Spontaneous Mobility of GABA<sub>A</sub> Receptor M2 Extracellular Half Relative to Noncompetitive Antagonist Action*

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The γ-aminobutyric acid type A receptor β<sub>3</sub> homopentamer is spontaneously open and highly sensitive to many noncompetitive antagonists (NCAs) and Zn<sup>2+</sup>. Our earlier study of the M2 cytoplasmic half (−1' to 10') established a model in which NCAs bind at pore-lining residues Ala<sup>23</sup>, Thr<sup>94</sup>, and Leu<sup>99</sup>. To further define transmembrane 2 (M2) structure relative to NCA action, we extended the Cys scanning to the extracellular half of the β<sub>3</sub> homopentamer (11' to 20').

Spontaneous disulfides formed with T13'C, L18'C, and E20'C from M2/M2 cross-linking and with I14'C (weak), H17'C, and R19'C on bridging M2/M3 intersubunits, based on single (M2 Cys only) and dual (M2 Cys plus M3 C2895) mutations. Induced disulfides also formed with T16'C, but there were few or none with M11'C, T12'C, and N15'C. These findings show conformational flexibility/mobility in the M2 extracellular half 17' to 20' region interpreted as a deformed β-like conformation in the open channel. The NCA radioligands used were [3H]1-(4-ethynylphenyl)-4-aminocyclohexane-1,2-dicarbonitrile and [3H]3,3-bis-trifluoromethylbicyclo[2.2.1]heptane-2,2-di-carbonitrile with essentially the same results. NCA binding was disrupted by individual Cys substitutions at 13', 14', 16', 17', and 19'. The inactivity of T13'C/T13'S may have been due to disturbance of the channel gate; I14'S and T16'S showed much better binding activity than their Cys counterparts, and the low activities of H17'C and R19'C were reversed by dithiothreitol. Zn<sup>2+</sup> potency for inhibition of [3H]EBOB binding was lowered 346-fold by the mutation H17'A. We propose that NCAs enter their binding site both directly, through the channel pore, and indirectly, through the water cavity of adjacent subunits.

The GABA<sub>A</sub> receptor (GABA<sub>A</sub>R) of the ligand-gated ion channel (LGIC) superfamily (1, 2) is blocked by noncompetitive antagonists (NCAs) of widely diverse structures (3) including some of the most important insecticides (4) and, at another site, by Zn<sup>2+</sup> (5). Complete mutational scanning of the 15 amino acids in the cytoplasmic half and adjacent regions of the M2 segments of the β<sub>3</sub> homopentamer (Ala<sup>−4</sup> to Thr<sup>100</sup>) establishes that eight are directly or indirectly involved in NCA binding with Ala<sup>27</sup>, Thr<sup>94</sup>, and Leu<sup>99</sup> of greatest importance (3). The extracellular half of the M2 segments (Met<sup>111</sup> to Glu<sup>201</sup>) has a proposed NCA interaction site at S17’C in an α<sub>1</sub> subunit (6) and a Zn<sup>2+</sup> site at His<sup>177</sup> and Glu<sup>207</sup> in the β<sub>3</sub> subunit (5).

Disulfide trapping experiments with heteromeric receptors (α<sub>1</sub>β<sub>2</sub> or α<sub>2</sub>β<sub>1</sub>γ<sub>2</sub>) have helped to define the structure and mobility of the extracellular half of M2. The following cases of spontaneous or induced disulfide bond formation have been observed (in M2 unless stated otherwise): α<sub>1</sub>2C’ with several Cys mutants in the intrasubunit M3 (7); γ<sub>3</sub>14'C with both α<sub>1</sub>15'C and α<sub>1</sub>16'C and also γ<sub>3</sub>13'C and α<sub>1</sub>13'C (8); α<sub>1</sub>β<sub>1</sub>β<sub>1</sub> spontaneous disulfide cross-linking at 17' and 20' (9); nonadjacent β-β disulfide bridging by β<sub>1</sub>20'C (10). These relationships indicate that the M2 segments can undergo significant rotational and translational movement.

