Gating-induced Conformational Rearrangement of the \(\gamma\)-Aminobutyric Acid Type A Receptor \(\beta-\alpha\) Subunit Interface in the Membrane-spanning Domain*

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Background: GABA\(_{\alpha}\) receptor transmembrane domain undergoes conformational changes during channel gating.

Results: Solvent accessibility increases in a cluster of \(\alpha\)1 subunit M1 segment residues facing the \(\beta2\) subunit M3 segment during channel gating.

Conclusion: Channel gating induces a conformational change in the subunit interface between \(\alpha1\)M1 and \(\beta2\)M3 and within the \(\alpha1\) subunit.

Significance: This work increases understanding of pentameric ligand-gated ion channel gating.

GABA\(_{\alpha}\) receptors mediate fast inhibitory synaptic transmission. The transmembrane ion channel is lined by a ring of five \(\alpha\) helices, M2 segments, one from each subunit. An outer ring of helices comprising the alternating M1, M3, and M4 segments from each subunit surrounds the inner ring and forms the interface with the lipid bilayer. The structural rearrangements that follow agonist binding and culminate in opening of the ion pore remain incompletely characterized. Propofol and other intravenous general anesthetics bind at the \(\beta2\)M2\(_{269}\) during gating. Thus, channel gating does not involve rigid body movements of the entire transmembrane domain. Channel gating causes changes in the relative position of transmembrane segments both within a single subunit and relative to the neighboring subunits.

GABA\(_{\alpha}\) receptors are members of the Cys-loop receptor superfamily of neurotransmitter-gated ion channels. They mediate fast synaptic inhibition in the brain and are a major target for general anesthetics and drugs used to treat anxiety disorders, insomnia, muscle spasms and epilepsy (1–5). Elucidating the conformational changes that occur during channel gating to understand the structural basis for chemoelectrical transduction and the mechanisms of drug action is the subject of active investigation.

In the central nervous system the most common synaptic GABA\(_{\alpha}\) receptors contain 2 \(\alpha\), 2 \(\beta\), and 1 \(\gamma\) subunit arranged in a \(\alpha\)\(\beta\)\(\alpha\)\(\gamma\) counterclockwise sequence around the central channel axis (6, 7). For each subunit, the extracellular domain contains ~200 amino acids forming a \(\beta\) sandwich structure. The transmembrane channel is lined by the five M2 segments, one from each subunit (8–11). This inner ring of \(\alpha\) helices is surrounded by an outer ring of alternating M1 and M3 segments that form the interface with the lipid bilayer membrane (12). The M4 segment lies in close proximity to the M1 and M3 segments of the same subunit beyond the outer helical ring. GABA binds in the extracellular domain at the \(\beta-\alpha\) subunit interface approximately 30 Å above the membrane surface (12–14). It induces a conformational change that propagates to open the channel gate in the middle of the transmembrane domain allowing anions to pass through the channel (15). In the presence of GABA the channels undergo rapid transitions among closed, open, and desensitized states. The GABA-activated open state is metastable, and the desensitized state is the most energetically favorable in the presence of GABA. Signal transduction from the extracellular ligand binding domain to the channel gate occurs through the interface region between the extracellular and transmembrane domains. The interface region is formed by two extracellular loops, namely the \(\beta1-\beta2\) loop and the \(\beta6-\beta7\) Cys-loop, that interact with the extracellular loop between the M2 and M3 transmembrane segments (14, 16–18). In addition, the extracellular \(\beta10\) strand connects the binding site C-loop to the extracellular end of the M1 membrane-spanning segment. Intravenous general anesthetics are thought to bind in the extracellular half of transmembrane domain subunit interface between the \(\beta3\)M3 and \(\alpha1\)M1 segments, the same interface where GABA binds in the extracellular...
domain (19–22). We sought to probe the conformational changes occurring in the β3-αM1 transmembrane subunit interface region that plays such a critical role in the mechanism of action of general anesthetics.

