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Defining the Propofol Binding Site Location on the $GABA_A$ Receptor

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ABSTRACT

The $GABA_A$ receptor is a target of many general anesthetics. The low affinity of general anesthetics has complicated the search for the location of anesthetic binding sites. Attention has focused on two pairs of residues near the extracellular ends of the M2 and M3 membrane-spanning segments, α_1Ser270/β_2Asn265 (15′ M2) and α_1Ala291/β_2Met286 (M3). In the 4-Å resolution acetylcholine receptor structure, the aligned positions are separated by ~10 Å. To determine whether these residues are part of a binding site for propofol, an intravenous anesthetic, we probed propofol's ability to protect cysteines substituted for these residues from modification by the sulfhydryl-specific reagent *p*-chloromercuribenzenesulfonate ($pCMBS^-$). $pCMBS^-$ reacted with cysteines substituted at the four positions in the absence and presence of GABA. Because propofol binding induces conformational change in the $GABA_A$ receptor, we needed to establish a reference state of the receptor to compare reaction rates in the absence and presence of propofol. We compared reaction rates in the presence of GABA with those in the presence of propofol + GABA. The GABA concentration was reduced to give a similar fraction of the maximal GABA current in both conditions. Propofol protected, in a concentration-dependent manner, the cysteine substituted for β_2Met286 from reaction with $pCMBS^-$. Propofol did not protect the cysteine substituted for the aligned α_1 subunit position or the 15′ M2 segment Cys mutants in either subunit. We infer that propofol may bind near the extracellular end of the β subunit M3 segment.

General anesthetic administration induces a state characterized by loss of consciousness, amnesia, analgesia, and immobility (Yamakura et al., 2001). Early anesthetic action theories hypothesized a lipid bilayer interaction, but recent findings indicate that anesthetics have low-affinity interactions with specific protein targets (Franks and Lieb, 1994; Krasowski and Harrison, 1999). Many anesthetics target $GABA_A$ receptors; some, however, such as ketamine, α_2 adrenergic agonists, and xenon, target other receptors (Franks et al., 1998; Belelli et al., 1999; Miller, 2002; Nelson et al., 2003). Thus, there may be multiple neural pathways to achieve general anesthesia. These may converge on the tuberomamillary nucleus (Nelson et al., 2002, 2003).

Intravenous anesthetic effects on $GABA_A$ receptors are concentration-dependent. At low concentrations, GABA-active anesthetics potentiate submaximal GABA-induced currents. At higher concentrations, they directly open channels in the absence of GABA (Belelli et al., 1999; Yamakura et al., 2001). At even higher concentrations, some anesthetics inhibit currents.

$GABA_A$ receptors are formed by five homologous subunits assembled around the central channel (Hevers and Luddens, 1998; Moss and Smart, 2001). A common subunit stoichiometry is 2α:2β:1γ subunits (Im et al., 1995; Sieghart and Sperk, 2002). Each subunit has an ~200-amino acid extracellular N-terminal domain and a C-terminal domain with four membrane-spanning segments (M1, M2, M3, M4). The extracellular domain structure is similar to that of the homologous acetylcholine binding protein, with the GABA-binding sites located at the β-α subunit interfaces (Brejc et al., 2001; Newell and Czajkowski, 2003). The 4-Å resolution structure of the homologous acetylcholine receptor (AChR) (Miyazawa et al., 2003) confirms that the channel is principally lined by the five M2 segments (Xu and Akabas, 1996) that we showed are two α helical turns longer than predicted by hydrophobicity analysis (Bera et al., 2002).

Potential anesthetic binding site residues have been identified. The amino acid in the β subunit M2 segment 15′ position,[1] $\beta_{2/3}$265, determines etomidate's efficacy (Belelli et al., 1997). Etomidate's anesthetic efficacy is eliminated in

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[1] Residues in the M2 segment are identified using an index numbering system to facilitate comparisons with other subunits in the gene superfamily. The 0′ residue is an absolutely conserved positively charged residue near the cytoplasmic end of M2, α_1Arg255 and β_2Arg250.

ABBREVIATIONS: AchR, acetylcholine receptor; propofol, 2,6-di-isopropylphenol; $pCMBS^-$, *p*-chloromercuribenzenesulfonate; CFFR, calcium-free frog Ringer's; MMTS, methylmethanethiosulfonate; MTS, methanethiosulfonate.

β_3N265M knock-in mice (Jurd et al., 2003). This residue is on the protein-facing side of M2 (Xu and Akabas, 1996; Miyazawa et al., 2003). Mutations at aligned positions α_1Ala291 and β_2M286, near the M3 segment extracellular end, also affect anesthetic efficacy (Mihic et al., 1997). Mutations at the two α subunit positions affect volatile anesthetic efficacy, whereas mutations at the two β subunit positions affect intravenous anesthetic efficacy (Belelli et al., 1997; Mihic et al., 1997; Mascia et al., 2000; Krasowski et al., 2001; Nishikawa et al., 2002). The residues aligned with these two positions are separated by ~10 Å in the AChR structure (Fig. 1) (Miyazawa et al., 2003). Given the separation, it is unclear whether they form part of the same anesthetic binding site.

We sought to determine which of these residues participate in forming a binding site for the intravenous anesthetic 2,6-di-isopropylphenol (propofol). We investigated two pairs of aligned positions α_1Ser270 and β_2Asn265 in M2 (15′), and α_1Ala291 and β_2Met286 in M3. We used cysteine (Cys) accessibility experiments to determine whether propofol could protect a Cys substituted for these residues from modification by the sulfhydryl-reactive reagent *p*-chloromercuribenzenesulfonate ($pCMBS^-$). The $pCMBS^-$ reaction rate with an engineered Cys depends on two major factors: 1) accessibility of the Cys to bulk solution and 2) reactivity of the Cys with the sulfhydryl reagents. Accessibility depends on steric and electrostatic factors in the access pathway from bulk solution to the site of the Cys. Reactivity depends on the local Cys environment; factors that affect reaction rate include steric constraints and Cys ionization state, which is influenced by fractional time on the water-accessible surface and local electrostatic potential (Karlin and Akabas, 1998). Similar approaches have identified other ligand binding sites (Xu et al., 1995; Pascual and Karlin, 1998; Shi and Javitch, 2002).

