GABA_\text{A} Receptor $\beta_2$ Tyr$^{97}$ and Leu$^{99}$ Line the GABA-binding Site

INSIGHTS INTO MECHANISMS OF AGONIST AND ANTAGONIST ACTIONS*

Andrew J. Boileau‡, J. Glen Newell‡, and Cynthia Czajkowski§

From the Department of Physiology, University of Wisconsin-Madison, Madison, Wisconsin 53706

The identification of residues that line neurotransmitter-binding sites and catalyze allosteric transitions that result in channel gating is crucial for understanding ligand-gated ion channel function. In this study, we used the substituted cysteine accessibility method and two-electrode voltage clamp to identify novel $\gamma$-aminobutyric acid (GABA)-binding site residues and to elucidate the secondary structure of the Trp$^{92}$-Asp$^{101}$ region of the $\beta_2$ subunit. Each residue was mutated individually to cysteine and expressed with wild-type $\alpha_1$ subunits in Xenopus oocytes. GABA-gated currents ($I_{\text{GABA}}$) were measured before and after exposure to the sulfhydryl reagent, N-biotinylaminoethyl methanethiosulfonate (MTS). V93C, D95C, Y97C, and L99C are accessible to derivatization. This pattern of accessibility is consistent with $\beta_2$Val$^{93}$-Leu$^{99}$ adopting a $\beta$-strand conformation. Both GABA and SR95531 protect Y97C and L99C from modification, indicating that these two residues line the GABA-binding site. In D95C-containing receptors, application of MTS in the presence of SR95531 causes a greater effect on $I_{\text{GABA}}$ than MTS alone, suggesting that binding of a competitive antagonist can cause movements in the binding site. In addition, we present evidence that $\beta_2$L99C homomers form spontaneously open channels. Thus, mutation of a binding site residue can alter channel gating, which implies that Leu$^{99}$ may be important for coupling agonist binding to channel gating.

Central to the understanding of neurotransmission is the nature of allosteric transitions, such as the movements required for an apoprotein to grasp its ligand, or the movements that govern the shift from a closed to an open conformation in an ion channel protein. Among members of the superfamily of ligand-gated ion channels (LGICs$^1$), both allosteric transitions are required, and must be linked together in a coordinated fashion, for the ligand binding to effect channel gating. The association of the neurotransmitter with the receptor represents the initial step in the induction of conformational movements that result in channel activation, deactivation, and desensitization. Therefore, an understanding of the structure of the ligand-binding site is necessary to comprehend these processes.

The $\gamma$-aminobutyric acid type A receptor (GABA$_{\text{A}}$R) is a member of the LGIC superfamily of neurotransmitter receptors that includes nicotinic acetylcholine, glycine, and serotonin type 3 receptors (see Fig. 1) (1–4). The GABA$_{\text{A}}$R is a heteromeric protein complex that is composed of five subunits arranged in a pseudo-symmetric manner around a central Cl$^-$/selective channel. Putative stoichiometries for recombinant GABA$_{\text{A}}$R include 2$\alpha$:2$\beta$:1$\gamma$ for $\alpha$/$\beta$ receptors (5–8) and 3$\alpha$:2$\beta$ for $\alpha$/$\beta$ receptors (5); however, other stoichiometries such as 2$\alpha$:3$\beta$ may exist (8, 9).

Multiple amino acid residues from both $\beta$ and $\alpha$ subunits have been identified that are important determinants for GABA binding. A widely accepted model of ligand recognition is based on the nicotinic acetylcholine receptor (nAChR) (10). The ligand-binding site is formed at subunit-subunit interfaces with clusters of binding site residues (which are, by arbitrary convention, designated “loops” A–F) found in several distinct regions of neighboring subunits. In the GABA$_{\text{A}}$R-binding site, these residues include: Phe$^{84}$, Arg$^{86}$, Ser$^{86}$ (loop D), Arg$^{119}$ and Ile$^{120}$ (loop E) of the $\alpha_1$ subunit (11–15) and Tyr$^{205}$, Thr$^{207}$ (loop B), Thr$^{207}$, Ser$^{204}$, Tyr$^{205}$, Arg$^{207}$, and Ser$^{209}$ (loop C) of the $\beta_2$ subunit (16, 17).