A variety of experimental approaches have been used to investigate conformational changes in functional ion channels and transporters including the substituted cysteine accessibility method (SCAM)2 and disulfide cross-linking (8, 21, 23, 24). SCAM experiments measure the reactivity of engineered Cys residues to reaction with charged sulfhydryl-reactive reagents (8, 23). The reactivity of a Cys depends on multiple factors, including (i) the local concentration of the sulfhydryl reagent in proximity to the Cys, (ii) local steric factors that may affect the access of the reagent to the Cys and the collision frequency between the sulfhydryl reagent and the Cys, and (iii) the ionization state of the Cys. The local concentration of the sulfhydryl reagent depends on (i) concentration in bulk solution, (ii) the access pathway from bulk solution to the Cys, and (iii) if the reagent is charged, the local electrostatic potential in the region of the Cys. The access pathway from bulk solution to the Cys may limit the ability of the reagent to reach the Cys. In addition, the local electrostatic potential at the Cys residue will influence both the local concentration of the reagent, if it is charged, and the ionization state of the Cys thiol. The local concentration, which in turn depends on the proximity and relative mobility of the two protein regions. Protein structural changes that affect the reaction rate, mutation of residues along the access pathway traversed by pCMBS or in proximity to the engineered Cys can affect measured reaction rates. Furthermore, if the reaction rate of charged sulfhydryl-reactive reagents is different in two states of a channel, it suggests that either the access pathway to the engineered Cys residue or its local environment, steric or electrostatic, has changed.

In contrast to SCAM, disulfide cross-linking experiments test the ability of two engineered Cys residues to form a disulfide bond (25–27). The rate of disulfide bond formation depends on collision frequency between the pair of Cys residues, which in turn depends on the proximity and relative mobility of the two protein regions. Protein structural changes during channel gating can also be detected by alterations in the rate or ability to form disulfide bonds. In the current work we have used SCAM and disulfide cross-linking experiments to investigate the conformational changes occurring in the β3-αM1 transmembrane subunit interface upon channel activation.

EXPERIMENTAL PROCEDURES

In Vitro Transcription and Oocyte Expression—The pGEMHE plasmids coding for the rat GABAAα1, β2, and γ2S subunits and the various mutants were described previously (21). Of note, the endogenous cysteines in the GABAAα1 and β2 subunit transmembrane domains were mutated to create a “Cys-light” background. The mutations were α1C234S and α1C293S in M1 and α1C293S in M3, and β2C288A in M3; the γ2S subunit was not modified. Preparation and injection of mRNA in oocytes were performed as described previously (28). Capped mRNA was synthesized by in vitro transcription using T7 RNA polymerase mMessage mMachine kit (Applied Biosystems/ Ambion, Austin, TX) from Nhel-linearized (New England Biolabs, Ipswich, MA) plasmids. Xenopus laevis oocytes were harvested and defolliculated with 0.2% collagenase (Sigma-Aldrich) for 60 min at room temperature in SOS medium (100 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, and 5 mM HEPES, pH 7.5, with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Invitrogen) supplemented with 5% horse serum (Sigma-Aldrich)). Oocytes were injected with 50 nl of 200 ng/μl mRNA 24–48 h after isolation and maintained at 16 °C. The ratio of mRNA α1;β2;γ2 injected was 1:1:2. We previously showed based on Zn2+ and diazepam sensitivity that this ratio gives uniform incorporation of γ2 into the functional cell surface receptors (29).

Electrophysiology—Two-electrode voltage clamp experiments were done at room temperature 3–5 days after mRNA injection as described previously (15). Oocytes were perfused continuously in a ~200-μl chamber at 5–6 ml/min with calcium-free frog Ringer’s solution, containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM MgCl2, 10 mM HEPES, pH 7.5, adjusted with NaOH. Cells were impaled using 3 M KCl-filled glass electrodes (R < 2 megohms) and voltage clamped at ~60 mV using a TEV-200 amplifier (Dagan Instruments, Minneapolis, MN) controlled by pClamp 8.2 software (Molecular Devices, Sunnyvale, CA). Currents were digitized at 200 Hz using a Digidata 1322A analog-digital converter (Molecular Devices) and stored in a PC before being analyzed using the same software. The ground electrode was connected to the bath via a 3 M KCl agar bridge. All experiments were performed on at least three oocytes from at least two different batches.

Determination of pCMBS Reaction Rates—For most of the mutants used in this study we had previously determined the GABA concentration that produces 50% of the maximal current (EC50) (21). Measurement of EC50 for the newly designed mutants was performed as described previously (21).