The β<sub>3</sub> homopentamer is ideal for defining the open state channel structure relative to NCA action because it is self-assembling, spontaneously open, and symmetrical with high sensitivities to NCAs and Zn<sup>2+</sup> (3, 11, 12). To further understand M2 structure and NCA action, here we have examined the role of the extracellular half of the M2 segments in NCA and Zn<sup>2+</sup> binding involving site-directed mutagenesis with Cys, Ser, and Ala replacements in M2 and M3 using two NCA radioligands. The structural aspects were studied with SCAM (substituted Cys accessibility method) and disulfide trapping (3, 13). This investigation establishes the conformational flexibility of the extracellular half of M2 segments in the β<sub>3</sub> homopentamer and suggests that NCAs access their binding sites both directly, through the channel pore, and indirectly, through the interface of adjacent subunits. The relationships considered lead to a model of the β<sub>3</sub> homopentameric GABA<sub>A</sub> receptor M2 segments relative to NCA action (shown in Fig. 1).

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Transfection, and Expression**—Alignment of M2, M3, and flanking sequences of various LGICs are shown in

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*Positions in the M2 segment are identified by a system of index numbers in which the absolutely conserved basic residue at the N-terminal end of M2 is numbered 0', i.e. GABA<sub>A</sub>β<sub>3</sub>Arg<sup>930</sup>. Residues toward the C terminus are numbered 1', 2', etc., and toward the N terminus are numbered −1', −2', etc.
Fig. 2 (1, 3, 7, 14, 15). Single Cys mutations were introduced for the entire 10 residues Met11 to Glu20 of the β3 M2 extracellular half. Four Ser mutations, i.e. T13’S, T14’S, N15’S, and T16’S, were generated for comparison with the corresponding Cys mutations. In addition, two Ala mutations, H17’A and E20’A, allowed further study of the sites of Zn2+ interaction. It was possible that the engineered M2 Cys might interact with the single endogenous Cys (Cys289) in M3. To avoid this possible complication, some dual mutations (13 and 19) were expressed in insect Sf9 cells. The methods for site-directed mutagenesis of M2 of mutants thereof were expressed in insect Sf9 cells. The meth-
ods for site-directed mutagenesis of M2 were described previously (3, 12). NCA binding was determined with [3H]-[1-(4-ethylphenyl)-4-n-propyl-2,6,7-trioxabicyclo[2.2.2]octane ([3H]EBOB) (3, 16) or [3H]-3-bis-trifluoromethylbicyclo[2.2.2]heptane-2,2-dicarbonitrile ([3H]BIDN) (3, 17) at 1 and 3 nM, respectively, in pH 7.4 buffer (see below) containing 100 mM NaCl with incubation for 90 min at 25 °C. Specific binding was 75–85% of total binding using 1 μM α-endosulfan or 3 μM unlabeled BIDN to determine nonspecific binding of [3H]EBOB and [3H]BIDN, respectively. The buffer was 3 mM NaH2PO4/1 mM K2HPO4 except with studies involving Zn2+ when 20 mM Tris-HCl was used to avoid precipitation.

Flow Cytometry Analysis—Flow cytometric analysis was used to determine receptor surface expression measured as membrane immunofluorescence (18). Sf9 cells were transfected with recombinant baculovirus for 48 h. Cells were harvested and washed with phosphate-buffered saline (PBS), and then 106 cells in 100 μl of PBS were incubated for 30 min on ice with anti-GABAAR antibody as described above or, as a negative control, with PBS only. After washing twice with PBS, Alexa Fluor 488 goat anti-mouse IgG (1:200) (Invitrogen Molecular Probes) was added to each sample on ice for 30 min. Following three washes with PBS, the fluorescence density was measured using a Coulter Elite instrument (Beckman-Coulter) and analyzed with WinMDI 2.8 software provided by Duke University.
(Durham, NC). The expression of \( \beta_3 \) homomer was evaluated as the mean fluorescence value.

