thus, we conclude that diazepam binding induces a new conformation of the receptor that is structurally distinct from the conformations induced by GABA binding.

Our previous results suggested that GABA-induced gating produced changes in the conformations of both the M2 and M3 membrane-spanning segments (Xu and Akabas, 1996; Williams and Akabas, 1999). In addition to opening the channel, this GABA-induced conformational change appears to create a water-filled crevice into the interior of the membrane-spanning domain to expose the residues in the M3 segment and allow pCMBS$^-$ penetration into the interior of the membrane-spanning domain. Creation of this water-filled crevice suggests that the membrane-spanning segments are less tightly packed in the presence of GABA and diazepam compared with the closed state of the receptor. Whether this crevice extends from the extracellular surface or from the channel lumen is uncertain (Williams and Akabas, 1999). The diazepam-induced conformational change creates a more limited water-filled crevice that also extends into the interior of the membrane-spanning domain in the region of the α_1 M3 segment, thus allowing pCMBS$^-$ access to only three of the M3 residues. This more limited diazepam-induced conformational change is insufficient to open the channel gate. The diazepam-bound conformation may represent an intermediate state between the closed and open states: the conformation of the GABA-binding site has been shifted to the higher affinity state and the membrane-spanning domain has undergone a portion of the changes induced by GABA activation. These conformational changes would permit easier activation by GABA and suggests that diazepam may increase the spontaneous open probability of the channel.

The transduction of benzodiazepine binding in the extracellular domain to the conformational changes in the vicinity of the M3 segment may involve interactions between the extracellular domain and the M2-M3 loop. Consistent with this hypothesis, Boileau and Czajkowski (1999) reported that benzodiazepine potentiation, but not binding, was reduced by mutation of three residues in the γ_2-subunit M2-M3 loop. Similar interactions also appear to be important for agonist efficacy. Mutations of M2-M3 loop residues altered agonist

TABLE 3

Second order reaction rates (l/mol-s) of pCMBS$^-$ applied in the presence of the indicated reagent with the Cys-substitution mutants
Data given as means ± S.E.M. (*n*).

	No GABA	Saturating GABA	100 nM Diazepam	100 nM Flumazenil
A291C	353 ± 162 (3)	274 ± 118 (3)	N.D.	N.D.
Y294C	44 ± 4 (3)	153 ± 35 (4)	50 ± 8 (3)	53 ± 6 (3)
F296C	—	N.D.	47 ± 22 (3)	N.D.
F298C	—	36 ± 7 (4)	41 ± 12 (4)	46 ± 11 (3)
L301C	—	29 ± 4 (3)	61 ± 15 (3)	28 ± 9 (3)
E303C	—	198 ± 58 (5)	—	—

N.D., not determined; —, no reaction.

efficacy in the GABA, glycine, and acetylcholine receptors (Campos-Caro et al., 1996; Lynch et al., 1997; Sigel et al., 1999). A further indication of the importance of interactions between the M2 and M3 segments in channel gating is that mutation of residues near the extracellular ends of the $GABA_A$ receptor M2 and M3 segments, aligned with α_1Ser270 and α_1Ala291, alter the ability of general anesthetics to potentiate GABA-induced currents (Belelli et al., 1997; Mihic et al., 1997). These two residues are on the water-accessible surface of the protein (Williams and Akabas, 1999), although α_1Ser270 is not on the channel-lining face of the M2 segment (Xu and Akabas, 1996). Thus, these two residues may form a portion of the lining of a water-filled crevice that extends into the interior of the protein. Anesthetics may act by intercalating into the water-filled crevice between these two segments, destabilizing interactions between the M2 and M3 segments, thereby initiating a conformational change similar to that induced by GABA (Belelli et al., 1997; Mihic et al., 1997). Alternatively, these residues may be part of the machinery that transduces the effects of anesthetic binding rather than part of an anesthetic binding site. Further studies will be necessary to determine whether potentiating concentrations of general anesthetics induced conformational changes similar to those induced by diazepam or whether the molecular basis of potentiation by different drugs is different.

It was surprising that in the presence of both flumazenil and β-CCE $pCMBS^-$ reacted with some of the M3 substituted Cys residues that were accessible in the presence of diazepam. In the presence of 100 nM flumazenil, $pCMBS^-$ reacted with α_1F298C and α_1L301C. In the presence of a 10-fold lower flumazenil concentration (10 nM) $pCMBS^-$ still reacted with α_1L301C but not with α_1F298C (Table 2). At low concentrations, below 10 nM, flumazenil acts as a benzodiazepine antagonist, but at higher concentrations (>1 μM), it acts as a weak potentiator of GABA-induced currents (Chan and Farb, 1985; Sigel and Baur, 1988; Mehta and Ticku, 1989; Yakushiji et al., 1989). In addition, some experiments have suggested that even at low concentrations flumazenil is not a strict antagonist (Chiu and Rosenberg, 1983). The potentiating activity of flumazenil presumably results from binding at a site distinct from the benzodiazepine-binding site. The location of the potentiating binding site has not been identified. It is uncertain whether the conformational changes induced by flumazenil occur because of binding at the benzodiazepine site or at the other site; however, we did not observe an effect of 100 nM flumazenil on the GABA-induced current, although this concentration was sufficient to increase the accessibility of the two residues. Similarly, 100 nM β-CCE increased the accessibility of α_1F298C to react with $pCMBS^-$, whereas 10 nM β-CCE did not (Table 2). β-CCE is a benzodiazepine inverse agonist at low concentrations (>10 nM) and potentiates GABA-induced currents at higher concentrations (>1 μM), consistent with binding at two sites of different affinity (Sigel and Baur, 1988; Yakushiji et al., 1989; Stevenson et al., 1995). Thus, for flumazenil and β-CCE, it is not possible to determine which binding site is mediating their actions to increase the accessibility of these M3 residues. Further complicating the interpretation of the flumazenil and β-CCE results is the possibility that some of the Cys mutations might alter the affinity or the efficacy of action at one binding site but not at the other. Alternatively, flumazenil binding at the benzodiazepine-binding site may induce conformational changes in the membrane-spanning domains that are not sufficiently extensive to result in alteration of GABA-induced currents but are sufficient to alter the accessibility of some of the M3 substituted Cys mutants. This raises the question as to whether flumazenil is a strict benzodiazepine antagonist causing no conformational change but blocking the binding site, or whether flumazenil binding does alter the receptor conformation but the energy imparted to the receptor by flumazenil binding is insufficient to cause the total conformational change necessary for potentiation of GABA-induced currents.

In summary, diazepam binding induces a conformational change in the $GABA_A$ receptor. This conformational change extends into the membrane-spanning domain of the receptor. Diazepam does not simply induce a state similar to one of the predominant GABA bound states, open or desensitized. Rather, our results indicate that the diazepam bound state is a distinct structural state of the receptor. This has important implications for potential kinetic models of the actions of benzodiazepines on $GABA_A$ receptors.

TABLE 4
Summary of the effect on subsequent GABA-induced currents of $pCMBS^-$ application in the presence of various reagents

Mutant	−GABA[a]	+GABA[a]	+Diazepam
WT	—	—	—
Y294C	↓	↓	↓
F296C	—	↓	↑
V297C	—	—	—
F298C	—	↑	↑
S299C	—	—	—
A300C	—	↑	—
L301C	—	↑	↑
I302C	—	—	—
E303C	—	↓	—

—, no effect; ↑, potentiation of subsequent GABA-induced currents; ↓, inhibition of subsequent GABA-induced currents.
[a] Results in the presence and in the absence of GABA taken from Williams and Akabas (1999).

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