To measure pCMBS (Toronto Research Chemicals, North York, ON, Canada) reaction, covalent modification of the receptor must have an effect on the macroscopic channel function. To determine reaction rates for each construct, a submaximal concentration of pCMBS was chosen based on preliminary experiments so that the reaction would proceed to completion during a 1–2 min cumulative application. pCMBS was applied repeatedly (15–30 s/application) either in the absence of GABA for closed state rates or in the presence of a saturating GABA concentration for activated state rates. The pCMBS applications were alternated with EC50 GABA test pulses. Note that the receptor EC50 before modification does not necessarily remain unchanged after pCMBS modification. The GABA EC50-induced current amplitudes were plotted as a function of the cumulative pCMBS exposure time and fitted with a monoexponential function (pClamp 8.2 or Prism 5,
GraphPad Software, La Jolla, CA) to calculate the pseudo-first-order time constant (in s⁻¹). In some mutants, pCMBS modification increased the holding current in the absence of GABA presumably due to an increase in spontaneous activation of the pCMBS modified channels. In these cases, the magnitude of the GABA-induced test currents was measured from level of the holding current in the absence of GABA and not from I = 0. For several cells the currents were measured from both I = 0 and from the level of the base-line holding current, and the pCMBS reaction rates calculated were the same. The second-order rate of reaction (in M⁻¹s⁻¹) was calculated by dividing the pseudo-first-order time constant by the pCMBS concentration used. Data are reported as means ± S.E. Statistical analysis (t tests or one-way analysis of variance) was performed with Prism 5 software.

RESULTS

To probe for conformational changes during channel gating in the β2M3-α1M1 transmembrane interface region we measured the reaction rate of pCMBS in the absence and presence of GABA with Cys substituted for residues in this region. We measured the pCMBS reaction rate by applying 4–6 pulses of pCMBS at a submaximal concentration and recorded the subsequent EC₅₀ GABA-induced test currents. pCMBS was applied to the resting receptors, in the absence of GABA, and, in separated sets of experiments, to the “activated state” of the receptors, in the presence of 200 μM GABA, a saturating concentration. This activated state includes a mixed population of open and desensitized receptors. As reported previously, the GABA EC₅₀ for the Cys-light WT construct was 4.0 μM, and the GABA EC₅₀ for the Cys mutants constructed ranged from 1.5 μM for α1L232C to 16 μM for α1T230C (21). The EC₅₀ value for the newly generated receptor α1β2L285Cγ2 was 0.5 ± 0.1 μM, n_H = 1.2 ± 0.2 (n = 4).

Fig. 1 illustrates the measurements of reaction rates in the closed state and in the activated state for the α1Q229Cβ2γ2 receptor. Note that pCMBS reaction with this Cys increased both the holding current in the absence of GABA and the magnitude of the GABA-activated current. The increase in holding current was most likely due to an increase in the spontaneous open probability for this pCMBS-modified mutant channel, an effect we have observed at other positions in previous studies (15, 21). The values of the reaction rates for all the receptors studied are presented in Table 1. The closed state reaction rates ranged from 500 ± 40 (α1Y225C) to 72,000 ± 13,000 M⁻¹s⁻¹ (α1G224C). In the activated state, the reaction rates ranged from 625 ± 195 (α1V227C) to 465,000 ± 175,000 M⁻¹s⁻¹ (α1L228C). Of note, pCMBS did not react with α1I228C at a measurable rate in the absence of GABA, but did react at the fastest measured rate in the presence of GABA.

For the mutants α1F226C, α1I228C, and β2F289C the pCMBS reaction rate increased >10-fold in the presence of GABA (Fig. 2). For the mutants α1L232C, α1M236C, and β2L285C the pCMBS reaction rate increased between 5- and 10-fold in the presence of GABA (Fig. 2). For the mutants α1Y225C, α1Q229C, β2M286C, and β2G287C the reaction rates increased 2–5-fold in the presence of GABA. For the mutants α1V227C and α1T230C the reaction rates were not significantly different in the absence and presence of GABA. In contrast, for α1G224C the pCMBS reaction rate decreased by 60-fold in the presence of GABA (Fig. 2).