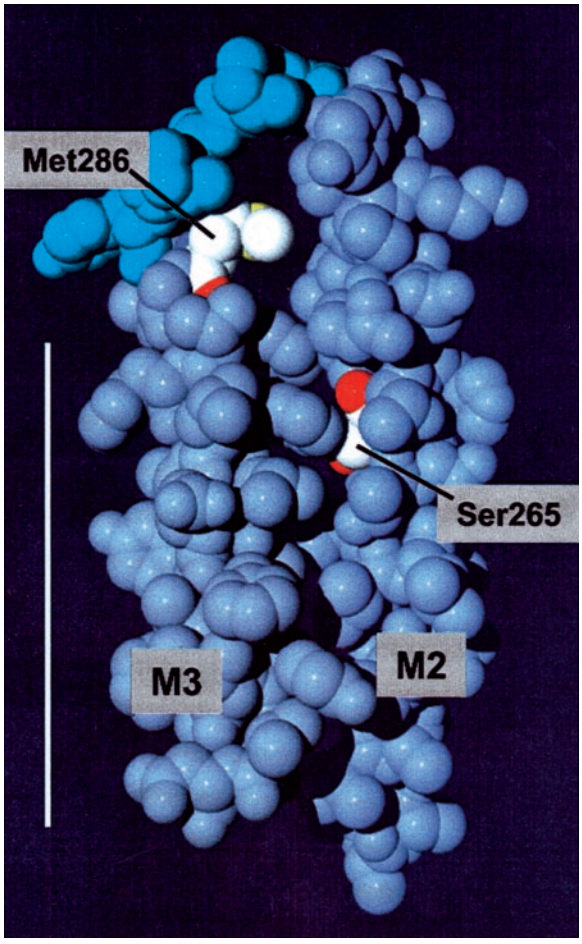

Fig. 1. Homology model structure of the $GABA_A$ receptor β_1 subunit M2 and M3 membrane-spanning segments based on the 4-Å resolution cryo electron microscopic structure of the nicotinic acetylcholine receptor (Miyazawa et al., 2003). The M2 residue β_1Ser265, aligned with β_2Asn265, and the M3 residue β_1Met286 are shown in Corey/Pauling/Koltun (CPK) color scheme: carbons, white; oxygen, red; nitrogen, blue. The rest of the M2 and M3 residues are colored blue-gray. The non–α-helical M2-M3 loop residues are colored blue. The white line at the left indicates the position of the membrane. Note that β_1Ser265 and β_1Met286 are separated by ~10 Å.

A fundamental issue in conducting these experiments was to define a reference state that allowed comparison of the $pCMBS^-$ reaction rates in the absence and presence of propofol. This issue arose because we have shown that propofol binding induces a conformational change that alters reaction rates with engineered M3 Cys (Williams and Akabas, 2002). Thus, we could not compare the reaction rates in propofol's absence and presence. Our solution was to compare reaction rates in the presence of GABA with those in the presence of propofol + GABA, where the GABA concentration was adjusted to give a similar fraction of the maximal GABA current in both conditions. The similarity of the single channel characteristics of GABA- and GABA + propofol-activated channels (Hales and Lambert, 1991; Orser et al., 1994) implies that the open channel structures are also similar. Our experiments indicate that propofol protects β_2M286C from modification by $pCMBS^-$. We infer that this residue lies near the propofol-binding site.

Materials and Methods

In Vitro Transcription and Oocyte Expression. The rat $GABA_A$ receptor subunit cDNAs in the pGEMHE vector were linearized with NheI for in vitro transcription using T7 RNA polymerase (Promega, Madison, WI) (Williams and Akabas, 2002). mRNA was stored at −80°C in diethylpyrocarbamate-treated water at a concentration of 200 μg/ml. Stage V-VI *Xenopus laevis* oocytes were defolliculated by treatment with 3 mg/ml collagenase type 1A (Sigma, St. Louis, MO) for 45 min. They were washed thoroughly in OR2 (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES; pH adjusted to 7.5 with NaOH) and kept in OR3 [70% Leibovitz L-15 medium (Invitrogen, Carlsbad, CA) supplemented with 10 mM HEPES, 50 μg/ml tetracycline, and 50 μg/ml gentamicin]. Oocytes were injected 24 h after isolation with 50 nl of a 1:1:1 mixture of $\alpha_1/\beta_2/\gamma_{2S}$ subunit mRNA and were kept in OR3 medium for 2 to 4 days at 17°C. Mutant subunit mRNA was substituted for wild-type subunits where necessary.

Electrophysiological Recordings. The electrophysiological recordings were conducted at room temperature in an ~250-μl chamber continuously perfused at a rate of 5 to 6 ml/min with nominally calcium-free frog Ringer's (CFFR) solution composed of 115 mM NaCl, 2.5 mM KCl, 1.8 mM $MgCl_2$, and 10 mM HEPES, pH adjusted to 7.5 with NaOH. Buffer reservoirs were glass. Teflon perfusion tubing was used throughout. Currents were recorded from *Xenopus laevis* oocytes using two-electrode voltage-clamp recording at a holding potential of −60 mV. The ground electrode was connected to the bath via a 3 M KCl/Agar bridge. Glass microelectrodes filled with 3 M KCl had a resistance of <2 MΩ. Data were acquired and analyzed using a TEV-200 amplifier (Dagan Instruments, Minneapolis, MN), a Digidata 1322A data interface (Axon Instruments, Union City, CA), and pClamp 8 software (Axon Instruments). Currents were elicited by 10- to 20-s applications of GABA separated by at least 4

min of CFFR wash to allow complete recovery from desensitization. Currents were judged to be stable if the variation between consecutive GABA pulses was <10%.

GABA and Propofol Concentration-Response Relationships. To determine the GABA concentration-response relationship, progressively increasing GABA concentrations ranging from 0.05 to 500 μM were applied to oocytes expressing wild-type or mutant receptors. The currents were normalized to the maximal GABA-induced current. The GABA concentration-response relationship was calculated for each mutant by least-squares fitting of the currents to a logistic equation of the form: $I/I_{max} = 1/(EC_{50}{}^{nH}/ [GABA]^{nH})$, where n_H is the Hill coefficient and EC_{50} is the GABA concentration that gives rise to 50% of the maximal current. Parameters from several oocytes were averaged to obtain the mean EC_{50} and Hill coefficient (Fig. 2 and Table 1). Data are presented as mean ± S.E.M.