**Disulfide Cross-linking**—Disulfide cross-linking profiles were determined for the single and dual mutants comparing spontaneous, oxidative, and reductive conditions (3, 7, 9). CuSO\(_4\) was prepared as a 100 mM stock solution in water and \( \sigma \)-phenanthroline (Sigma) as a 200 mM stock solution in ethanol. CuSO\(_4\) and \( \sigma \)-phenanthroline were freshly mixed to obtain the Cu:phen solution, which was added to samples to give a final concentration of 100:200 \( \mu \)M. For oxidation, membrane protein (100 \( \mu \)g) was incubated with Cu:phen for 5 min. Then the reaction was terminated by adding 10 mM \( \sigma \)-ethylmaleimide and 1 mM EDTA. Following this treatment sequence, sample buffer (3) was added with or without 10 mM dithiothreitol (DTT). Finally, the cross-linked subunits were detected by immunoblotting. The results were compared with control samples without any treatment.

**DTT Reduction, Cu:phen Oxidation, and NCA Binding**—Membranes as described above were subjected to reduction and oxidation to determine the effect of the disulfide on receptor NCA binding activity. A similar procedure was used by others for cells with electrophysiology assay (7, 9). Samples were incubated with 10 mM DTT for 15 min or 100:200 \( \mu \)M Cu:phen for 5 min at room temperature. Samples with Cu:phen were also treated with 1 mM EDTA following oxidation to chelate the Cu\(^{2+}\) in solution. Controls were samples without any treatment. Finally, each sample was subjected to the \(^{3}\text{H}\)EBOB binding assay. Activity values were expressed as percent of wild type (WT) binding level.

**Sulphydryl Modification and NCA Binding**—2-Aminoethylmethanethiosulfonate hydrochloride (MTSEA\(^{-}\)) (Toronto Research Chemical, North York, Canada) was used under previously described conditions (19) with analysis for its effect on \(^{3}\text{H}\)EBOB binding. Briefly, selected Cys mutants (more than 30% WT binding level) and WT were labeled for 10 min at room temperature with 2.5 mM MTSEA\(^{-}\). Following incubation, each sample was centrifuged at 20,000 \( \times \) g for 15 min and the supernatant removed completely. The pellet was resuspended in PBS and subjected to a binding assay.

**Molecular Modeling**—Disulfide cross-linking interactions were examined with the \( \beta_3 \) homopentamer model based on the homologous nAChR with \( \alpha \)-helical structure throughout the transmembrane segments (3, 20). All modeling was done with Maestro 6.5 (Schrödinger LLC). The M2 residues from 11 to 20 were mutated to Cys and the distances measured between sulfur centers.

**RESULTS**

**Mutagenesis, Transfection, and Expression**—PCR analysis indicated a recombination efficiency of nearly 100% for all mutants (Fig. 3A) with their identities confirmed by sequencing. Western blotting established similar expression levels for each mutant (Fig. 3B). A faint dimer from T13’C was resistant to reduction. Similar transfection efficiencies and protein expression levels were also observed for dual M2 and M3 mutations, i.e. T13’C, I14’C, T16’C, H17’C, L18’C, R19’C, and E20’C with C289S (data not shown). Flow cytometry was used to further characterize protein localization and quantity with special respect to mutant T13’S (with little activity for NCA binding, as considered later) using non-fixed, transfected Sf9 cells (Fig. 3C). Mean fluorescence values were similar for the WT and mutant T13’S, i.e. values of 16.1 and 16.4, respectively. This result shows strong \( \beta_3 \) homopentamer surface expression, i.e. the mutation did not disturb the subcellular location of the homomer. 

**Disulfide Cross-linking Profiles**—The 10 single Cys mutants (11’ to 20’ were exposed to ambient oxygen in the presence and absence of Cu:phen catalyst (Fig. 4). Mutants forming disulfide bonds were evident by conversion of some or most of the band at \( \sim \)55 to \( \sim \)130 kDa based on Western blot analysis. The WT (3) and the M11’C mutant did not form any disulfide bonds. There were no dimers formed at positions 12’ and 15’ without Cu:phen, but extremely weak dimers were induced by Cu:phen. T16’C formed a strong dimer with the oxidant. Five positions (T13’C, H17’C, R18’C, L19’C, and E20’C) formed...
disulfides spontaneously in the absence of Cu:phen, and there was some evidence of low spontaneous disulfide formation with 114°C. Cu:phen strongly increased the amount of dimer at these six positions, especially from 17' to 20', where only a small amount of monomer remained. The dramatic increase in dimer formation at 17' to 20' indicated that intersubunit disulfide bonds dominated compared with possible intrasubunit M2/M3 disulfides, which would make the free Cys less available. The effect of DTT reduction on dimer yield varied with the mutants. All of the dimers at about 130 kDa were almost completely reduced by DTT except for T13'C and E20'C with faint resistant bands. A similar observation has been noticed in the 6' position (3, 9).

