Channel Opening by Anesthetics and GABA Induces Similar Changes in the $GABA_A$ Receptor M2 Segment

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ABSTRACT For many general anesthetics, their molecular basis of action involves interactions with $GABA_A$ receptors. Anesthetics produce concentration-dependent effects on $GABA_A$ receptors. Low concentrations potentiate submaximal GABA-induced currents. Higher concentrations directly activate the receptors. Functional effects of anesthetics have been characterized, but little is known about the conformational changes they induce. We probed anesthetic-induced conformational changes in the M2 membrane-spanning, channel-lining segment using disulfide trapping between engineered cysteines. Previously, we showed that oxidation by copper phenanthroline in the presence of GABA of the M2 6′ cysteine mutants, α_1T261Cβ_1T256C and $\alpha_1\beta_1$T256C resulted in formation of an intersubunit disulfide bond between the adjacent β-subunits that significantly increased the channels' spontaneous open probability. Oxidation in GABA's absence had no effect. We examined the effect on α_1T261Cβ_1T256C and on $\alpha_1\beta_1$T256C of oxidation by copper phenanthroline in the presence of potentiating and directly activating concentrations of the general anesthetics propofol, pentobarbital, and isoflurane. Oxidation in the presence of potentiating concentration of anesthetics had little effect. Oxidation in the presence of directly activating anesthetic concentrations significantly increased the channels' spontaneous open probability. We infer that activation by anesthetics and GABA induces a similar conformational change at the M2 segment 6′ position that is related to channel opening.

INTRODUCTION

The $GABA_A$ receptors are a major molecular target for general anesthetics such as pentobarbital, propofol, and isoflurane (1–5). Each of these anesthetics has three separate effects on $GABA_A$ receptors. Low concentrations potentiate currents induced by submaximal GABA concentrations. Higher anesthetic concentrations directly activate receptors in GABA's absence. At still higher concentrations many anesthetics inhibit both anesthetic and GABA-induced currents (4,5). These distinct actions imply that $GABA_A$ receptors contain several distinct binding sites for each anesthetic. At least for some anesthetics, these sites are distinct from the GABA binding sites (6–8). Occupancy of these sites stabilizes different receptor states or ensembles of states (9,10). Consistent with this, we showed that the conformation of the M3 membrane-spanning segment or the protein domains surrounding it are different in the presence of potentiating and activating concentrations of propofol, a commonly used intravenous general anesthetic (11). Single-channel studies have shown similar conductances but different kinetics after activation by GABA and by general anesthetics (12,13). This has led to the hypothesis that although the anesthetic binding sites are distinct from the GABA binding sites, the open-state channel structure is similar in the presence of GABA and the anesthetics. There is, however, little structural information available to support this hypothesis.

$GABA_A$ receptors are formed by five homologous subunits assembled around the central channel (14). A common in vivo subunit stoichiometry is 2α:2β:1γ subunits (15,16), but functional receptors are also formed by coexpression of just the α- and β-subunits with evidence supporting a stoichiometry of 2α:3β (17–19) and of 3α:2β (20,21). Each subunit has an ~200 amino acid extracellular N-terminal domain and a similar sized 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 (22–24). The transmembrane channel is principally lined by the five largely α-helical M2 segments (25,26). (To facilitate comparisons with other receptors in the gene superfamily, we will refer to M2 segment residues using an index numbering system in which the conserved positively charged residue at the M2 cytoplasmic end is the 0′ position, and residues toward the C-terminus are numbered consecutively 0′, 1′, 2′, ... (27) (Fig. 1).) The position and extent of the channel gate are uncertain, but all agree that it lies somewhere between the middle and cytoplasmic end of M2 (26,28–30).

We sought to determine whether the general anesthetics pentobarbital, propofol, and isoflurane induced a similar conformational change in the M2 channel-lining segments as that induced during GABA activation. As a reporter for the conformational state of the M2 segments, we used disulfide

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Abbreviations used: CFFR, calcium-free-frog Ringers; Cu:phen, copper phenanthroline; $I_{GABA,max}$, maximal GABA-induced current; SCAM, substituted cysteine accessibility method.

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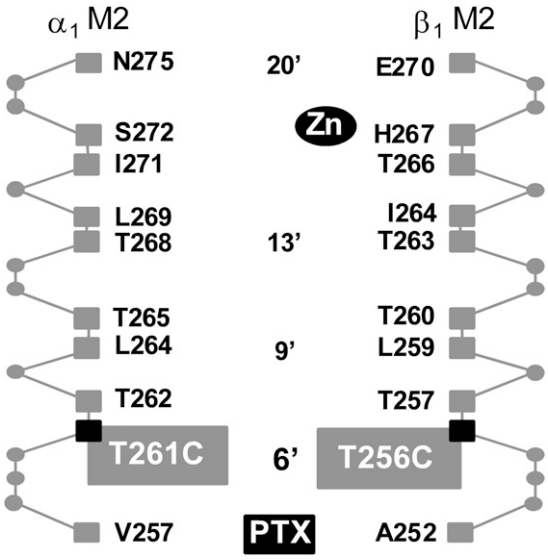

FIGURE 1 Aligned channel-lining residues in the α_1 and β_1 M2 membrane-spanning segments. Position of the 6′ residues is highlighted in reverse contrast. Index numbers are shown in the center to facilitate comparisons with other members of the gene superfamily (27). Solid squares indicate channel-lining positions, solid circles non-channel-lining positions based on SCAM experiments (25). Zn indicates the Zn^{2+} binding site location at β_1His267 (18), and PTX indicates the picrotoxin binding site location at the 2′ level (41).