The propofol concentration-response relationships for potentiation and direct activation were determined using EC_{10}-EC_{20} GABA as a test concentration. The oocytes were exposed to propofol alone for 10 to 20 s and then to propofol + EC_{10}-EC_{20} GABA for 10 s. Pretreatment with propofol was shown to enhance the potentiation of GABA-induced current (Sanna et al., 1995). This procedure also allowed us to measure the current induced by propofol in the absence of GABA. Propofol applications were separated by washes lasting at least 5 min to allow for complete propofol washout and full recovery from desensitization. It should be noted that propofol removal, particularly at high concentrations, was not always perfect, probably because the oocyte membrane acts as a reservoir for this hydrophobic drug. The currents induced by propofol alone and by propofol + GABA were normalized to the GABA test pulse, which was assigned a value of 100, and were plotted as a function of propofol concentration (Fig. 3).

pCMBS$^-$ Reaction Rate. The sulfhydryl-reactive reagent used in these experiments was pCMBS$^-$. pCMBS$^-$ reacts with Cys and covalently couples -$HgC_6H_4SO_3^-$ onto the sulfhydryl. pCMBS$^-$ reacts 1000 times faster with ionized thiolates (S^-) than with thiols (SH) (Hasinoff et al., 1971); thus, reaction is much more likely with water-accessible Cys, which can ionize. For the purposes of the present studies, the covalent modification must have an effect on the channel's macroscopic properties so that we can monitor the reaction rate. Whether modification causes inhibition or potentiation of the subsequent GABA-induced current or increases the spontaneous open probability of the channel does not matter for the purposes of these experiments as long as it causes a measurable functional effect. If an engineered Cys forms part of a propofol-binding site, then the presence of propofol should reduce the ability of pCMBS$^-$ to react with the Cys, and the measured reaction rate in propofol's presence should be decreased.

The following protocol was used to measure the pCMBS$^-$ reaction rate with an engineered Cys. Once stable GABA test currents were achieved, pCMBS$^-$ was applied repeatedly in the extracellular bath in the absence or in the presence of the appropriate test solutions (GABA or propofol + GABA) for brief periods. Between each application of pCMBS$^-$, the GABA-induced current was determined. For each mutant, the pCMBS$^-$ concentration to be used was chosen based on preliminary experiments so that the reaction would proceed to completion in 1 to 2 min of cumulative pCMBS$^-$ application time. The peak GABA test currents were normalized by the initial GABA current, plotted as a function of the cumulative pCMBS$^-$ application

TABLE 1
GABA EC_{50} and Hill Coefficient, n_H, for wild type and the Cys mutants
Data are presented as mean ± S.E.M.; n is number of oocytes.

	EC_{50}	n_H	n
	μM		
Wild type	1.67 ± 0.49	1.17 ± 0.10	4
$\alpha_1\beta_2$M286Cγ_{2S}	15.07 ± 1.52	1.215 ± 0.02	4
α_1A291C $\beta_2\gamma_{2S}$	2.78 ± 0.29	1.13 ± 0.09	3
α_1S270C $\beta_2\gamma_{2S}$	2.70 ± 0.72	1.23 ± 0.08	11
$\alpha_1\beta_2$N265Cγ_{2S}	6.31 ± 0.94	0.87 ± 0.03	8

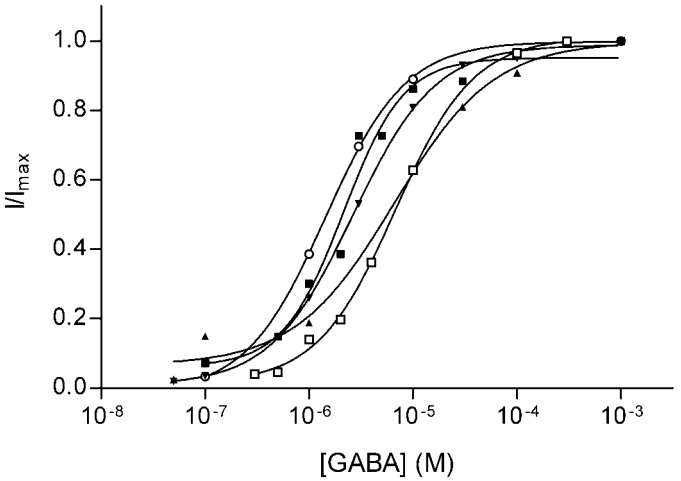

Fig. 2. GABA concentration-response relationships for wild type and the four Cys-substitution mutants. ○, wild type $\alpha_1\beta_2\gamma_{2S}$; ■, α_1S270C$\beta_2\gamma_{2S}$; ▼, α_1A291C$\beta_2\gamma_{2S}$; ▲, $\alpha_1\beta_2$N265Cγ_{2S}; □, $\alpha_1\beta_2$M286Cγ_{2S}. Solid lines are fits of the Hill equation to the data. Averaged data for GABA EC_{50}, and n for each mutant and wild type are in Table 1.