To determine M2/M2 versus possible M2/M3 intersubunit disulfide cross-bridging, dual mutants were generated by combining M3 C289S with M2 Cys mutants 13', 14', and 16' to 20' (Fig. 4). The selection of mutants was based on the spontaneous or induced disulfide formation and spatiality to M3 Cys289 based on the GABAAR model (3). Dual M2 mutants 13', 16', 18', and 20' showed strong spontaneous or oxidant-induced disulfide bond formation similar to that of the corresponding single Cys mutants establishing M2/M2 cross-linking. Spontaneous disulfide bond formation was greatly reduced in the dual Cys mutations 17' and 19', and the slight spontaneous dimer at single Cys mutant 114'C was also diminished indicating that spontaneous disulfide bond cross-linking by these M2 single Cys mutants largely involves M3 Cys289. However, for unknown reasons, oxidation also led to extensive protein loss at these three positions.

Effect of Site-specific Mutations on NCA Binding—The site-specific mutations had very different effects on NCA binding. [3H]EBOB and [3H]BiDN gave essentially the same results (Fig. 5), indicating that they bind at the same site within the extracellular portion of the GABAAR as noted before for the cytoplasmic half (3). Five single Cys mutants (T13'C, I14'C, T16'C, H17'C, and R19'C) showed only 10–35% of WT activity. The remaining Cys positions (11', 12', 15', 18', and 20') conferred binding activity more than 50% relative to the WT. Ser at 13' and 15' maintained similar activity to the Cys mutations, whereas Ser at 14' and 16' greatly enhanced activity relative to Cys. The seven M2/M3 dual mutations showed different patterns compared with their single M2 Cys counterparts. The binding activity of T13'C/C289S was the same as the single mutants T13'C and T13'S. Dual mutations I14'C/C289S and T16'C/C289S did not give the high activity of the corresponding Ser mutations I14'S and T16'S. On the other hand, H17'C/C289S and R19'C/C289S showed much better binding activity (60–70%) than the corresponding single Cys mutants (20–35%).

Effect of DTT Reduction and Cu:phen Oxidation on NCA Binding—Some single Cys mutants undergo partial spontaneous disulfide formation, which could affect [3H]EBOB binding and might be reversed with DTT or increased with Cu:phen. Any of these structural changes might either have no effect or alter [3H]EBOB binding. These relationships were further examined with the five single Cys mutants that undergo high spontaneous disulfide bond formation, i.e., 13', 17', 18', 19', and 20' (Fig. 6); all other Cys mutants showed no significant increase in binding activity with treatment (data not shown). DTT at 10 mM had essentially no effect or slightly decreased the binding level of [3H]EBOB with WT, L18'C, and E20'C. T13'C with DTT showed an increased but still low level of binding. In contrast, mutants H17'C and R19'C showed much greater binding activity on the addition of DTT, with recovery to near the WT level. To check the effect of the oxidant, WT Cys mutants 13' and 17' to 20' were treated with Cu:phen and EDTA. Before the addition of EDTA, Cu:phen inhibited the binding activity of WT, H17'C, L18'C, and E20'C about 15–40% but only slightly affected the binding of T13'C and R19'C. After the addition of EDTA to chelate the Cu2+ and withdraw the oxidative effect of Cu:phen, all of them returned almost to the binding levels without treatment. The stronger oxidative environment with Cu:phen did not reduce the binding level of these Cys mutants. DTT did not recover any significant activity for the seven dual mutants (data not shown).