trapping experiments with the 6′ engineered cysteine (Cys) mutants α_1T261Cβ_1T256C and $\alpha_1\beta_1$T256C. Previously, we showed that disulfide bond formation between engineered M2 segment cysteine (Cys) residues at the 6′ level was state dependent (Fig. 1) (31). Oxidation in the closed state had no effect. In contrast, in the presence of GABA, oxidation by copper phenanthroline (Cu:phen) caused disulfide bond formation between the adjacent β-subunits that resulted in a significant increase in the macroscopic holding current after GABA washout (31). This increased holding current presumably resulted from an increase in the channel's spontaneous open probability. We inferred that the disulfide bond formed between adjacent β-subunits because if it formed between nonadjacent β-subunits, the channel lumen would have been obliterated and that would be inconsistent with the increased holding current that we observed. A corollary to this conclusion is that with our expression conditions most of the receptors had a subunit stoichiometry of 2α:3β subunits. Furthermore, we inferred that in the presence of GABA, the proximity and orientation of the engineered Cys residues in the adjacent β-subunits were more favorable than in the closed state. Thus, the ability to form the 6′ disulfide bond provides a reporter for the open state structure of the M2 segments in this region of the channel.

Cu:phen promotes oxidation by catalyzing the formation of hydroxyl radicals and superoxide; it does not require direct contact between the Cu:phen and the sulfhydryls (32,33). Disulfide trapping has been used to study protein proximity and mobility relationships between residues in both water-soluble and integral-membrane proteins (32,34,35). The average α-carbon separation of disulfide-bonded Cys residues is ~5.6 Å in proteins of known structure (32). The disulfide bond formation rate depends on the collision frequency of the sulfhydryls, the energy of the collision, and the presence of an oxidizing environment (32). The collision frequency depends on the average separation distance of the sulfhydryls, their relative orientation in the protein, and the mobility and/or flexibility of the protein, especially in the regions containing the Cys residues.

Our results show that GABA induced a conformational change that allows disulfide bond formation between M2 6′ engineered cysteine residues. At potentiating concentrations, the anesthetics do not induce a similar conformational change, but at directly activating concentrations, the anesthetics induce a conformational change similar to that induced by GABA.

MATERIALS AND METHODS

Mutants and expression

The rat α_1 and β_1 M2 segment cysteine-substitution mutants in the pGEMHE plasmid were generated and characterized previously (31). Plasmid DNA was linearized with NheI for mRNA template synthesis. mRNA was synthesized by in vitro transcription using the T7 AmpliScribe kit (Epicenter, Madison, WI). *Xenopus laevis* oocyte preparation and injection were as described previously (31). Oocytes were injected with 50 nl of a 200 pg/nl solution of mRNA in a 1:1 ratio of α:β subunits and maintained at 17°C in OR3 medium as described previously (31).

Electrophysiology

Two-electrode voltage-clamp recording from *Xenopus* oocytes and data acquisition and analysis were performed as described previously (25,31). Briefly, the data acquisition system utilized a TEV-200 amplifier (Dagan, Minneapolis, MN), Digidata 1322A interface, and pClamp 8.2 software (Axon Instruments, Union City, CA). Electrodes were filled with 3 M KCl and had resistances of less than 2 MΩ. Bath electrode was connected via a 3 M KCl/agar bridge. Oocytes, in a 250-μl recording chamber, were continuously perfused at 5 ml/min with calcium-free frog Ringer's (CFFR) (115 mM NaCl, 2.5 mM KCl, 1.8 mM $MgCl_2$, 10 mM HEPES pH 7.5 with NaOH) at room temperature. Holding potential was −60 mV. Before experiments were performed on each oocyte, GABA test pulses were applied at 5-min intervals until the successive current amplitudes varied by less than 5%.

Oxidation was induced by a 1-min application of 100:400 μM Cu:phen prepared freshly as described previously (31). Because the magnitude of the change in holding current (ΔI_{hold}) induced by Cu:phen application will depend on the maximal GABA-induced current ($I_{GABA,max}$) for a given oocyte, we normalized ΔI_{hold} by $I_{GABA,max}$ determined before anesthetic application so that the changes in holding current after Cu:phen application are reported as $(\Delta I_{hold}/I_{GABA,max}) \times 100$.

Anesthetic concentration-response relationships

The anesthetic 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 anesthetic alone for 10 to 20 s and then to anesthetic + EC_{10}-EC_{20} GABA for 10 s. Pretreatment with potentiating concentrations of anesthetic enhances the potentiation of GABA-induced current. When applied at activating concentrations, it allowed us to measure the current induced by anesthetic in the absence of GABA. Anesthetic applications were separated by washes lasting at least 5 min to allow for anesthetic washout and full recovery from desensitization. It should be noted that, particularly at high concentrations, anesthetic removal was not always complete within a time frame of 10–20 min, probably because the oocyte membrane acts as a reservoir for these hydrophobic drugs. This was evident from the potentiation of subsequent submaximal GABA applications. This was an issue for the higher concentrations used but does not result in increased holding currents and thus does not affect the interpretation of our experiments.