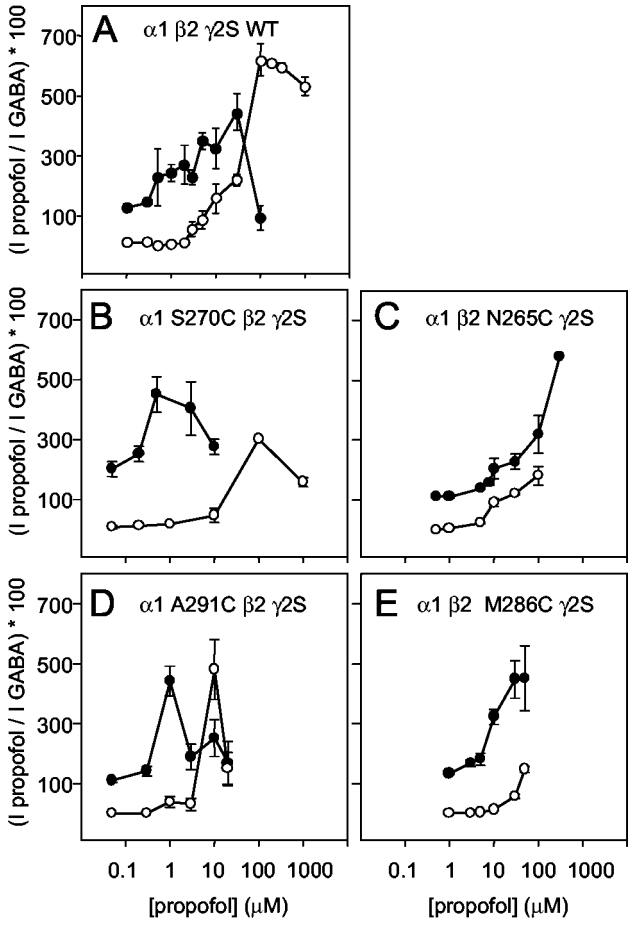

Fig. 3. Propofol concentration-response relationships for potentiation and direct activation of the four Cys mutants. ●, potentiation of GABA-induced currents. ○, direct activation current. A, wild-type; B, α_1S270C$\beta_2\gamma_{2S}$; C, $\alpha_1\beta_2$N265Cγ_{2S}; D, α_1A291C$\beta_2\gamma_{2S}$; E, $\alpha_1\beta_2$M286Cγ_{2S}. EC_{10}-EC_{20} GABA was used as a test concentration. The currents induced by propofol alone and by propofol + GABA were normalized to the GABA test pulse, which was assigned a value of 100 and are plotted as a function of propofol concentration. n = ≥3 cells for each data point.

time and fitted with a monoexponential function of the form $I = (I_0 - I_\infty)e^{-t\tau'} + I_\infty$, where I_0 is the value of the GABA-induced current amplitude before modification, I_∞ is the current amplitude at the end of the reaction, t is the cumulative $pCMBS^-$ application time, and τ' is the pseudo–first-order rate constant (s^{-1}). The second-order rate constants, τ, were calculated by dividing the pseudo–first-order time constants τ' by the $pCMBS^-$ concentration. The second-order rate constants were not dependent upon the $pCMBS^-$ concentration used in the experiments.

It should be noted that all reaction rates were well fit by a monoexponential function. For each mutant receptor, however, there are two engineered Cys residues, because the subunit stoichiometry is $2\alpha:2\beta:1\gamma$. Either the two Cys residues from each receptor reacted at the same rate, or reaction at only one residue gave the complete effect. Because of the symmetric arrangement of the receptor, the first hypothesis is the most likely, but we cannot discriminate between these two possibilities.

Chemicals. GABA (Sigma Chemical Co., St. Louis, MO) was prepared as a 100 mM stock solution in water. Propofol (ICN Biomedicals, Costa Mesa, CA) was prepared as a 100 mM stock solution in dimethyl sulfoxide. It was diluted into CFFR before each experiment. Dimethyl sulfoxide concentration was never higher than 0.1% (v/v) and did not alter the GABA-induced current at this concentration (data not shown). Methylmethanethiosulfonate (MMTS) (Sigma) was diluted in CFFR directly before use. A 5 mM $pCMBS^-$ (Sigma) stock solution was prepared in CFFR daily and diluted to the various concentrations used. MTS-tetramethylrhodamine was obtained from Biotium, Inc. (Haywood, CA).

M2-M3 Segment Structure. Using the Swiss-Model program (Schwede et al., 2003), the $GABA_A$ receptor β_2 subunit sequence for the M1 through M3 membrane-spanning segments was aligned with the corresponding region from the *Torpedo californica* AChR γ subunit, which contains the same number of residues in the M1-M2 loop as the $GABA_A$ receptor subunit. The $GABA_A$ receptor residues were then substituted for the aligned positions in the AChR atomic coordinates file (1OED) in the Protein Data Base (Miyazawa et al., 2003). Figure 1 was prepared using Deep View/Swiss-PdbViewer 3.7 (Schwede et al., 2003) and rendered with POV-Ray 3.0 (http://www.povray.org).

Results

Characterization of the Receptors. For the experiments described below, $GABA_A$ receptors formed by the coexpression of $\alpha_1\beta_2\gamma_{2S}$ subunits in *Xenopus laevis* oocytes were used. For mutant expression, the Cys-substitution mutant subunit replaced the corresponding wild-type subunit. GABA-induced currents were observed for wild type and the four Cys mutants. The mutant receptor EC_{50} values were within an order of magnitude of the wild-type receptor (Table 1 and Fig. 2). The GABA EC_{50} values of both β_2N265C and β_2M286C receptors were significantly higher than the wild-type receptor EC_{50} ($p < 0.05$). The EC_{50} values of the α_1-mutated receptors were not significantly different from wild type ($p > 0.05$).

The sensitivity of wild-type and mutant receptors to potentiation and direct activation by propofol were determined. The currents induced by propofol alone and by propofol + GABA were normalized to the GABA test pulse amplitude, which was assigned a value of 100, and were plotted as a function of propofol concentration (Fig. 3). It was not possible to calculate an EC_{50} for propofol potentiation because as the propofol concentration increased, potentiation effects ran into direct activation and inhibition, making it impossible to determine the maximum potentiation.

Propofol potentiated GABA-induced currents for wild type and all of the Cys mutants (Fig. 3, ●). The propofol concentrations that caused detectable and peak potentiation differed among the mutants (Fig. 3). In general, comparable levels of potentiation required higher propofol concentrations in the β_2 subunit mutants than in the α_1 mutants (Fig. 3), but the maximum potentiation of EC_{10}-EC_{20} GABA currents was at least 5-fold for wild type and all of the mutants. The bell-shaped potentiation curve for wild type and the mutated α_1 receptors presumably arises because of inhibition exerted by high concentrations of propofol (Orser et al., 1994). This inhibition is likely to be present in the β_2-mutated receptor but it is not evident, probably because it overlaps with the right-shifted potentiating effect.

Propofol also directly activated wild type and all of the mutant receptors (Fig. 3, ○). Comparable levels of direct activation required higher propofol concentrations in the β_2 subunit mutants than in the α_1 mutants (Fig. 3). At propofol concentrations greater than 50 to 100 μM, the direct activation current also declined consistent with an inhibitory effect at high propofol concentrations (Orser et al., 1994). The mechanism of inhibition is unknown.