Effect of Sulphydryl Modification on NCA Binding of Single Cys Mutants—Membrane-impermeable MTSEA+ was used to explore the accessibility of M2 single Cys mutants to sulphydryl modification. Mutants T13'C, I14'C, T16'C, and R19'C with less than 30% of WT [3H]EBOB binding activity could not be...
assessed accurately, but all others were examined (Fig. 7). MTSEA$^+$ inhibited $[^3]$H]EBOB binding activity for the WT by 20%, but for mutants T12$^C$, N15$^C$, and L18$^C$ the inhibition was more than 70%. The low inhibition (10–30%) for H17$^A$ and E20$^C$ may come from the strong, spontaneous, disulfide bond formation, but L18$^C$ still could be modified by MTSEA$^+$. This indicates the greater availability of its free thiol moiety.

Effect of $Zn^{2+}$ on NCA Binding—$[^3]$H]EBOB binding in the WT is very sensitive to $Zn^{2+}$ ($IC_{50}$ 1.3 $\mu$M) (Fig. 8). In the $\beta_3$ subunit of the $\alpha$$_3$$\beta_3$ GABA$\_A$ receptor, the $Zn^{2+}$ binding site is reported as M2 His$^{177}$ and Glu$^{201}$ plus extracellular domain residue Glu$^{182}$, which in turn is coordinated with the $\alpha_3$ subunit (5). These sites were therefore mutated by introducing Ala to give H17$^A$, E20$^A$, and E182A. H17$^A$ and E182A retained 95–100% of the WT binding level, whereas E20$^A$ reduced the binding activity to 60% of the WT. These mutations had very different effects on $Zn^{2+}$ sensitivity of $[^3]$H]EBOB binding with $IC_{50}$ values of 2.8 $\mu$M for Glu$^{182}$, 5.7 $\mu$M for E20$^A$, and 450 $\mu$M for H17$^A$. The 346-fold $IC_{50}$ increase for H17$^A$ established that the blocking effect of $Zn^{2+}$ mainly comes from occupying H17$^A$ instead of E20$^A$ or Glu$^{182}$.

### DISCUSSION

#### Proposed M2 Structure of Spontaneously Open $\beta_3$ Homopentameric Channel with High NCA Sensitivity

Findings are summarized in Table 1 and illustrated in Fig. 1 as a model for the proposed M2 structure and interaction sites. A detailed electrophysiological characterization of the functional $\beta_3$ homopentameric GABA$\_A$R has shown that this subunit forms a spontaneously open channel, which can be inhibited by picrotoxin (PTX) and $Zn^{2+}$ and is insensitive to GABA (11, 21). The cytoplasmic end of the channel may act as a fixed fulcrum to open the narrow domain of the pore between 9$^C$ and 14$^C$ (1, 8, 22) with little change in conformation during gating (23). Low mobility and limited accessibility from 2$^C$ to 6$^C$ are evident for the $\beta_1/\beta_2$ subunit (3, 13). Disulfide cross-linking at the 9$^C$ position supports its role as an important component of the channel gate (3). The current investigation defines how NCAs access their binding site at the cytoplasmic end by studying the conformational flexibility of the M2 extracellular half.

#### Important Role of 11$^C$ to 16$^C$ Region Relative to the Channel Gate

The ease of disulfide bridging depends on the average distances of Cys residues, the orientation of thiol moieties, and the mobility/flexibility of the protein region (9, 24). Positions M11$^C$, T12$^C$, and N15$^C$, with little or no oxidant-induced disulfide formation, are probably not exposed in the channel lumen, although T12$^C$ and N15$^C$ can be accessed by sulfhydryl modification reagents (13). T13$^C$ is the only position in the −1$^C$ to 16$^C$ region that spontaneously forms a strong disulfide bond, implying its flexibility in rapid opening and closing of the gate and/or the proximity of its thiol substituents in the narrowest region of the pore. The sulfurs in a disulfide bond are
separated, center-to-center, by 2 Å (24), and those for T13’C on adjacent subunits are only 6.8 Å apart (Fig. 9) consistent with their spontaneous formation of a strong disulfide cross-link. Disulfide bonds also form spontaneously between \( \gamma_213’C \) and \( \alpha_113’C \) (8) but not at \( \beta \) subunits (9). 114’C of the \( \beta_3 \) homopentamer gives very weak spontaneous disulfides associated with little M2 I14’C/M3 C289 cross-linking because of the considerably greater distance and unaligned orientation from M3 Cys289. Unaligned disulfides are also observed between \( \gamma_214’C \) and both \( \alpha_115’C \) and \( \alpha_116’C \) (8). Asn157 is not in the pore lumen and is important in alcohol or general anesthetic action (25). The induced but not spontaneous disulfide bond formation observed here with the \( \beta_3 \) homopentamer places Thr110 in the pore lumen consistent with a previous SCAM study (14), suggesting its relatively low mobility and longer sulfur–sulfur distance on adjacent M2, measured as 10.0 Å (Fig. 9).