Expression in human embryonic kidney 293 cells and single channel recording

Human embryonic kidney cells (HEK293T; American Tissue Culture Collection, Manassas, VA) were grown at 37°C in DMEM supplemented with 10% fetal calf serum, 2.5 mM L-glutamine, 100 IU of penicillin, and 170 μM streptomycin in an atmosphere of 5% CO_2/95% air. Cells were seeded in 100-mm plates at a density of 1.2–1.5 $\times$ 10^6 cells and transfected 24 h later for 12 h using the calcium phosphate precipitation technique (36) with 5–7 μg of plasmid DNA coding for $GABA_A$ α1 subunit, WT or T261C, and 5–7 μg of plasmid DNA coding for $GABA_A$ β1 subunit, WT or T256C. The plasmids were pXOON, a modified version of pXOOM (37), which encodes a neomycin-enhanced green fluorescent protein (eGFP) fusion protein for the visual identification of expression in transfected cells. Cells were washed with PBS and detached with trypsin before reseeding at low density in 35-mm polylysine-treated dishes that were mounted directly on the stage of an inverted microscope (Zeiss IM; Zeiss, Thornwood, NY) for patch-clamp experiments 24-48 h later. For single-cell recording in the cell-attached configuration of the patch clamp technique, pipettes were pulled from thick-walled borosilicate glass, coated with Sylgard, and fire-polished to a resistance of 10–14 MΩ when filled with the internal solution. The pipette contained 140 mM NaCl, 5 mM CsCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH = 7.4 adjusted with NaOH. Where necessary, 5 μM GABA was diluted in the pipette solution. The same medium was used for the bathing solution. The transfected cells chosen for the experiments had similar GFP fluorescence intensity. The pipette holding potential was +60 mV (hyperpolarization of the cell). Currents were low-pass filtered at 8 kHz (eight-pole Bessel filter) and acquired at 20 kHz using Pulse software interfaced with an EPC-9 amplifier (HEKA, Darmstadt, Germany). Where applicable, cells were treated for 20 min with Cu:phen (100 μM:400 μM) in the presence of 5 μM GABA and thoroughly washed before patching.

Reagents

Stock solutions of propofol (2,6-di-isopropylphenol) (ICN Biomedicals, Aurora, OH) in DMSO were diluted into CFFR immediately before application. The percentage of DMSO was never greater than 0.1% and had no effect on GABA-induced currents (data not shown). Pentobarbital (Sigma Chemical, St. Louis, MO) and isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) (Halocarbon Laboratories, River Edge, NJ) were dissolved directly into CFFR buffer. Isoflurane solutions were prepared in sealed plastic IV bags that contained no air bubbles immediately before use (38). Teflon tubing was used for all perfusion tubing. Cu:phen was prepared by diluting stock solutions of 100 mM $CuSO_4$ and 1 M phenanthroline (Sigma) in DMSO into CFFR immediately before use. DTT (Sigma) was dissolved in CFFR immediately before use. Oocytes were perfused for 3–5 min between applications of GABA or reagents to allow complete recovery from desensitization.

Data

Data are presented as mean $\pm$ SE. Statistical significance was determined by Student's *t*-test except in Table 2, where one-way ANOVA with Dunnett's post hoc test was used.

RESULTS

Effects of disulfide bond formation between 6′ cysteines at the single-channel level

Previously, we reported that oxidation of either α_1T261Cβ_1 T256C (6′) or $\alpha_1\beta_1$T256C (6′) $GABA_A$ receptors by a 1-min application of 100:400 μM Cu:phen + GABA caused a significant increase in the subsequent holding current as measured by two-electrode voltage-clamp recording from *Xenopus* oocytes (31). We inferred that the increased holding current was caused by an increase in the spontaneous open probability of the channels after disulfide bond formation. To investigate the basis for the increased holding current, we performed single-channel patch-clamp recordings from HEK293T cells expressing $\alpha_1\beta_1$T256C receptors or from cells transfected with empty pXOON vector. Transfected cells were identified by GFP fluorescence. As a control for effects of Cu:phen application, we performed patch-clamp recordings from cells transfected with empty vector, with no GABA in the pipette. Under these conditions, no $GABA_A$ receptor-like channels were observed in cell-attached patches either before or after application of Cu:phen + GABA (n = 7) (data not shown).

With cells expressing $\alpha_1\beta_1$T256C receptors, with GABA in the pipette, we observed currents from $GABA_A$ receptor channels in 88% of patches (43 of 49). The slope conductance of these channels was 23 $\pm$ 1 pS (n = 6). With no GABA in the pipette, no GABA receptor-like channels were observed in seven patches from cells expressing $\alpha_1\beta_1$T256C receptors (Fig. 2 *A*). In contrast, after cells expressing $\alpha_1\beta_1$T256C receptors were treated with Cu:phen + GABA, with no GABA in the patch pipette, $GABA_A$ receptor channels were present in 78% of patches (35 of 45) from these cells (Fig. 2 *B*). The slope conductance of these channels was 12 $\pm$ 1 pS (n = 4). The channels show bursts of openings and flickering between open and closed states. Thus, the 6′ disulfide bond increased the spontaneous open probability of the channels, but they could still undergo rapid transitions between the open and closed states.

Characterization of anesthetic effects on the 6′ Cys mutants

The GABA EC_{50} reported previously for wild-type $\alpha_1\beta_1$ $GABA_A$ receptors was 3.4 μM, and for the Cys mutants α_1T261Cβ_1 1.2 μM, $\alpha_1\beta_1$T256C 1.0 μM, and α_1T261Cβ_1 T256C 1.9 μM (31). For each anesthetic we identified two concentrations, one that gave maximal potentiation of