These results were used to choose the propofol concentration that would induce a similar level of potentiation among the receptors but have the least direct-activating effect possible. As described below, the propofol concentrations were chosen so that EC_{10} GABA currents would be doubled so that the EC_{10} GABA + propofol currents would be of the same magnitude as EC_{20} GABA currents. The propofol concentrations used were: 0.2 μM with α_1S270C$\beta_2\gamma_{2S}$, 1 μM with α_1A291C$\beta_2\gamma_{2S}$, 7 to 10 μM with $\alpha_1\beta_2$N265Cγ_{2S}, and 5 μM with $\alpha_1\beta_2$M286Cγ_{2S}.

$pCMBS^-$ Reaction Rate in the Closed State. A 1-min application of 0.5 mM $pCMBS^-$ had no functional effect on wild-type receptors (Xu and Akabas, 1996). Thus, all irreversible functional effects of $pCMBS^-$ application to the Cys mutants were assumed to be caused by covalent modification of the engineered Cys residues. $pCMBS^-$ applied in the absence of GABA or propofol reacted with each of the engineered Cys mutants. The reaction rates were significantly different among the four mutants (Table 2). $pCMBS^-$ reacted significantly faster with the M2 Cys mutants compared with the M3 Cys mutants. This may be caused by an electrostatic attraction between the positively charged arginine residue at the M2 19′ position and the negative charge of the sulfonate of $pCMBS^-$. This electrostatic interaction may effectively increase the local $pCMBS^-$ concentration or its dwell time in the site. The arginine lies on the same helical face as the 15′ residue but one helical turn closer to the extracellular surface (Xu and Akabas, 1996).

$pCMBS^-$ reaction with the M2 15′ Cys mutants caused an increase in the holding current at −60 mV (Fig. 4, A and B). The rate of current increase during $pCMBS^-$ application was monoexponential and reached a plateau level. After washout of the $pCMBS^-$, the current remained stable or relaxed slowly on a time scale of minutes. The $pCMBS^-$ reaction rate was calculated from a monoexponential fit to the current data. Second-order reaction rates are given in Table 2. The $pCMBS^-$ reaction rate was similar when $pCMBS^-$ was applied in the presence of 50 μM bicuculline (Table 2). Thus, we infer that $pCMBS^-$ is not acting as an agonist at the GABA binding sites. A 3-min pretreatment of the receptors with 10

mM MMTS, which covalently adds a $-SCH_3$ to reactive Cys, caused a small increase (<10% of maximal GABA current) in the holding current but blocked the effect of $pCMBS^-$ added subsequently ($n = 3$, data not shown). Thus, we infer that the $pCMBS^-$-induced activation required covalent reaction with the 15′-engineered Cys residue. Access to the 15′ site seems to be sterically limited because the larger MTS reagent, MTS-tetramethylrhodamine, did not react with α_1S270C, presumably because of tetramethylrhodamine's size (data not shown). The $pCMBS^-$-induced current was blocked by 50 μM picrotoxin ($n = 3$; data not shown). Thus, we infer that the current was passing through the $GABA_A$ receptor channels. We infer that $pCMBS^-$ modification of the 15′ engineered Cys in either the α or β subunits increased the spontaneous open probability of the receptors, resulting in the increased holding current in the absence of GABA.

$pCMBS^-$ modification of the two M3 Cys mutants caused different effects on the subsequent GABA-induced currents. There was little or no effect on the holding current in the absence of GABA. For the α_1A291C mutant, complete $pCMBS^-$ modification reduced the subsequent GABA-induced currents by 60 ± 3% ($n = 4$). In contrast, $pCMBS^-$ modification of the β_2M286C mutant increased the subsequent GABA-induced currents by 75 ± 15% ($n = 7$). The basis of this difference between the effect of $pCMBS^-$ modification of the α and β subunits at this position is not known, nor is it relevant for the focus of this work; which is to determine whether propofol can protect any of these Cys residues from modification by $pCMBS^-$. The important issue is that $pCMBS^-$ reacted with all of the Cys mutants and the reaction rates can be measured.

$pCMBS^-$ Reaction Rates in the Presence of Propofol. Our previous work demonstrated that propofol binding alters the $GABA_A$ receptor structure and changes the reaction rates of α_1 subunit M3 substituted Cys residues (Williams and Akabas, 2002). Thus, to determine whether propofol could protect a Cys mutant from covalent reaction with $pCMBS^-$, we had to choose an appropriate reference state to compare the reaction rates in the absence and in the presence of propofol. We chose to compare the rates with receptors in which the current was the same fraction of the maximal current. We did this because single-channel studies by others had shown that the single-channel conductance and open time constants were similar whether the channels were activated by GABA or GABA + propofol (Hales and Lambert, 1991; Orser et al., 1994). This implies that the open state structure is likely to be the same in the presence of GABA and GABA + propofol. We compared the reaction rate in the presence of EC_{20} GABA with the reaction rate in the presence of EC_{10} GABA + propofol to induce 100% potentiation of the GABA current. Thus, in both cases, the current was ~20% of the maximal GABA-induced current.

In the presence of EC_{20} GABA, the $pCMBS^-$ reaction rate with the β_2 M3 Cys residue in $\alpha_1\beta_2$M286Cγ_{2S} receptors was 5000 ± 1000 M^{-1}/s, ($n = 10$) (Fig. 5). In the presence of 5 μM propofol + EC_{10} GABA, the $pCMBS^-$ reaction rate was significantly slower, 2030 ± 340 M^{-1}/s ($n = 8$; $p < 0.05$). This rate is similar to the rate with this mutant in the absence of GABA, 1750 ± 170 M^{-1}/s ($n = 7$). It is important to recognize that the concentration of propofol used in these experiments does not give maximal potentiation (Fig. 3). This implies that only a fraction of the propofol binding sites will be occupied at any specific time; given propofol's low affinity, its dwell time on the receptor is likely to be relatively short. Protection experiments are thus a competition between a rapidly reversible blocker, propofol, and an irreversible covalent reaction, $pCMBS^-$ modification. We do not expect to see complete protection of the engineered Cys, only a reduction in the $pCMBS^-$ reaction rate if there is protection. We sought to demonstrate a relationship between propofol concentration and the extent of protection. We measured the $pCMBS^-$ reaction rate in the presence of different propofol concentrations (Fig. 5D). The relationship between the reaction rate and propofol concentration was linear, with a correlation coefficient r^2 equal to 0.96. The maximum protection was achieved using 5 μM propofol. Note that the lowest reaction rate achieved, 2030 ± 340 M^{-1}/s, is near the closed state reaction rate, 1750 ± 170 M^{-1}/s.