To date, no residues in the region defined by Trp$^{92}$-Asp$^{101}$ (loop A) of the $\beta_2$ subunit have been implicated in the formation of the GABA-binding site. A residue in this domain, $\beta_2$Leu$^{99}$, is found in the homologous position as His$^{103}$ of the $\alpha_1$ subunit, which participates in the formation of the GABA$_{\text{A}}$R benzodiazepine-binding site (Fig. 1) (18–20). In addition, $\beta_2$Leu$^{99}$ aligns with Tyr$^{205}$ of the nAChR $\alpha$ subunit, which has been identified as forming part of the acetylcholine-binding site (21–24). On the basis of LGIC-binding site homology, we reasoned that the $\beta_2$Trp$^{92}$-Asp$^{101}$ region is likely to contribute to formation of part of the GABA-binding site. To test this hypothesis, we used the substituted cysteine accessibility method (SCAM) to identify potential binding site residues and examine the secondary structure of this domain.

The development of SCAM (25) has proved to be a powerful tool to gain information about the structure and dynamics of protein domains and has been utilized to study LGICs (26, 27), G-protein coupled receptors (28), voltage-gated ion channels (29), and transporters (30). Specifically, it has been used to identify amino acid residues that line ion channels (31, 32) as well as residues that line binding sites (12, 17, 24, 28, 33, 34). We have previously used this technique to 1) gain information about the secondary structure of benzodiazepine-binding and GABA-binding domains of specific GABA$_{\text{A}}$R subunits (12, 17, 34), 2) identify residues that line these binding sites, and 3) examine...
been implicated in the formation of recognition sites for acetylcholine modification by MTS after cysteine substitution. The mutant receptors were similar to wild-type receptors (1–Leu99, Asn100, and Asp101 (Fig. 1). The region of the GABA AR, which was injected with excess cysteine mutant constructs were made using recombinant PCR (36), as a template, we present a structural description of this region of the GABA AR

binding sites conformational dynamics (17, 34). SCAM entails introducing cysteine residues at individual positions within the protein and testing the effect (if any) of modifying these introduced cysteines with sulphydryl-specific reagents. We use two criteria to determine if a cysteine-substituted residue lines part of the binding site: (i) reaction with a sulphydryl-specific reagent alters function and (ii) binding site ligands (agonists and antagonists) protect the engineered cysteine from covalent modification.

In this report, using SCAM analysis of the β2Trp92–Asp101 region of the GABA_R, we found that this domain adopts a β strand conformation and identified two novel amino acid residues, Leu299 and Tyr302, that participate in the formation of the GABA-binding site. These observations mark the initial structural description of this region of the GABA_R β2 subunit. Using the recently determined crystal structure of the related acetylcholine binding protein (35) as a template, we present a model of this domain of the GABA-binding site. Furthermore, we illustrate that the binding of a competitive antagonist, SR95531, triggers conformational movement near the binding site and that mutation of a binding-site residue (Leu299) can perturb channel gating.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and Expression in Oocytes—Wild-type rat cDNAs for the α1 and β2 subunits of the GABA_A receptor were used. The β2 cysteine mutant constructs were made using recombinant PCR (36), as described previously (37, 38). Cysteine substitutions were made in the β2 subunit at positions Trp92, Val95, Pro96, Asp97, Thr98, Tyr99, Phe99, Leu99, Asn100, and Asp101 (Fig. 1). The β2-cysteine-substituted mutants were subcloned into pGHis (39, 40) for expression in Xenopus laevis oocytes. All cysteine mutants were verified by double-stranded DNA sequencing. The β2-cysteine mutants have been named using the single-letter code, as (wild-type residue) (residue number) (cysteine), e.g. L99C.

Oocytes were prepared as previously described (41). GABA_A receptor α1 and β2 or β3 mutant subunits were expressed by injection of cRNA (0.7–2.7 ng/subunit cRNA) in a 1:1 ratio (μl) with the exception of L99C, which was injected with excess subunit cRNA in a 4:1 ratio (see “Results” and “Discussion”). The maximal currents for the majority of the mutant receptors were similar to wild-type receptors (1–5 μA). Expression of F94C and F98C produced receptors in which the peak current amplitudes were significantly reduced (<100 nA), even with 7-nM GABA injection. The oocytes were maintained in modified ND96 recording solution in 96 mM NaCl, 2.0 KCl, 1.0 MgCl2, 1.8 CaCl2, and 5 HEPES, pH 7.4, that had been supplemented with 100 μg/ml gentamicin and 100 μg/ml bovine serum albumin. Oocytes were used 2–14 days after injection for electrophysiological recordings.