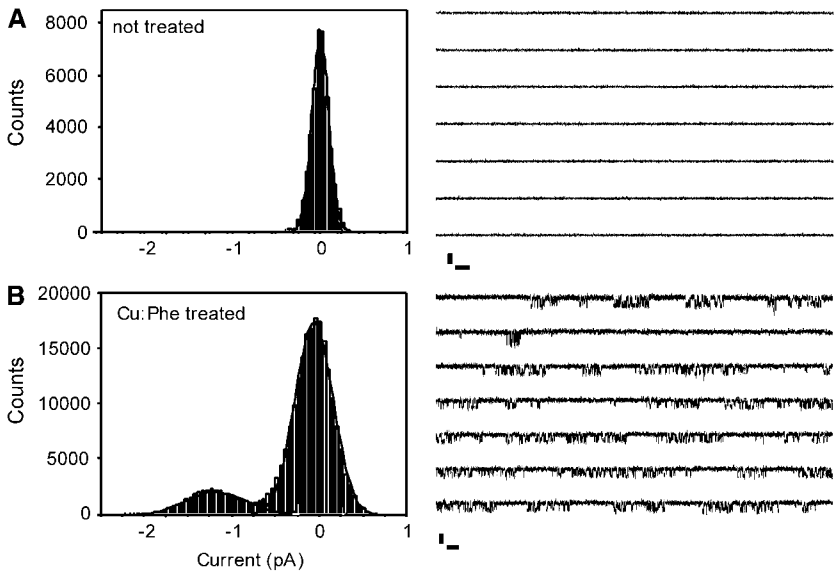

FIGURE 2 Effect of Cu:phen-induced oxidation in the presence of GABA on single-channel currents recorded from $\alpha_1\beta_1$T256C-containing receptors. (*A* and *B*) All-points histogram of currents recorded from an HEK 293 cell in the cell-attached configuration (*left*) and the corresponding current recordings (*right*). The pipette did not contain GABA. The pipette voltage was clamped to +60 mV (hyperpolarization of the cell). Data traces were numerically filtered to 1 kHz for display using QuB Software (59). The scale bars represent 2 pA and 50 ms. (*A*) Cell-attached patch recording from a cell expressing $\alpha_1\beta_1$T256C receptors. No $GABA_A$ receptor channel activity was observed. The current amplitude histogram was fitted by a Gaussian distribution centered around -0.05 ± 0.1 pA. (*B*) Cell-attached patch recording from an $\alpha_1\beta_1$T256C-transfected cell that was treated with Cu:phen in the presence of 5 μM GABA for 10–15 min. Before patch formation, GABA and Cu:phen were thoroughly washed out of the dish. After oxidation in the presence of GABA, a significant amount of single-channel activity was observed with no GABA in the pipette. The current amplitudes were best fitted using the sum of two Gaussian distributions centered around -0.06 ± 0.22 pA, relative area = 82%, and 1.19 ± 0.34 pA, relative area = 18%.

GABA-induced currents with little or no direct activation and the second that gave significant direct activation with minimal inhibition.

As the propofol concentration was increased, direct activation became significant at ~10 μM propofol and increased up to 40 μM propofol (Fig. 3 *A*). At higher concentrations the directly activated current diminished, presumably because of channel block (Fig. 3 *A*). The extent of potentiation of GABA currents peaked at 10 μM propofol and diminished at high concentrations (Fig. 3 *A*). In the current traces at 20 μM propofol and above, we can see an increase in current as GABA and propofol were washed out. Transient washout currents like this usually indicate that some fraction of the channels were blocked by the drug, which washed out more rapidly than GABA. This suggests that even at 20 μM propofol there is some channel block. Quantification of the anesthetic concentration-response relationship is difficult because potentiation effects continue to increase even after direct activation begins. Furthermore, at higher concentrations, inhibition makes it difficult to determine the maximal extent of potentiation and activation. The results of similar experiments with pentobarbital (Fig. 3 *B*) and isoflurane (Fig. 3 *C*) are shown. There was very little direct activation with isoflurane at 3 mM, although there was significant potentiation (Fig. 3 *C*). At 20 mM isoflurane, there was direct activation but a significant amount of inhibition as can be seen by the large washout currents when isoflurane was applied by itself and with GABA (Fig. 3 *C*). The anesthetic concentrations that we used in the subsequent experiments are shown in Table 1.

Effect of Cu:phen oxidation in the presence of potentiating anesthetic concentrations

We tested whether application of 100:400 μM Cu:phen in the presence of a potentiating concentration of propofol or pentobarbital altered the holding current or the subsequent GABA-induced currents of the double Cys mutant α_1T261Cβ_1T256C (Fig. 4). A 1-min application had little or no effect on subsequent GABA-induced currents or on the holding current at −60 mV (Table 2). In the presence of propofol or pentobarbital, the holding current increased by 12% or 1% of $I_{GABA,max}$, respectively. Similar results were obtained in oocytes expressing $\alpha_1\beta_1$T256C receptors (data not shown). Likewise, a 1-min coapplication of 100:400 μM Cu:phen and a potentiating concentration of isoflurane (1 mM) to oocytes expressing $\alpha_1\beta_1$T256C receptors caused the holding current to increase by $13 \pm 3\%$ ($n = 3$) of $I_{GABA,max}$ (Table 2). Thus, we infer that there is not a significant amount of disulfide bond formation during oxidation in the presence of potentiating concentrations of these anesthetics. The small increases in holding current may arise because of a small amount of direct activation at the anesthetic concentrations used.

Effect of Cu:phen oxidation in the presence of activating anesthetic concentrations

A 1-min application of Cu:phen in the presence of directly activating concentrations of propofol and pentobarbital had two effects on the double Cys mutant α_1T261Cβ_1T256C (Fig. 5 and Table 2). It increased the subsequent holding