We performed similar experiments with the Cys mutant at the aligned position in the α_1 subunit, α_1A291C. $pCMBS^-$ application inhibited the subsequent GABA-induced currents in α_1A291C$\beta_2\gamma_{2S}$ receptors (Fig. 6). The $pCMBS^-$ reaction rate in the presence of EC_{20} GABA was 1000 ± 300 M^{-1}/s ($n = 6$) and in the presence of EC_{10} GABA + propofol, the reaction rate was 1600 ± 130 M^{-1}/s ($n = 3$). These results are not significantly different. They suggest that, in contrast to the β_2M286C results, propofol did not protect α_1A291C from covalent modification by $pCMBS^-$.

$pCMBS^-$ reaction with the M2 15′ Cys mutants also did not show protection by propofol. The reaction rates in the presence of GABA were significantly faster than in the absence of GABA (Table 2). This implies that channel activation induced conformational changes either in the access pathway or in the region of these residues. The reaction rates in the presence of GABA and in the presence of GABA + propofol were not significantly different, indicating that propofol did

TABLE 2

Second-order reaction rate constants for $pCMBS^-$ with the Cys mutants in the presence of the indicated reagents

Concentrations of GABA and propofol for each mutant are given in the text. Data are presented as mean ± S.E.M.

	Basal	+ GABA	+ GABA & propofol	Effect of $pCMBS^-$ modification
		M^{-1}/s		
$\alpha_1\beta_2$M286Cγ_{2S}	1750 ± 50 ($n = 7$)	5000 ± 1000 ($n = 10$)	2300 ± 300 ($n = 13$)	Increase of GABA-induced current
α_1A291C$\beta_2\gamma_{2S}$	300 ± 30 ($n = 4$)	1000 ± 300 ($n = 6$)	1600 ± 130 ($n = 3$)	Decrease of GABA-induced current
α_1S270C$\beta_2\gamma_{2S}$	6400 ± 600[a] ($n = 20$)	45,000 ± 9500 ($n = 5$)	43,000 ± 4000 ($n = 22$)	Direct inward current
$\alpha_1\beta_2$N265Cγ_{2S}	4800 ± 470 ($n = 8$)	9500 ± 800 ($n = 8$)	9500 ± 500 ($n = 9$)	Direct inward current

[a] Reaction rate of $pCMBS^-$ in the presence of 50 μM bicuculline with α_1S270Cβ_2 γ_{2S} was 8200 ± 550 M^{-1}/s ($n = 4$), not significantly different from the rate in the absence of GABA.

not protect the engineered Cys at these sites from covalent modification by $pCMBS^-$ (Fig. 7 and Table 2).

Discussion

In this work, we sought to test the hypothesis that one or more of the four residues, α_1S270, β_2N265, α_1A291, and β_2M286, hypothesized to form anesthetic binding sites, was near the binding site for the intravenous anesthetic propofol. We assayed the ability of propofol to protect engineered Cys residues from covalent modification by $pCMBS^-$. Because propofol binding induces conformational changes in the $GABA_A$ receptor membrane-spanning domain (Williams and Akabas, 2002) the major technical problem in performing these experiments is to define a reference state that allows one to compare $pCMBS^-$ reaction rates in the absence and presence of propofol. We used the open channel state because previous work by others had shown that the single-channel conductance and open time constants were indistinguishable whether $GABA_A$ receptors were activated by GABA or GABA + propofol (Hales and Lambert, 1991; Orser et al., 1994). The similarity of single channel conductance and open time distribution implies that the channel's open state structure is likely to be the same in the presence of GABA and GABA + propofol. Our results showed that the $pCMBS^-$ reaction rate with the M3 segment mutant β_2M286C was reduced in the presence of propofol in a concentration-dependent manner (Fig. 5). The reaction rate at the aligned α_1 position, α_1A291C, was unaffected by the presence of propofol (Fig. 6). Furthermore, propofol did not affect the reaction rate with either the α_1 or β_2 15′ M2 segment positions tested (Fig. 7). We infer from the reduction in reaction rate that propofol protects the engineered Cys at position β_2M286C from covalent modification. Equally important was the lack of protection at the other three positions, which argues that they are not part of the propofol binding site(s).

The question is whether this protection is steric (i.e., caused by the presence of propofol near this residue) or allosteric (i.e., a propofol-induced conformational change that reduces access or reactivity at this position). We argue that the observed protection of β_2M286C by propofol was caused by a local steric effect. The assumption underlying this conclusion is that the open channel structure is similar in channels activated by GABA and by GABA + propofol (i.e., that the reaction rates in the absence and presence of propofol are measured in states with similar structures). The assumption is supported by the fact that at the single channel level, the conductances and open time distributions are similar in channels activated by GABA and by GABA + propofol (Hales and Lambert, 1991; Orser et al., 1994). The functional similarity is most probably based on structural similarity, although there is little structural data to support this conclusion. Because GABA increased the $pCMBS^-$ reaction rate with all four Cys mutants compared with the resting state rate, we infer that the conformational change from the rest-

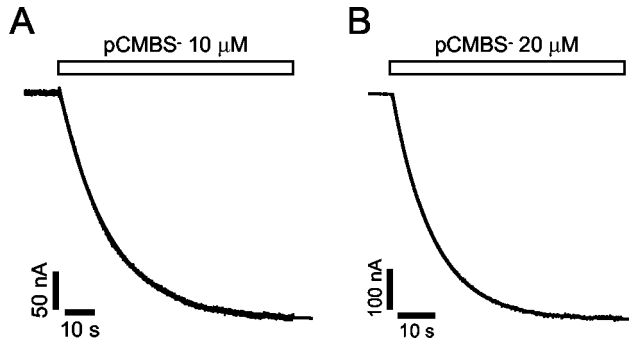

Fig. 4. $pCMBS^-$ modification of the M2 segment 15′ Cys mutants causes a marked increase in spontaneous activation of the receptors, resulting in a large holding current. A, $pCMBS^-$ alone on α_1S270C$\beta_2\gamma_{2S}$. B, $pCMBS^-$ alone on $\alpha_1\beta_2$N265Cγ_{2S}.