The receptor α1I228Cβ2γ2 showed a unique feature. In the closed state, when using pCMBS concentrations <0.1 μM, the receptor was not modified during a 2-min pCMBS application. However, when pCMBS concentrations >1.0 μM were used, pCMBS appeared to react with α1I228C, and the measured rate approached the very high reaction rate measured in the GABA-activated state. We interpreted this behavior as follows. In the

![Example of rate determination in the α1Q229Cβ2γ2 receptor.](image-url)

**FIGURE 1.** Example of rate determination in the α1Q229Cβ2γ2 receptor. A, the oocyte voltage clamped at −60 mV was alternately exposed to an EC₅₀ GABA concentration (2 μM, rectangles) and to a submaximal concentration of pCMBS (5 μM, arrow, data not shown) and the resulting currents recorded. The **double bar** represents a gap of ~5 min. B, the GABA-induced test current amplitudes from A are plotted as a function of cumulative pCMBS exposure time and fitted with a monoexponential function to obtain the pseudo-first-order rate constant.

**TABLE 1**

| Subunit | Mutation | Basal rate (−GABA) | Rate of reaction |
|---------|----------|--------------------|------------------|
| α1      | G224C    | 72.000 ± 15.000    | +GABA 1,200 ± 310* |
|         | (+ R269Q)| 4,200 ± 700        | 1,400 ± 340*     |
|         | Y225C    | 500 ± 40           | 1,800 ± 900*     |
|         | F226C    | 900 ± 90           | 25,000 ± 7,000*  |
|         | V227C    | 780 ± 80           | 625 ± 195        |
|         | L228C    | No reaction        | 465,000 ± 175,000*|
|         | (+ R269Q)| 1,400 ± 230        | 16,700 ± 500*    |
|         | Q229C    | 25,000 ± 8,000     | 61,500 ± 10,000* |
|         | (+ R269Q)| 20,500 ± 3,400     | 25,000 ± 6,300   |
|         | T230C    | 231C               |                 |
|         | L232C    | 1,800 ± 350        | 9,000 ± 700*     |
|         | M236     | 820 ± 50           | 8,100 ± 2,250*   |
|         | L285C    | 1,500 ± 500        | 11,000 ± 400*    |
|         | M286C    | 1,700 ± 500        | 5,000 ± 1,000*   |
|         | G287C    | 245 ± 15           | 950 ± 80*        |
|         | F289C    | 380 ± 10           | 4,000 ± 350*     |

* Statistically significantly different from the rate in the absence of GABA (p < 0.05).

* Currents too small to measure rates.
GABA Alters GABAA Receptor β-α Subunit Interface Conformation

FIGURE 2. Ratio of the pCMBS reaction rates in the presence and absence of GABA. Note that for α1I228C (hatched bar) we assumed that the reaction rate in the closed state was 10 M⁻¹s⁻¹ for the purposes of the figure, but there was no evidence of reaction in the closed state on the time scale of our experiments.

closed state pCMBS did not react. At pCMBS concentrations >0.1 μM, pCMBS has two actions: it behaves as a weak agonist activating the receptors, and then other pCMBS molecules can react with the now accessible Cys of α1I228C at a very high rate. We have previously observed agonist effects of pCMBS on other GABAA receptor Cys mutants (15, 21).

Following pCMBS application, three mutant receptors, α1Q229C, α1T230C, and α1M236C, showed an increase in their spontaneous open probability (Fig. 3). For the α1T230C and α1M236C mutants, we could measure the pCMBS reaction rate in the absence of GABA by the rate of rise of the spontaneous current. The rate of reaction in the closed state measured either by fitting the pCMBS-induced current or by plotting the amplitude of the GABA-induced EC₅₀ test currents following submaximal pCMBS applications gave the same rate of reaction (Fig. 3). As noted above, pCMBS also caused an increase, albeit much smaller, in the spontaneous open probability for the α1Q229Cβ2y2 receptor (Fig. 1).

Electrostatic Effect of α1R269 on pCMBS Reaction Rates—The pCMBS reaction rate was very high, >50,000 M⁻¹s⁻¹, at α1G224C in the absence of GABA and at α1I228C and α1Q229C in the presence of GABA. One of the factors affecting the reaction rate is the local concentration of pCMBS in close proximity to the Cys. Because pCMBS is negatively charged, the local electrostatic environment can affect its local concentration and thus, its apparent reaction rate. We hypothesized that the very high reaction rates seen at these three positions might be explained by an electrostatic effect from a positively charged amino acid in the vicinity. In the crystal structures of both the nAChR and GluCl the M2 segment 19 residue, β2R269 in the GABAA receptor, faces toward the interface formed by α1M1

and β2M3 (12, 14). Therefore, it was a candidate for the observed effect.