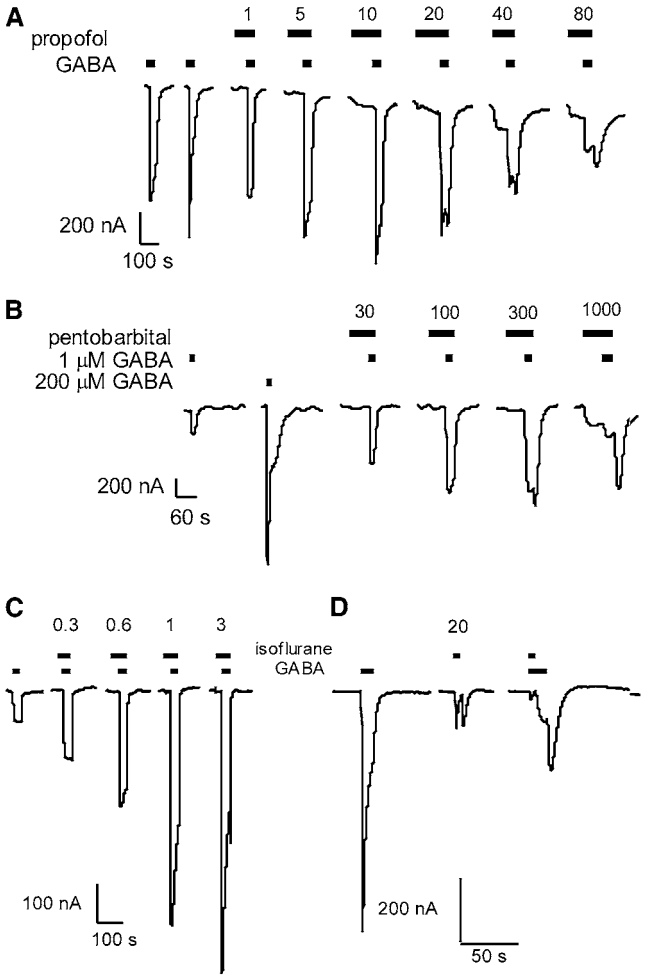

FIGURE 3 Anesthetic concentration-response relationships for potentiation and direct activation. (*A*) Current traces from an oocyte expressing α_1T261Cβ_1T256C receptors. Periods of GABA and propofol application are indicated by the black bars above the current traces. Propofol concentration (μM) is indicated above bars. GABA concentration was 0.4 μM except for the second trace, where it was 20 μM. Traces are separated by 5-min washout periods. (*B*) Pentobarbital direct activation and potentiation of GABA currents from an oocyte expressing α_1T261Cβ_1T256C receptors. Periods of GABA and pentobarbital application are indicated by the black bars above the current traces. Pentobarbital concentration (μM) is indicated above bars. The prominent rebound currents seen after pentobarbital and GABA washout, particularly in the 300 and 1000 μM pentobarbital applications, represent relief of inhibition by pentobarbital. Traces are separated by 5-min washout periods. (*C*) Potentiating isoflurane concentration responses on current traces from an oocyte expressing $\alpha_1\beta_1$T256C receptors. Isoflurane concentration (μM) above the bars. GABA concentration was 0.5 μM. (*D*) Direct activation and inhibition by isoflurane. Current traces from an oocyte expressing $\alpha_1\beta_1$T256C receptors. Isoflurane concentration was 20 mM, GABA concentration was 5 μM.

current and decreased the subsequent GABA-induced currents. In the presence of 40 μM propofol, Cu:phen application increased the holding current to 44 $\pm$ 17% ($n = 5$) of the initial $I_{GABA,max}$. In the presence of 1 mM pentobarbital, Cu:phen application increased the holding current to 28 $\pm$ 7% ($n = 3$) of the initial $I_{GABA,max}$. We infer that the increase in the holding current after Cu:phen application in the presence of activating concentrations of these anesthetics was caused by disulfide bond formation that significantly increased the channels' spontaneous open probability. As a percentage of the maximal GABA current, the holding current increase was similar regardless of whether Cu:phen was coapplied with GABA, propofol, or pentobarbital. The increase was significantly greater than the effect of application of Cu:phen alone (Table 2). After washout of the anesthetic and Cu:phen, in some experiments, the holding current relaxed over a 10- to 20-min period to a smaller value, although rarely back to the original baseline value. This relaxation may be the result of endocytosis of receptors from the cell surface and their replacement by unmodified receptors, as was previously reported (39). This was rarely observed when Cu:phen oxidation was performed in the presence of GABA (31). The basis for this difference in recovery is uncertain, but the variability in the rates of endocytosis of cell surface proteins in batches of *Xenopus* oocytes could account for this difference (39,40).

Similar results were observed with propofol and pentobarbital on the $\alpha_1\beta_1$T256C mutant (data not shown). With these two anesthetics there was no effect of Cu:phen on the α_1T261Cβ_1 mutant (data not shown). Thus, disulfide bond formation in the presence of directly activating concentrations of the anesthetics required only the Cys in the β-subunit, just as with disulfide bond formation in the presence of GABA. At the 6′ level in the channel, it appears that the α-subunit is incapable of participating in disulfide bond formation. We believe that this is because there are only two α-subunits, and they are in nonadjacent positions around the central channel axis.

Fig. 5 *C* illustrates the typical effect of coapplication of an activating concentration of isoflurane and Cu:phen on $\alpha_1\beta_1$ T256C receptors. As can be seen in the current traces in Fig. 5 *C*, the holding current increased and remained elevated after washout of isoflurane and Cu:phen. After the subsequent application of GABA, however, there was a further increase in the holding current. This was seen consistently in the isoflurane + Cu:phen experiments. After a 1-min application of Cu:phen in the presence of 20 mM isoflurane, the holding current increased to 32 $\pm$ 22% ($n = 4$) of $I_{GABA,max}$ for $\alpha_1\beta_1$T256C receptors (Fig. 5 *C* and Table 2).

We previously showed in control experiments that Cu:phen application to uninjected oocytes or to oocytes expressing wild-type $\alpha_1\beta_1$ receptors in the absence or in the presence of GABA did not produce any significant change in the oocyte holding currents (31). Thus, we infer that the observed increase in holding current is caused by disulfide bond formation in the Cys mutant $GABA_A$ receptors. We previously noted that this increased holding current could not be blocked by either picrotoxin or penicillin (31). We believe that the disulfide bond may prevent these open channel blockers from gaining access to their binding sites, which for picrotoxin is thought to be at the 2′ level (41). We also

TABLE 1 Anesthetic concentrations used

	Potentiating concentraion (mM)			Activating conc. (mM)		
Mutant	Propofol	Pentobarbital	Isoflurane	Propofol	Pentobarbital	Isoflurane
α_1T261Cβ_1T256C	0.002	0.03	ND	0.04	1	ND
$\alpha_1\beta_1$T256C	0.002	0.03	1	0.04	1	20*

ND, not done.