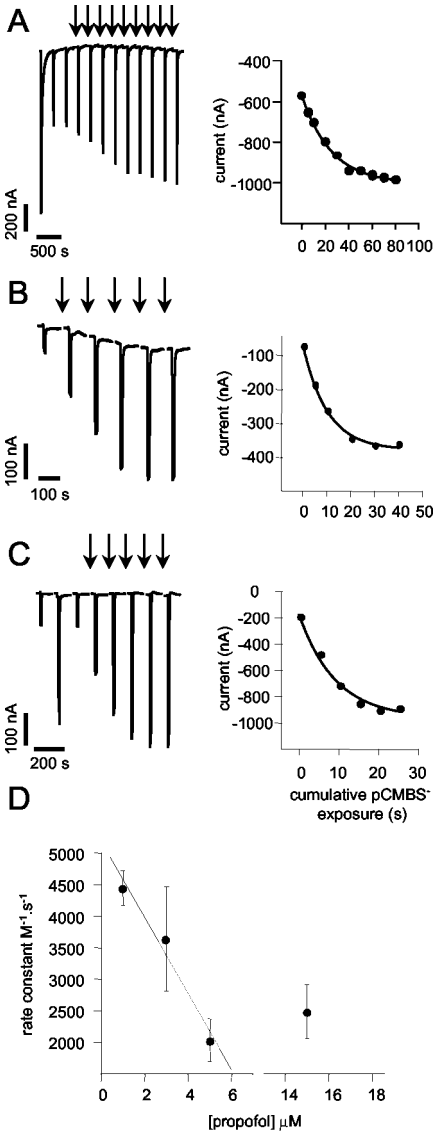

Fig. 5. Propofol reduces the $pCMBS^-$ reaction rate with $\alpha_1\beta_2$M286Cγ_{2S} receptors. A, 40 μM $pCMBS^-$ reaction in the absence of GABA and propofol. B, 25 μM $pCMBS^-$ reaction in the presence of EC_{20} GABA (6 μM). C, 40 μM $pCMBS^-$ reaction in the presence of 15 μM propofol + EC_{10} GABA (3 μM). Left, currents recorded from oocytes. In A to C, $pCMBS^-$ was applied ($\downarrow$) for 5 s in the presence of the indicated reagents for the first four and 10 s for the last two. Currents during $pCMBS^-$ application are not shown. The GABA test pulses between the $pCMBS^-$ applications are shown. Right, monoexponential fits to the peak GABA test currents as a function of cumulative duration of $pCMBS^-$ application. The average reaction rates are shown in Table 2. D, propofol concentration dependence of the $pCMBS^-$ reaction rates with $\alpha_1\beta_2$M286Cγ_{2S} receptors. Data points represent the average reaction rates determined on at least three oocytes. Straight line was fit to the rates for propofol concentrations less than 6 μM. The effect of increasing propofol on reaction rate saturated at 5 μM propofol.

ing to the open/desensitized states increases the accessibility/reactivity of these substituted Cys residues. At three positions, α_1S270C, α_1A291C, and β_2N265C, propofol + GABA had the same effect on the pCMBS$^-$ reaction rate as GABA alone, suggesting that the structure in the region of these Cys is similar in the absence and presence of propofol. In contrast, at β_2M286C, the rate in the presence of propofol + GABA was significantly slower. Based on the evidence cited above, we think that it is unlikely that propofol is inducing a conformational change at this position different from that induced by GABA. Thus, we infer that propofol protected the substituted Cys at β_2M286C by a steric effect caused by the local presence of propofol. We infer that β_2Met286 is near the propofol binding site. We cannot determine, however, whether this residue is in contact with propofol or forms a structural element in the binding site.

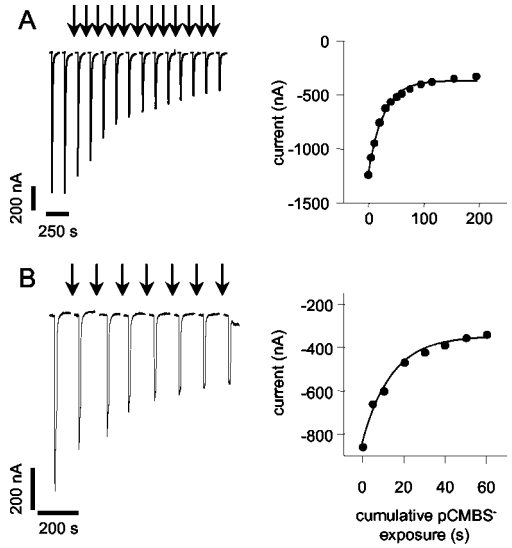

Fig. 6. Propofol does not protect α_1A291C$\beta_2\gamma_{2S}$ receptors from covalent reaction with pCMBS$^-$. A, 50 μM pCMBS$^-$ reaction in the presence of EC_{20} GABA (2 μM). B, 50 μM pCMBS$^-$ reaction in the presence of 1 μM propofol + EC_{10} GABA (1 μM). Left, currents recorded from oocytes. In both panels, pCMBS$^-$ was applied for 5 s in the presence of the indicated reagents for the first two applications ($\downarrow$) and the subsequent applications were 10 s. Currents during pCMBS$^-$ application are not shown. The GABA test pulses between the pCMBS$^-$ applications are shown. Right, mono-exponential fits to the peak GABA test currents as a function of cumulative duration of pCMBS$^-$ application. The average reaction rates are shown in Table 2.