To test this hypothesis, we generated the β2R269Q mutant. We measured the pCMBS reaction rate with the α1M1 Cys using β2R269Q in lieu of the β2 WT. For α1G224C, the pCMBS reaction rate in the absence of GABA was 72,000 ± 13,000 M⁻¹s⁻¹ with WT β2, but replacing the β2-19’ Arg with Gln reduced the reaction rate 17-fold to 4,200 ± 400 M⁻¹s⁻¹ in the α1G224Cβ2R269Qy2 receptors (Table 1). At α1G224C in the presence of GABA the pCMBS reaction rate is significantly slower, 1,200 ± 310 M⁻¹s⁻¹, and was not affected significantly by the β2R269Q substitution (Table 1). This suggests that the distance between β2R269 and β1G224 is different in the closed and GABA-activated states.

pCMBS did not react with α1I228C in the absence of GABA, but in the presence of GABA the β2R269Q substitution reduced the pCMBS reaction rate by a factor of 12. The reaction rate decreased from 465,000 ± 175,000 to 37,000 ± 3,400 M⁻¹s⁻¹ (Table 1). For α1Q229C, replacing WT β2 with β2R269Q reduced the pCMBS reaction rate in the absence of GABA by 17-fold from 25,000 ± 8,000 to 1,400 ± 230 M⁻¹s⁻¹ (Table 1). However, in the presence of GABA the effect of the β2R269Q substitution was smaller, reducing the pCMBS reaction rate from 61,500 ± 10,000 to 16,700 ± 500 M⁻¹s⁻¹ (a factor of 3.6, Table 1). These results indicate that β2R269 is positively charged in both the closed and in the activated state and that the positive charge influences the pCMBS reaction rate with the α1 Cys mutants G224C, I228C, and Q229C.
GABA Alters GABA$_A$ Receptor $\beta$-$\alpha$ Subunit Interface Conformation

![Figure 4. Representative current recording from an oocyte expressing the $\alpha_{11228C}$$\beta_{2R269C}$$\gamma_2$ voltage clamped at $-60$ mV. Oocytes were superfused sequentially with a saturating GABA concentration (200 $\mu$M), then to the oxidizing agent 100-200 $\mu$M copper phenanthroline (Cuphen), then to 200 $\mu$M GABA, then to 2 mM reducing agent DTT, and finally to 200 $\mu$M GABA. Between reagent applications the oocyte was continuously superfused with buffer solution.](image)

**DISCUSSION**

Agonist binding to Cys-loop receptors induces conformational changes that result in channel opening and desensitization. Despite the number of recent x-ray crystal structures of Cys-loop receptors, the nature of the molecular movements associated with channel gating remains incompletely understood. In this paper, we investigated whether conformational change occurs in the transmembrane domain at the subunit interface formed by the $\beta_2M3$ segment and the $\alpha_1M1$ segment by measuring the $p$CMBS reaction rates with Cys substituted for residues forming this subunit-subunit interface. This interface is of particular interest because amino acid $\beta_2M286$ in this interface is part of or in the immediate vicinity of the general anesthetic binding site for propofol and etomidate (19, 20, 22, 31). The region is also of interest because it is in close proximity to the lipid membrane. Conformational changes in this region would likely alter lipid-protein interactions.

**Reaction in the Closed, Resting Channel State**—In the resting state, we found that all of the residues examined, except $\alpha_{11228C}$, were accessible to $p$CMBS modification (Table 1). $p$CMBS is negatively charged and would fit into a right cylinder about 6 Å in diameter and 10 Å in length. The ability of $p$CMBS to react with almost all of the $\alpha_1M1$ Cys suggests that in the resting state the extracellular half of $\alpha_1M1$ is not tightly packed against either the surrounding transmembrane segments or the lipid bilayer. Alternatively, $p$CMBS may gain access to the substituted Cys residues as a result of thermal mobility of the extracellular half of M1 that may create crevices from the extracellular aqueous surface down to the substituted Cys (32–34). It should be noted that the access pathway for $p$CMBS from bulk solution to these $\alpha_1M1$ residues is unknown, although it is probably not through the ion channel.