*Although the mean current induced by 20 mM isoflurane was only 3 ± 0.6% ($n = 7$) of the maximal GABA-induced current, the current was not corrected for the significant amount of inhibition that was also observed at this isoflurane concentration.

previously showed that the reducing agent dithiothreitol (DTT) did not reverse the effects of disulfide bond formation at the 6′ level, nor did EDTA (31). Presumably, once formed, the 6′ disulfide bond is inaccessible to DTT in intact channels.

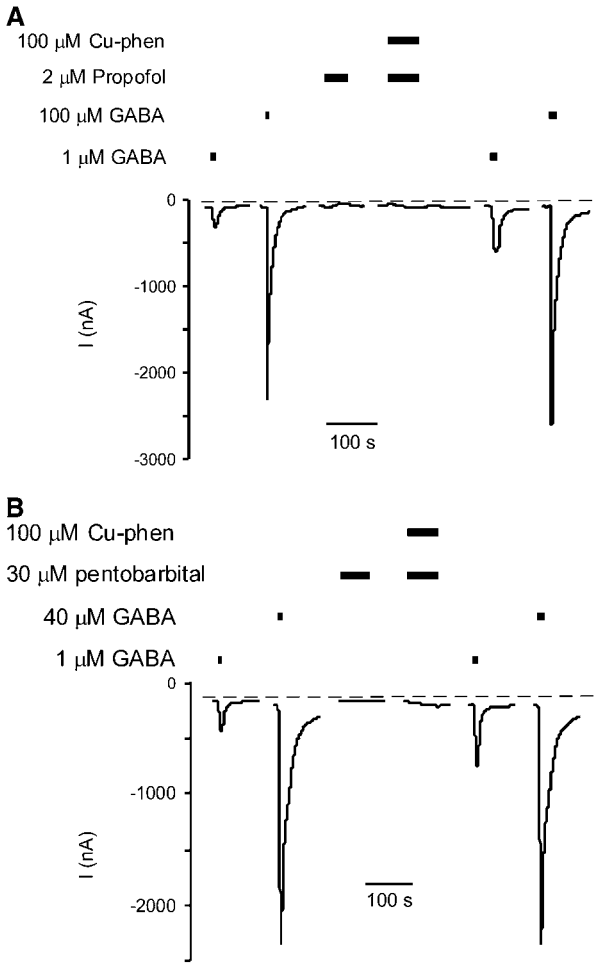

FIGURE 4 Effect of Cu:phen-induced oxidation in the presence of potentiating concentrations of propofol and pentobarbital on currents from oocytes expressing α_1T261Cβ_1T256C receptors. Dotted line indicates the initial holding current level. Note that the resting holding currents indicated by the initial currents at the start of the traces after Cu:phen application are similar to the initial holding currents before Cu:phen application. Periods of reagent application are indicated by the black bars above the current traces. (*A*) Oxidation in the presence of a potentiating concentration of propofol, 2 μM. (*B*) Oxidation in the presence of a potentiating concentration of pentobarbital, 30 μM.

We know that a disulfide bond is formed because dimers were present on Western blots that in SDS could be reduced with DTT to monomers (31).

DISCUSSION

These experiments sought to elucidate the structural consequences of general anesthetic binding to $GABA_A$ receptors in the region of the ion channel. We used disulfide-trapping experiments to investigate the conformational changes in the M2 channel-lining segment after anesthetic binding. Although this provides only low-resolution structural information, it does provide a basis for comparing conformational changes induced by GABA with those induced by general anesthetics. We used the state dependence of disulfide bond formation between engineered Cys residues at the M2 segment 6′ position as a reporter for anesthetic-induced conformational changes in this channel-lining region. We showed previously that disulfide bonds formed at a measurable rate between Cys residues in α_1T261Cβ_1T256C receptors only when Cu:phen-induced oxidation occurred in the presence of GABA (31). This resulted in an increase in the holding current after Cu:phen washout. We have now shown that the increased macroscopic holding current resulted from a significant increase in the channel's spontaneous open probability (Fig. 2). Strikingly, even with a disulfide bond at the 6′ level between two adjacent channel-lining M2 segments, the channels can still fluctuate between the open and closed states, as reflected by the flickering activity during bursts (Fig. 2 *B*). Furthermore, the channels can enter longer-lasting desensitized states that likely account for the long nonconducting intervals that we observed between bursts (Fig. 2 *B*). This implies that even with the 6′ disulfide bond there is sufficient mobility in the remaining three subunits and/or in the more extracellular portions of the M2 segments of the disulfide-linked pair to allow opening and closing of the channel gate(s).

We infer that the increase in spontaneous openings occurs because the disulfide bond distorts the channel structure sufficiently to reduce the energy barrier for channels entering the open state in the absence of agonist. We hypothesize that channel opening may induce a conformational change at the 6′ level that brings the engineered Cys on adjacent subunits into close proximity. This allows disulfide bond formation to occur. The disulfide bond presumably stabilizes this region

TABLE 2 Initial leak and change in leak after Cu:phen as a percentage of maximal GABA current ($I_{GABA,max}$) for α_1T261Cβ_1T256C receptors

	Initial leak (%)*	Leak after Cu:phen, potentiating conc. (%)*	Leak after Cu:phen, activating conc. (%)*
Alone	10 ± 6 (9)	9 ± 8 (10)	NA
GABA	11 ± 4 (6)	NA	29 ± 10 (6) †
Propofol	10 ± 9 (12)	12 ± 10 (7)	44 ± 17 (5) †
Pentobarbital	11 ± 4 (6)	1 ± 0 (3)	28 ± 7 (3) †
Isoflurane	6 ± 4 (10)	13 ± 3 (3)	32 ± 22 (4) †

Data given as mean ± SE (number of experiments). NA, not applicable.
*Percentage of $I_{GABA,\ max}$.
†Significantly different ($P < 0.01$) from initial leak by one-way ANOVA with Dunnett's post hoc test.

of the channels in a conformation similar to the open state, thus increasing the spontaneous open probability. The fact that the single-channel conductance is reduced in the disulfide-linked channels suggests that their structure is not identical to the open channel. Furthermore, neither picrotoxin nor penicillin blocks the disulfide-cross-linked channels (31), perhaps because the disulfide bond narrows the lumen or reduces the flexibility in this region and does not allow picrotoxin to reach its binding site at the 2′ level (41,42). The disulfide bond may distort the structure of the more cytoplasmic portion of M2 that lines the narrowest portion of the open channel. In the homologous ACh receptor, the −1′ to 2′ region appears to be the narrowest region of the open channel (43–45). This region is likely to have a major impact on single-channel conductance, but other more cytoplasmic regions may also affect conductance as well (46).