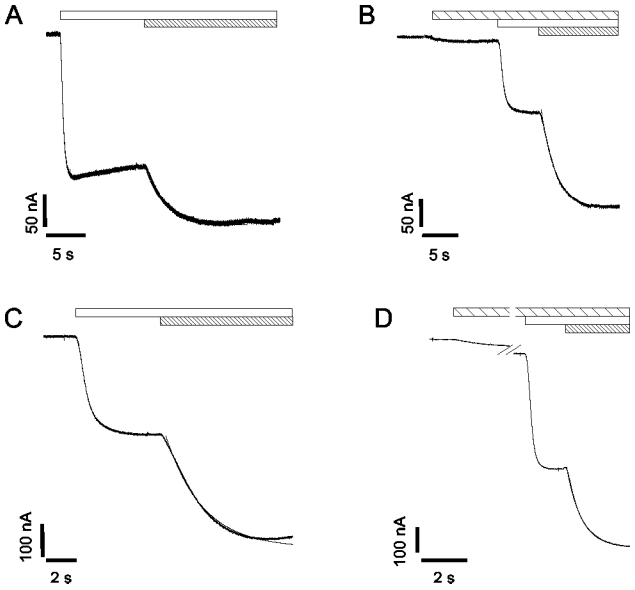

Fig. 7. Propofol does not protect either α_1S270C$\beta_2\gamma_{2S}$ or $\alpha_1\beta_2$N265Cγ_{2S} receptors from modification by pCMBS$^-$. Effect of pCMBS$^-$ applied in the presence of GABA or propofol + GABA on currents recorded from oocytes. Current increase after pCMBS$^-$ application was fit to a single exponential function. The fitted line is superimposed on the current trace as a thin line. In several panels, it is not visible because it completely overlaps the current trace. The bars above the current traces indicate reagents added. Open bar, GABA; fine dashed bar, pCMBS$^-$; and slashed bar, propofol. A and B, α_1S270C$\beta_2\gamma_{2S}$ receptors. C and D, $\alpha_1\beta_2$N265Cγ_{2S} receptors.

Previous efforts to identify anesthetic binding sites have relied on the functional effects of mutagenesis or chimera construction to locate regions or residues that alter anesthetic efficacy. The $GABA_A$ receptor is an allosteric protein and anesthetic binding alters $GABA_A$ receptor structure (Williams and Akabas, 2002). Therefore, it was difficult for these approaches to distinguish residues in a binding site from residues that alter transduction of anesthetic binding when mutated (Colquhoun, 1998). The low affinity of general anesthetics for $GABA_A$ receptors, in the micromolar to millimolar concentration range, has also limited efforts to identify binding sites. Despite these limitations, the working hypothesis is that general anesthetics interact with $GABA_A$ receptors in a region near the extracellular end of the membrane-spanning domain (Belelli et al., 1999; Krasowski and Harrison, 1999; Miller, 2002). This region seems to be lined by the nonchannel-lining face of the M2 segment (Xu and Akabas, 1996) and by residues from M3 and possibly also M1 and M4 (Belelli et al., 1997; Mihic et al., 1997; Krasowski and Harrison, 1999; Jenkins et al., 2001; Krasowski et al., 2001; Nishikawa et al., 2002). Our results provide additional support for the hypothesis that propofol binds in a region near the extracellular end of the M3 segment near β_2Met286. This site probably mediates propofol's potentiating action, because mutation of this residue to tryptophan reportedly eliminated potentiation but not direct activation (Krasowski et al., 1998). In contrast, mutation of a GABA binding site residue, β_2Tyr157, reduced direct activation but did not affect propofol's modulatory actions (Fukami et al., 1999). These results suggest that for propofol, potentiation and direct activation may involve binding to distinct sites. In contrast, the GABA-binding site mutation reportedly had no effect on either potentiation or activation by pentobarbital and etomidate (Fukami et al., 1999).

The other β_2 subunit residue, β_2N265C, was not protected by propofol. In the 4-Å resolution structure of the homologous AChR, presumably in the closed state but possibly in the desensitized state, the aligned positions are separated by ~10 Å (Fig. 1) (Miyazawa et al., 2003). Thus, it might have been surprising if propofol had protected both positions. It is somewhat perplexing that methionine substitution at the aligned β_3 M2 position, β_3Asn265, virtually eliminates propofol's anesthetic efficacy in a knock-in mouse (Jurd et al., 2003). Methionine occupies a volume 43 $Å^3$ greater than asparagine and is more hydrophobic. Thus, steric bulk at position 265 can alter propofol binding, perhaps by inducing a conformational change at the propofol binding site ~10 Å away. Both β subunit Cys mutants caused significant right shifts in the propofol potentiation-response curve (Fig. 3). This contrasts with the smaller effects of the α subunit Cys

mutants on the propofol potentiation-response relationships. These results and those discussed above are consistent with previous work indicating that intravenous anesthetics, such as propofol and etomidate, mainly interact with the β subunits, whereas volatile anesthetics largely interact with α subunit sites (Belelli et al., 1997; Mihic et al., 1997; Nishikawa et al., 2002).

Covalent modification by $pCMBS^-$ of the 15′ M2 Cys mutants in both α_1 and β_2 caused an increase in the spontaneous open probability manifested as a picrotoxin-blockable holding current in the absence of GABA (Fig. 4). A previous study reported that an α_1S270I mutation decreased the GABA EC_{50} because of stabilization of the open state. The mutation decreased the closing rate constant, resulting in increased spontaneous opening (Scheller and Forman, 2002). It is likely that $pCMBS^-$ modification causes a much larger decrease in the closing rate than the serine-to-isoleucine mutation, thus resulting in a greater degree of spontaneous opening. Covalent modification by the smaller, uncharged MMTS caused a minimal increase in holding current. Modification of α_2S270C by MTS-propanol decreased GABA EC_{50} but was not reported to cause spontaneous opening (Mascia et al., 2000).

Insights into the residues that might form a propofol binding site can be gleaned from the crystal structure of two propofol molecules bound to human serum albumin. The crystal structure of bound propofol reveals several interesting features about the binding sites (Bhattacharya et al., 2000). First, a wide range of amino acids are in direct contact with propofol, including Asn, Cys, Gly, Ala, Leu, Ile, Val, Phe, Tyr, Arg, and Glu. Second, the phenolic hydroxyl hydrogen bonds to a backbone carbonyl at one site and to a serine hydroxyl at the other site. Thus, it is difficult to predict, a priori, which $GABA_A$ receptor residues might form the propofol binding site. Finally, at both albumin sites, propofol bound in preformed pockets or clefts and did not significantly alter the protein structure. It seems unlikely that this will occur when propofol binds to the $GABA_A$ receptor. Propofol binding alters the $GABA_A$ receptor structure in the M3 membrane-spanning segment region (Williams and Akabas, 2002). Furthermore, kinetic studies suggest that propofol stabilizes a doubly liganded preopen state (Bai et al., 1999). This suggests that propofol binds to this state with higher affinity than to the resting state, further suggesting that the binding site structure also changes as the receptor changes conformation consistent with the allosteric nature of the $GABA_A$ receptor. Further work will be necessary to understand the conformational changes that are induced by anesthetic binding to elucidate the molecular basis of their actions.

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