In the closed resting state, the $p$CMBS reaction rate with Cys substituted at different positions varied by $\geq 2$ orders of magnitude. Closed state reaction rates were $<1,000$ M$^{-1}$s$^{-1}$ for five mutants, $\alpha_{1Y225C}$, $\alpha_{1F226C}$, $\alpha_{1V227C}$, $\alpha_{1M236C}$, $\beta_2G287C$, and $\beta_2F289C$ (Fig. 5A, cyan). These residues are located in two different clusters. One cluster, including $\alpha_{1M236C}$, $\beta_2G287C$, and $\beta_2F289C$, lies in the subunit interface but is located furthest from the membrane surface, four helical turns from the top of M1 (Fig. 5A, cyan). The other cluster, including $\alpha_{1Y225C}$, $\alpha_{1F226C}$, and $\alpha_{1V227C}$, is near the top of the $\alpha_1M1$ helix. They do not face the subunit interface but rather face toward either the lipid bilayer or into the center of the $\alpha_1$ subunit four-helix bundle (Fig. 5A, cyan). It is not possible to know whether the slow $p$CMBS reaction rates are due to steric factors or limited exposure on the aqueous protein surface.

Reaction rates were slightly faster, between 1,000 and 2,000 M$^{-1}$s$^{-1}$, at three positions, $\alpha_{1L232C}$, $\beta_2L285C$, and $\beta_2M286C$ (Fig. 5A, salmon pink). These residues lie in the subunit interface, about three helical turns down from the extracellular ends of M1 or M3. The faster reaction rates may arise due to their location in the protein-protein interface and the ability of water to penetrate into this region.

For three residues the reaction rate was $>20,000$ M$^{-1}$s$^{-1}$ (Fig. 5A, red). One of these residues, $\alpha_{1G224C}$, is near the extracellular end of M1. The second, $\alpha_{1Q229C}$, faces into the subunit interface, close to $\beta_2R269$, which is on the backside of the $\beta_2M2$ segment. The third residue, $\alpha_{1T230C}$, is near the subunit interface but points toward the backside of the $\alpha_1M2$ segment in the same subunit. We showed that the fast reaction rates with $\alpha_{1G224C}$ and $\alpha_{1Q229C}$ in the closed state were due, in part, to an electrostatic attraction between the positive charge of $\beta_2R269$ and the negative charge of $p$CMBS. This electrostatic interaction may have increased the local $p$CMBS con-

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**Notes:**

- $\beta_2R269C$ mutant was reversed using DTT (Fig. 4). Application of the heavy metal-chelating agent EGTA did not reverse the inhibition following GABA application (data not shown). This suggests that metal-chelating agent EGTA did not reverse the inhibition following GABA application (data not shown).

- No GABA-induced currents were observed in oocytes expressing $\alpha_{1Q229C}$$\beta_2R269C$$\gamma_2$ receptors. Pentobarbital (100 $\mu$M) also did not induce any current in these oocytes. Furthermore, no GABA-induced current was observed after a 2-min application of 5 mM DTT, a reducing agent (data not shown). Either the double mutant is not functional, or a disulfide bond forms that prevents it from opening and/or trafficking to the cell surface.

- In contrast, in oocytes expressing $\alpha_{1L228C}$$\beta_2R269C$$\gamma_2$ the initial GABA-induced current was robust. Interestingly, the GABA-induced current in oocytes expressing $\alpha_{11228C}$$\beta_2R269C$$\gamma_2$ decreased upon GABA application (Fig. 4). The decrease of current was reversed using DTT (Fig. 4). Application of the heavy metal-chelating agent EGTA did not reverse the inhibition following GABA application (data not shown). This suggests that GABA activation induced disulfide bond formation presumably by increasing the collision frequency and/or proximity between $\beta_2R269C$ and $\alpha_{11228C}$.

- We also sought to investigate whether the negatively charged residue in the M2-M3 loop, $\beta_2D282$, that is located in close proximity in the GluCl crystal structure, had any effect on the $p$CMBS reaction rates. However, receptors that included either $\beta_2D282C$ or $\beta_2D282N$ were nonfunctional. We do not know why neither of these substitutions was tolerated. Of note, mutation of the aligned residue in the $\alpha_1$ subunit was tolerated, but the current amplitude was only 10% of WT (30).
centration/residence time and/or oriented pCMBS to increase its apparent reaction rate with the Cys at these two positions in the resting state. Eliminating the positive charge, via the 2R269Q mutation, reduced the pCMBS reaction rate by 20-fold at 1G224C and 1Q229C. This suggests that in the closed state 2R269 is close to these two 1M positions. In the GluCl structure (Protein Data Bank ID 3RHW), these two residues are separated from the 2R269 carbon by 12 and 4 Å, respectively (Fig. 5, A and D). These distances may be affected by the specific rotamer position of the 2R269 carbon.