The M2 and M3 cross-links apparently yield only dimers but not trimers, quatermers, or pentamers. This finding is consistent with the observation that endogenous Cys289, both in WT (3) and M2 mutant M11’C (Fig. 4), does not form a spontaneous or Cu:phen-induced dimer or multimer, indicating that M3 Cys289 will not cross-link with itself. The major product from the mutated Cys in M2 and endogenous M3 Cys289 cross-linking is a dimer that probably “locks” the channel in an unfavorable conformation preventing further cross-linking to form multimers.

Conformational Mobility of 17’ to 20’ Region—The nAChR-based GABA\(_{\alpha}\)R model with an \( \alpha \)-helical M2 structure places 17’ and 20’ in pore-facing positions (14, 20) and gives average M2/M2 sulfur–sulfur distances for 17’, 18’, 19’, and 20’ of 6.7, 13.6, 16.3, and 9.3 Å, respectively (Fig. 9). The pore-facing residues are spatially preferred to form disulfide bonds with relatively small movement asymmetrically (9), but this movement would rotate the non-pore-facing residues like 18’ and 19’ away from each other. However, all single Cys mutants in this region of the \( \beta_3 \) homopentamer form disulfide bonds spontaneously, to a similar extent. The strong and newly observed spontaneous disulfide formations at L18’C and R19’C are therefore not consistent with the helical conformation. The \( \beta_3 \) E20’C even forms a nonadjacent disulfide bond in the \( \alpha_1\beta_1\gamma_2 \) receptor, suggesting a possible structural difference from the nAChR in this region (10). The M2 H17’C/M3 Cys289 and M2 R19’C/M3 Cys289 intersubunit sulfur–sulfur distances are quite long, ranging from 18 to 27 Å (Fig. 10). The observed M2/M3 intersubunit bridging by H17’C and R19’C further indicates that they probably trap in different directions from L18’C and E20’C and face out of the channel lumen (Fig. 1). Their cross-linking would be greatly facilitated by a \( \beta \)-like conformation, allowing closer proximity to the endogeneous M3 Cys.

We propose that in the \( \beta_3 \) homopentamer open channel, the 17’ to 20’ region assumes a \( \beta \)-like conformation (Fig. 1) based on the disulfide formation profile. The extended deconformation structure, directly connected with the channel activation domain M2/M3 loop (22, 26) (Fig. 10), may help to open or widen the channel gate. GABA activation alters the access of a sulfhydryl modification reagent in the 17’ to 20’ region (13) implying a conformational change that relies on channel functional states. The nAChR \( \delta \) subunit extracellular part of M2 undergoes a pronounced change between closed and open conformations (27). Significant backbone deformation occurs at positions 13’, 16’, and 19’ in nAChR \( \alpha \) subunit M2 during channel gating (28). If LGICs in the closed state assume a well ordered \( \alpha \)-helix structure (1), then the \( \beta_3 \) homopentamer may have undergone transition to a \( \beta \)-like conformation in this region on channel opening. The proposed \( \text{GABA}_{\alpha} \text{R} \) \( \beta \)-like conformation refers to the \( \beta \) subunit only, because it is distinctive in flexibility and channel function relative to the \( \alpha \) or \( \gamma \) subunit (10, 29, 30).