**Two-electrode Voltage Clamp Analysis—**Oocytes under two-electrode voltage clamp (V_blank = −80 mV) were perfused continuously with ND96 at a rate of 5–15 ml/min. The volume of the recording chamber was 200 μl. Standard two-electrode voltage clamp procedures were carried out using a GeneClamp500 Amplifier (Axon Instruments, Inc.). Borosilicate electrodes were filled with 3 M KCl and had resistances of 0.5–3.0 MΩ in ND96. Stock solutions of GABA (Sigma Chemical Co., St. Louis, MO) and SR95531 (Sigma) were prepared in water, whereas 0.1 M stock of N-(2-mercaptoethyl)maleimide (MTSEA-biotin, MTS hereafter; Biotium, Hayward, CA) was dissolved in MeSO. The MTSEA-biotin molecule (14.5 Å, unreacted; 11.2 Å reacted moeity) is longer than GABA (4.5 Å) but similar in length to SR95531 (13.5 Å). Furthermore, MTS is a relatively membrane impermeant compound (42, 43), allowing only for extracellular reactions. Taken together, these properties make MTS a reasonable choice for experimentation on water-soluble ligand-binding regions. All compounds were diluted appropriately in ND96 such that the final concentration of MeSO in MTS solutions was ≤5%. This concentration of solvent did not affect recombinant GABA_A receptor properties.

To measure the sensitivity to GABA, the agonist (0.0001–30 mM) was applied via gravity perfusion (~5–10 s) or by pipette application (150–200 μl) with a 3- to 15-min washout period between each application to ensure complete recovery from desensitization. Peak GABA-activated current (I_{GABA}) was recorded at each application. To correct for slow drift in the maximum amplitude of the response, concentration-response data were collected by a prior application of a low, non-desensitizing concentration of GABA (EC_{50}–EC_{max}) between which there was an approximate 10-s wash-out period and to which each test concentration of GABA was normalized. Concentration-response curves were generated for each recombinant receptor, and the data were fitted by nonlinear regression analysis using GraphPad Prism software (San Diego, CA, www.graphpad.com). Data were fitted to the equation: I = I_{max}/(1 + (IC_{50}/[Ant])^n), where I is the peak amplitude of the current for a given concentration of GABA ([Ant]), I_{max} is the maximum amplitude of the current, EC_{50} is the concentration required for half maximal receptor activation, and n is the Hill coefficient.

To measure the sensitivity to SR95531, GABA (EC_{50}) was applied via gravity perfusion followed by a brief (20 s) wash-out period before concomitant application of GABA (EC_{50}) and increasing concentrations of SR95531 by pipette application. The response to the application of SR95531 and GABA was normalized to the response elicited by the agonist alone. Concentration-inhibition curves were generated for each recombinant receptor, and the data were fitted by non-linear regression analysis using GraphPad Prism software. Data were fitted to the equation: I = 1 – 1/1 + (IC_{50}/[Ant])^n), where IC_{50} is the concentration of the antagonist ([Ant]) that reduces the amplitude of the GABA-evoked current by 50% and n is the Hill coefficient. KI values were calculated using the Cheng-Prusoff equation of correction: KI = IC_{50} + ([Ant]/IC_{50}), where IC_{50} is the concentration of GABA used in each experiment and EC_{50} is the concentration of GABA that elicits a half-maximal response for each receptor (44, 45).

**MTS Reactions and Agonist/Agonist Protection Assays—**Oocytes expressing either wild-type or mutant receptors were pulsed with solutions containing GABA concentrations corresponding to approximately EC_{50} of the particular receptor being studied every 5 min until IC_{50} varied by ~5% for two consecutive pulses. Then, cells were exposed to a high concentration of MTS (3 mM exposure for 3 min) and washed for 10 min, and GABA at EC_{50} was applied again to determine accessibility to the modified cysteine residues by the MTS reagent. Preliminary experiments showed that this concentration and time of MTS exposure were sufficient to achieve maximum inhibition of I_{GABA} in all accessible mutant residues. Percent inhibition was calculated as (1 – (I_{GABA} after MTS)/I_{GABA} before MTS) × 100%. Currents were further examined by multiple pulses of EC_{50} GABA after MTS reaction, and responses remained stable. All accessible residues were also tested for the specificity of the MTS reaction by treating the oocytes with the reducing reagent dithiothreitol (20 μM, 3 min), which reversed the inhibition caused by covalent modification.