The state dependence of the disulfide bond formation could result from two factors. As alluded to above, channel opening may induce a conformational change in the position of the engineered Cys to bring them into close proximity to allow disulfide bond formation. In addition, channel activation may open a closed channel gate in the 9′–14′ region (26,29), allowing access of oxidants to the 6′ engineered Cys residues. The fact that disulfide bond formation increases the spontaneous open probability implies that the channel conformation in the 6′ region is likely to be different from that in the closed state. This implies that channel gating induces conformational changes at the 6′ level, although the more cytoplasmic region of the channel may be more rigid (47,48).

In the current experiments, we observed a significant increase in holding current after oxidation only in the presence of activating concentrations of anesthetic. The magnitudes of the increases were similar to those seen after oxidation in the presence of GABA (Table 2). After oxidation in the presence of potentiating concentrations of propofol and isoflurane, there were small but not statistically significant increases in holding current. It is possible that potentiating concentrations that we used induced sufficient channel opening, i.e., low-level direct activation, to allow small amounts of disulfide

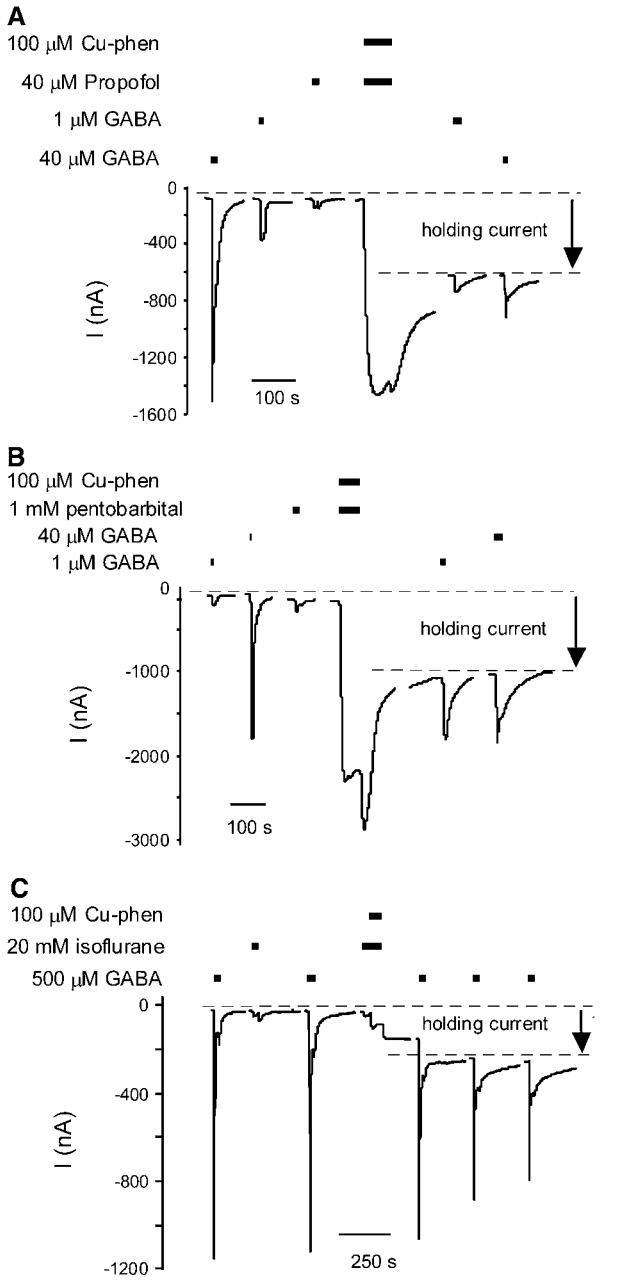

FIGURE 5 Effect of Cu:phen-induced oxidation in the presence of directly activating concentrations of the three anesthetics. Top dotted line indicates the initial holding current level. Lower dotted line indicates level of holding current after Cu:phen application. Note the increase in holding currents indicated by the arrows (between the *dotted lines* and indicated as *holding current*). Also note the decrease in the subsequent GABA-induced currents after application of Cu:phen. Periods of reagent applications are indicated by the black bars above the current traces. (*A*) Oxidation in the presence of an activating concentration of propofol, 40 μM. Oocyte expressing α_1T261Cβ_1T256C receptors. (*B*) Oxidation in the presence of an activating concentration of pentobarbital, 1 mM. Oocyte expressing α_1T261Cβ_1T256C receptors. (*C*) Oxidation in the presence of an activating concentration of isoflurane, 20 mM. At this isoflurane concentration there is also a significant amount of inhibition by isoflurane. Oocyte expressing $\alpha_1\beta_1$T256C receptors.

bond formation. Given that the ability to form disulfide bonds at this channel level appears to require channel activation either by GABA or by the anesthetics and that once formed the disulfide bonds increase the spontaneous open probability, we infer that the conformational change being detected is directly related to channel opening. Thus, we infer that activating concentrations of the anesthetics tested induced a conformational change in the M2 segment 6′ level similar to that induced by GABA.