Curiously, pCMBS did not react with 1I228C in the resting state. Using the GluCl structure, the aligned residue faces into...
the subunit interface and is surrounded by residues that reacted with pCMBS (Fig. 5, A and D). One possible explanation for the lack of reaction is that α1I228C may face the lipid in the closed state. This would imply that the GluCl structure may not be an ideal template for understanding the closed, resting state structure of the GABA<sub>α</sub> receptor; we will discuss this issue below. Alternatively, in the GluCl structure the residues aligned with α1I228 and β2D282 (M3) are in close proximity (Fig. 5A). Perhaps the aspartate negative charge may repel the negatively charged pCMBS. Unfortunately, we could not investigate this hypothesis because mutation of β2D282 to either asparagine or cysteine did not yield functional receptors.

Previous SCAM studies of the nicotinic acetylcholine receptor (nAChR) α and β M1 segments, by us and others, showed that a smaller subset of the residues in this region was accessible in the resting state (35, 36). The reason for the differences between nAChR and GABA<sub>α</sub> receptors is uncertain. It may relate to differences in the structure or packing of the receptors in this region. More likely it is due to the difference in the sulfhydryl reagents used. The nAChR studies used methanethiosulfonate derivatives, whereas the current study used the mercurial reagent pCMBS. The pCMBS reaction rate with simple thiols is several orders of magnitude faster and less sensitive to the ionization state of the thiol than the methanethiosulfonates (11, 23).

Changes with Channel Activation—In the activated states, pCMBS reacted at a similar or faster rate at all positions, except for α1G224C, the most extracellular residue tested (Table 1 and Figs. 2 and 5B). At α1G224C, pCMBS reacted 60-fold slower in the presence of GABA than in its absence. In the closed state, removal of the positive charge with the β2R269Q mutation reduced the pCMBS reaction rate with α1G224C by 17-fold, but in the presence of GABA the pCMBS reaction rate was unaffected by the β2R269Q mutation (Table 1 and Fig. 2). This indicates that channel activation altered the relative distance between β2R269 and α1G224. We infer that channel gating induces a conformational rearrangement of the transmembrane subunit interface.

The largest change in pCMBS reaction rate during channel activation occurred at position α1I228C. In the absence of GABA α1I228C was unreactive with pCMBS, whereas in the presence of GABA it reacted at the fastest rate, 465,000 M<sup>−1</sup>s<sup>−1</sup> (Table 1 and Fig. 2). The fast rate was due, in part, to electrostatic effects of the positive charge at β2R269. The pCMBS reaction rate decreased 12-fold with the β2R269Q mutation. Thus, in the activated state, α1I228C in close proximity to β2R269. In the GluCl crystal structure, the β2R269 guanadinium group is ~4 Å from the α1I228 γ carbon, the position of the sulfur in the α1I228C mutant (Fig. 5D). The lack of reaction of pCMBS with α1I228C in the resting state suggests that in the resting state the distance separating these two residues is greater than in the GluCl structure. The observation that a disulfide bond did not form spontaneously in the α1I228Cβ2R269Cγ2 double Cys mutant in the absence of GABA but did form during GABA activation provides further evidence supporting a GABA-induced conformational change during channel gating (Fig. 4). This is similar to the results we obtained for disulfide cross-linking between pairs of Cys in β2M3 and α1M1, where disulfide bonds only formed in the activated state (21). We conclude that during channel activation both the β2M2 and the β2M3 segments move toward the α1M1 segment.

The pCMBS reaction rate increased by >9-fold in the presence of GABA at four positions: α1F226C, α1Q229C in the context of the β2R269Q mutant, α1M236C and β2F289C (Table 1, Fig. 2, and Fig. 5C, red). All of these residues face the subunit interface except for α1F226C. At two other residues facing the subunit interface, α1L232C and β2L285C, the pCMBS reaction rate increased by >5-fold (Fig. 5C, salmon).

Model for Channel Activation and Relationship to Published Cys-loop Receptor Structures—Our current and past results demonstrate that in the transmembrane domain, channel activation by GABA does not occur as a rigid body movement of individual subunits. Channel gating changes the relative positions of transmembrane segments both within an individual subunit and between neighboring subunits. The question that arises is how to place these findings in the context of the x-ray crystal structures of homologous Cys-loop receptors that have recently been published (12, 14, 37–40). These structures provide static pictures of the channel structure; however, the conformational state of the receptors in these crystal structures is uncertain (11, 41, 42). The GLIC and GluCl structures are reported to be in the open state (14, 38, 39). The Erwinia chrysanthemi ligand-gated channel, ELIC, is reported to be in a closed state (37).