NCA Interactions with the Extracellular Half—The non-pore-facing M11’C, T12’C, and N15’C had little effect on NCA binding and were not considered further here. Mutants T13’C and T13’S in the \( \beta_3 \) homopentamer showed dramatically
reduced NCA binding, $\beta_1$T13’A in an $\alpha_1\beta_1$ receptor does not respond to GABA and loses muscimol binding, suggesting its essential role in receptor function (29), possibly by disturbing the channel gate. L9’ mutations change both the channel gating and NCA sensitivity (31), further indicating that NCA action is closely related with the gate domain. Cys mutations at Ile14’ and Thr16’ cause more damage than Ser on NCA binding, and DTT does not reverse the low binding activity; possibly the increased size of the Cys sulfur versus the Ser oxygen impedes NCA access to its binding site. With an $\alpha_1\beta_1\gamma_2$ receptor, a thiol-reactive probe related to the NCA fipronil irreversibly inhibits GABA-induced chloride current at a Cys-mutated 17’ position of the $\alpha_1$ subunit (6). However, $\beta_3$H17’A in the present study did not change the binding activity. Zn$^{2+}$ binding affinity was sharply reduced by H17’A, consistent with earlier studies (5, 21, 32). The inhibition of $[^3H]$EBOB binding by Zn$^{2+}$cross-linking by 17’KSH reduces NCA binding.

The inhibition of $[^3H]$EBOB binding by Zn$^{2+}$reduced by H17’A, PTX can reach its site through its access pathway.

The 17’ to 20’ region is particularly interesting relative to NCA binding. With the $\beta_1$ homopentamer, DTT reverses the low activity of single Cys mutants H17’C and R19’C but does not affect L18’C and E20’C, which have original high binding levels (Fig. 6), although these four single Cys mutants have similar levels of disulfide bond formation (Fig. 4). In contrast, disulfide formation at H17’C in the $\beta_1$ subunit of the $\alpha_1\beta_1$ receptor is much less damaging on Cl$^-$ conductance than that at E20’C (9). The blocking effect from M2/M3 intersubunit disulfide cross-linking by 17’C/Cys$^{289}$ and 19’C/Cys$^{289}$ indicates that the NCAs may prefer the exposed water cavity of 17’ and 19’ as an access pathway.

**NCA Access to the Binding Site**—The NCA PTX has been proposed to access its binding site either through open channels or with different efficacies through closed channels (34, 35). The present study suggests two pathways for NCA access to its $\beta_1$ homopentamer binding site at the cytoplasmic end of M2 (Fig. 10). One is directly through the pore and another one possibly through the water cavities between adjacent subunits, similar to the suggested general anesthetics binding pocket in the water crevice behind M2 (25). The M2 segments are well shielded from the lipid bilayer by an outer ring of the helices M1, M3, and M4 to form channel pore and water cavities around M2 (1) (Fig. 10). There is precedent for these proposals because access to the ligand binding site of G-protein-coupled receptors is via a water-filled crevice (36–39). The more cytoplasmic residues in M3 become available for modification by sulphydryl reagents on GABA activation (40, 41). The challenge is to define how the NCA can reach its binding site at the cytoplasmic end of M2 through this novel pathway. As an alternative interpretation, M2/M3 disulfide formation from 17’C/Cys$^{289}$ and 19’C/Cys$^{289}$ may lock the channel pore in an unfavorable conformation for NCA binding. Similarly, disulfide bond formation at M2/M2 intersubunits or M2/M3 intra-subunit disrupts the channel conformation to alter the Cl$^-$ current (7–10). Although we cannot rule out this possibility, our new pathway suggestion has important implications for NCA access.

GABA$_A$ receptor noncompetitive antagonism is a complex phenomenon (34, 35, 42, 43). PTX can reach its site through both hydrophilic and hydrophobic pathways (34). It is suggested to bind at an allosteric site to stabilize a closed or desensitized state of the channel (35) or to bind to both use-dependent and use-independent sites in GABA$_A$ receptors (43). The overall inference is that channel opening is not an absolute requirement for PTX to reach its site. The complexity of NCA antagonism probably is related in part to the conformational mobility of the M2 extracellular half and multiple access pathways to the binding site.

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