Our data suggest that channel activation by the intravenous anesthetics propofol and pentobarbital produced a conformational change that allowed disulfide bond formation between Cys residues substituted for the β-subunit M2 segment 6′ residues. Similar results were obtained for the volatile anesthetic isoflurane. With isoflurane, however, reopening the channels after disulfide bond formation seemed to increase the holding current (Fig. 5 *C*). This difference between isoflurane and the intravenous anesthetics may imply that isoflurane induced a somewhat different conformational change than GABA and the intravenous anesthetics. Alternatively, at the isoflurane concentration that we used, the extent of channel block was significantly greater than that with propofol or pentobarbital. It is possible that disulfide bond formation in isoflurane-blocked channels may cause isoflurane to become trapped in the channel at an inhibitory binding site. Subsequent activation by GABA may release the trapped isoflurane, allowing the increased spontaneous open probability to become apparent. Evidence indicates that the isoflurane inhibitory site may be near the M2 segment 2′ position (49). Thus, the disulfide bond at the 6′ position could affect isoflurane affinity at a channel site that is only 6 Å away. Consistent with the idea of an interaction between the 2′ and 6′ sites, covalent modification of α_1T261C$\beta_1\gamma_{2S}$ receptors markedly reduced the affinity for picrotoxin, which binds at the 2′ level (41).

We previously showed using both an electrophysiological assay and Western blots with epitope-tagged subunits that the disulfide bonds at the 6′ level formed between β-subunits; disulfide bonds did not involve the α-subunits (31). The results with the anesthetics using the electrophysiological assay were consistent with the previously observed β-subunit dependence. Oxidation in the presence of anesthetics of the double Cys mutant α_1T261Cβ_1T256C and the single β Cys mutant, $\alpha_1\beta_1$T256C, had similar effects. Oxidation in the presence of anesthetics did not affect the single α Cys mutant, α_1T261Cβ_1. Because the major subunit stoichiometry in $\alpha\beta$ receptors is two α- and three β-subunits (17–19), and because disulfide bond formation increased the spontaneous open probability, we inferred that the bond likely formed between Cys residues in adjacent β-subunits. We felt that disulfide bond formation between Cys in nonadjacent subunits would block the channel lumen at this level. Thus, the formation of the 6′ disulfide bond appeared to involve subunit 5, the one not involved in forming a GABA binding site, and the adjacent β subunit. Perhaps there is an asymmetry in the channel, two pairs of β-α subunits form GABA binding sites and effectively form functional units relative to subunit 5, which is a β-subunit in the case of $\alpha\beta$ receptors or the γ-subunit in the case of $\alpha\beta\gamma$ receptors. We suggested that the disulfide bond was able to form because channel opening involved an asymmetric movement at the β-β subunit interface either in time or in space (31). It is interesting that with the anesthetics as channel activators we observe a similar β-Cys subunit dependence for disulfide bond formation. Thus, regardless of whether the channel is opened by GABA binding in the GABA binding sites or by anesthetic binding at activation sites that remain to be identified, the open-state channel conformations appear structurally similar.

Our experimental evidence of the structural similarity of the GABA and anesthetic open-state conformations is consistent with functional studies that have shown similar single-channel conductances regardless of whether $GABA_A$ receptor channels are opened by GABA or by anesthetics, leading to the hypothesis that the open-channel structures would also be similar (12,13). Thus, although the binding sites for these various agonists, GABA, propofol, pentobarbital, and isoflurane, may be at different locations in the protein (6–8,50,51), the open-state conformation that they induce is similar. In the homologous nicotinic acetylcholine receptor, using linear free energy relationships, a conformational wave has been measured from the acetylcholine binding site in the extracellular domain to the cytoplasmic end of the channel (52,53). It would be interesting to determine whether the conformational wave induced by GABA binding is similar to that induced by the anesthetics. This may be particularly useful in determining whether activation by anesthetics occurs through binding in the membrane-spanning domain or in the extracellular domain.

Propofol, at potentiating concentrations, induced conformational change in the membrane-spanning domain that increased the accessibility of α_1 subunit, M3 segment, substituted Cys residues to the sulfhydryl reagent $pCMBS^-$ (11). It did not, however, facilitate a significant amount of disulfide bond formation at the M2 segment 6′ level. Thus, propofol binding at its potentiating site(s) stabilizes a membrane-spanning domain conformation or ensemble of conformations that is/are different from the closed and open states. This is consistent with the conclusions of studies of propofol's effects on channel kinetics. These indicate that propofol stabilizes a doubly liganded, preopen state (9). Current evidence suggests that the propofol potentiation binding site is located near the extracellular end of the M2 and M3 membrane-spanning segments (2,38). Similar locations have been suggested for isoflurane and pentobarbital (54,55).

Of note, Lynch and co-workers reported that in HEK293 cells they did not observe Cu:phen-induced increases in holding current (56). It is possible that the difference may arise because some HEK cells lines have been shown to express significant levels of endogenous wild-type β-subunit

(57,58). Given the β-subunit dependence that we observed at the 6′ level, the coexpression of endogenous wild-type β-subunits would have a major impact on the results. Further work will be necessary to clarify these issues.

In summary, the current experiments provide structural evidence to support the hypothesis that at concentrations that activate $GABA_A$ receptors, general anesthetics induce a similar ion channel conformation as that induced by GABA activation. These experiments also indicate that the conformational change that is required to facilitate 6′ disulfide bond formation is associated with channel opening. Anesthetic binding at the potentiating binding site(s) does not induce this M2 segment structural change to a significant extent. This implies that the structural changes in the M3 segment region detected in the presence of potentiating concentrations of propofol (11) represent states between the closed and open states. Thus, a picture is starting to emerge of the sequence of conformational changes that occur during channel gating by GABA and by anesthetics.

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