The channel gate is formed in the middle of the transmembrane channel by the close apposition of the five M2 segments (12, 15). Thus, in the closed state, the β2M2 and β2M3 segments must be a few angstroms closer to the central channel axis, i.e. further away from the α1M1 segment, than in the GLIC and GluCl crystal structures (see Fig. 5E, white arrow). Having β2M2 closer to the central channel axis in the closed state would be consistent with the ability of β2M2 channel-lining, engineered Cys residues to form spontaneous disulfide bonds in the GABA<sub>α</sub> receptor closed state (29, 43). If in the closed state, β2M2 is closer to the central channel axis, then, due to the short loop linking β2M2 and β2M3, it is likely that M3 is also closer to the central axis. If β2M2 and β2M3 are closer to the channel axis in the closed state this might reduce contact with the adjacent α1M1 segment and increase the surface area of α1M1 residues in contact with lipid rather than protein. The relatively slow pCMBS reaction rates that we observed in the absence of GABA might be due to these residues spending a greater fraction of the time in contact with lipid.

In the transition from the closed to the GABA-activated state, β2M2 and β2M3 must move away from the central channel axis, toward α1M1, in the adjacent subunit (Fig. 5E, white arrow). This motion would cause some of the β2M3/α1M1 interface residues to move from a protein-lipid interface in the closed state into a protein-protein interface in the GABA-activated state. Water and pCMBS can probably penetrate into protein-protein interface faster and more energetically favorably than into protein-lipid interface. This may account for the increased pCMBS reaction rate that we observed at most positions in the activated state (Fig. 5, B and E). The proposed movement of β2M2 and β2M3 during gating might displace
some lipid molecules that interact with αM1 residues in the closed state (Fig. 5E). Consistent with a conformational change occurring in this subunit interface region during channel gating, voltage clamp fluorometry experiments using rhodamine coupled to a Cys substituted for the αM1 β2R269 detected conformational changes during acetylcholine-induced gating (44). It should be noted that β2M2 and β2M3 do not move away from the channel axis during gating as a rigid helical hairpin. The disulfide bonds that form between pairs of engineered Cys in the M2 and M3 segments are different in the absence and presence of GABA (24). Thus, our data suggest that in the transmembrane domain, gating involves conformational changes both within and between subunits.

**Crystal Structures and Channel State**—Although the past 10 years have seen the publication of multiple medium to high resolution Cys-loop receptor structures, it is uncertain which functional state they represent. Part of the problem relates to the fact that the open state is a metastable state in functional receptors embedded in lipid membranes. In the presence of agonist, the desensitized state is the most stable state on a time course of seconds to minutes. Crystals are obtained on a time scale of days to weeks, making it difficult to know whether they are in an open or desensitized state. Furthermore, crystals are obtained in detergent-solubilized protein, usually in the absence of lipids. How the absence of lipids affects the protein structure is uncertain, but interactions with the lipids certainly affect channel function (45). A further issue is that cryopreservation of crystals induces tighter packing of the protein (46). Thus, the crystal structures may not accurately represent the proximity relationships in functional protein at physiological temperatures. For example, SCAM experiments on GLIC indicate that the structure is much less tightly packed than the crystal structure suggests (11, 38, 39). A final issue is that the crystal structures are often of family members that have not been extensively studied, requiring that homology models be generated potentially leading to a decrease in the likelihood that they reflect the actual structures in vivo.

The GluCl structure was inferred to represent the open state because it was crystallized in the presence of ligand and because picrotoxin, which binds in the open state, is localized in the pore of the crystal. We have shown previously that in the GABA<sub>A</sub> receptor, picrotoxin can be trapped in the closed state pore in its binding site near the cytoplasmic end of the channel (15, 47). Thus, the presence of picrotoxin in the GluCl pore does not necessarily imply that the channel is in the open state. None of the published crystal structures provides a basis to explain many of the experimental results obtained in functional Cys-loop receptors. They certainly provide a framework within which we can interpret the results, but much remains to be done before we have the detailed molecular understanding of Cys-loop receptor channel function that the field has sought for decades.

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