To determine whether the accessibility of the mutated cysteine could be altered by the presence of agonist (GABA) or antagonist (SR95531), the following protocol was used: After I_{GABA} at EC_{50} was stabilized, cells were exposed to a concentration of MTS that yielded approximately 50% of the full inhibitory effect seen with maximal MTS exposure. This concentration of MTS was determined separately for each accessible mutation. In a different set of cells, accessible residues were exposed to a concentration of MTS that yielded approximately 50% of the full inhibitory effect seen with maximal MTS exposure. This concentration of MTS was determined separately for each accessible mutation. In a different set of cells, accessible residues were exposed to a concentration of MTS that yielded approximately 50% of the full inhibitory effect seen with maximal MTS exposure. This concentration of MTS was determined separately for each accessible mutation. In a different set of cells, accessible residues were exposed to a concentration of MTS that yielded approximately 50% of the full inhibitory effect seen with maximal MTS exposure. This concentration of MTS was determined separately for each accessible mutation.
Agonist and Antagonist Actions at a Novel GABA-binding Domain

RESULTS

Cysteine substitutions were made individually at ten positions in the β2 subunit (Trp92, Val93, Pro94, Asp95, Thr96, Tyr97, Phe98, Leu99, Asn100, and Asp101) and tested for functional expression in Xenopus oocytes. Data represent the mean ± S.E. for at least three independent experiments. Concentration-inhibition data for SR95531 for wild-type receptors and those carrying mutations of the β2 subunit are summarized in Table I.

The competitive antagonist SR95531 reduced I_{GABA} in a concentration-dependent manner (Fig. 2B) with a K_I value of 330 nM at wild-type receptors (Table I). Only the Y97C substitution increased SR95531 K_I. For all other mutations, there was either no change in the K_I value for SR95531 or there was a moderate decrease. The K_I value was not determined for receptors carrying the F98C mutation, because small currents elicited by GABA at EC_{50} (~25 nA) precluded an accurate determination (see “Experimental Procedures”). None of the SR95531 Hill coefficients were significantly different from wild-type (Table I). Overall, these results indicate that cysteine substitution in this region is tolerated.

Wild-type and cysteine mutant receptors were then tested for modification by MTS (Fig. 3). After exposure to 3 mM MTS for 3 min, I_{GABA} peaks were measured and compared with current peaks prior to MTS exposure. I_{GABA} was significantly inhibited in only four mutant receptors (Fig. 3B); α_β2V93C was inhibited by 71.4 ± 2.1%, α_β2D95C by 98.9 ± 0.4%, α_β2Y97C by 66.9 ± 1.9%, and α_β2L99C by 98.2 ± 0.4%. MTS had no effect on wild-type GABA_A Rs and receptors containing P94C, T96C, F98C, N100C, and D101C. It should be noted that these receptors may have reacted with MTS, but the reactions had no functional effect. Nevertheless, a change in current amplitude following MTS treatment and reversibility of the sulfhydryl-specific reaction by dithiothreitol proves that cysteine modification has occurred. The pattern of accessibility (i.e. every other residue) is indicative of a β strand conformation.

MTS inhibition of I_{GABA} could be the result of a direct effect such as steric block and/or an indirect allosteric effect on the binding site. To determine if the accessible residues contribute to the lining of the GABA binding pocket, mutant receptors were tested for “protection” from the MTS reaction using co-application of agonist (GABA) or antagonist (SR95531) as “protections.” First, we determined a concentration and time of MTS exposure that resulted in an approximately half-maximal inhibition for each of the accessible mutants (Fig. 4). For α_β2V93C, normalized inhibition of 41.9 ± 3.6% (29.9% mean inhibition divided by mean maximal inhibition of 71.4%) required 1-min exposure to 3 mM MTS; α_β2D95C required 67 µM, 1 min (normalized inhibition 47.3 ± 3.6%), α_β2Y97C required 3 mM, 1.25 min (54.8 ± 2.9%), α_β2L99C required 100 µM, 1 min (56.2 ± 2.8%). Then, either GABA (at -500× the EC_{50} value for that mutant) or SR95531 (at -10× the K_I value) was co-applied with the half-maximal MTS in previously unexposed cells, and the change in I_{GABA} was measured. Mutant receptors containing L99C were completely protected from MTS reaction (0% MTS inhibition) by both agonist and antagonist (Fig. 4, A and B), even at concentrations of MTS (1 mM) that produce maximal inhibition (data not shown). α_β2Y97C receptors were significantly protected by both GABA and SR95531 as well (Fig. 4A). V93C- and D95C-containing receptors were not protected by either ligand, indicating that these residues do not line the binding site. Interestingly, SR95531 enhanced MTS inhibition of I_{GABA} in D95C-containing receptors (Fig. 4, A and C; 93 ± 1.7% inhibition in the presence of SR95531 versus 47.3 ± 3.6% inhibition in the absence). To test whether maximal MTS effects could still be attained, “protected” receptors were exposed to a high concentration of MTS after each protection assay (Fig. 4, B and C). In all cases, I_{GABA} in mutant receptors could be further inhibited to a level indistinguishable from maximal MTS exposure in naïve cells (data not shown). In summary, these data indicate that Tyr97 and Leu99 contribute to the lining of the GABA binding pocket, and Asp95 is a nearby protein.
residue that undergoes a change in environment in response to SR95531 binding.

It was interesting to note that, for studies using receptors containing L99C, it was necessary to express this mutant cRNA with an excess of \textalpha_1 subunit (4:1 ratio). Receptors expressed in a 1:1 \alpha_2\beta ratio exhibited a large “leak” current at the holding potential of ~80 mV. To determine the source of this leak current, we expressed the cRNA encoding \beta_2L99C alone. \beta_2L99C homomers consistently produced a leak current (500–8000 nA) that appeared to slowly desensitize and was “blocked” by picrotoxin (Fig. 5A). To extricate mutant homomer spontaneously open current from measurements of \(I_{\text{GABA}}\) in \alpha_1\beta_2L99C receptors, we biased the mutant receptors to assemble with \alpha_1 subunits by increasing the \alpha:\beta ratio.

The \beta_2L99C leak current was always much larger than the leak current in wild-type \beta_2 homomers (50–400 nA) injected even at 5- to 10-fold higher cRNA concentrations. Furthermore, like \beta_2 homomers, the \beta_2L99C channels formed were activated by pentobarbital (Fig. 5, B and C). The leak and pentobarbital-activated currents are both blocked by picrotoxin, providing further evidence that both the wild-type \beta_2 and mutant \beta_2L99C subunits form homomeric \text{GABA}_A receptors. The data suggest that mutation of Leu99 within the \text{GABA}-binding site is capable of initiating the initial allosteric transitions necessary to gate the ion channel open.

### DISCUSSION

In this study, we used SCAM to investigate the structure and function of the \text{GABA}_A\beta_2Trp^{22}-Asp^{101} region, which overlapsр the putative “loop A” agonist-binding domain. Because alternating residues from \beta_2Val^{96}-Leu^{100} reacted with MTS, we predict this region forms a \beta-strand. Our secondary structure prediction is consistent with the recently solved crystal structure of the acetylcholine binding protein (AChBP) (35). The AChBP is a homologue of the extracellular amino-terminal domain of the nAChR and binds acetylcholine; thus, its structure can be used as a homology model for the agonist-binding sites of the LGIC superfamily of receptors. Residues in the AChBP aligned with the loop A domain of the \text{GABA}_A\beta_2 homomer form a \beta-strand (Figs. 1 and 6). Based on our accessibility data, we would predict that the \beta strand in the \text{GABA}_A\beta_2 extends from \beta_2Val^{96} to \beta_2Leu^{103}, which is longer than the length of the homologous \beta strand in the AChBP structure. This small difference may reflect variation in the structure of the binding sites of these different proteins or may reflect limitations of the SCAM approach. The relative placement of the residue side chains over the AChBP crystal structure reveals that the homologous \beta_2 residues may still be accessible in an alternating pattern (Fig. 6), even if the underlying structure were not a strict \beta strand. Surprisingly, in the aligned region of the nAChR \alpha_1 subunit, the accessibility to sulfhydryl modification does not predict a \beta-strand conformation (20). The pharmacological specificity of these receptors reflects differences in structure and side-chain chemistry of the amino acid residues lining the binding pocket and thus may account for this lack of secondary structural homology between members of the LGIC superfamily.
superfamily.

GABA EC₅₀ values for seven of the cysteine mutations were >10-fold higher than wild-type receptors (Fig. 2, Table I), demonstrating that GABA EC₅₀ is quite sensitive to perturbation of this domain and points to the possible importance of this domain for GABA binding and/or activation. As in all mutagenesis experiments, one complication with this interpretation is that the overall structure of the mutant receptors and/or the orientation of the cysteine side chain may be unlike wild-type receptor structure. However, because the majority of the cysteine mutations caused either no change in the Kᵥ value for SR95531 or decreased SR95531 Kᵥ < 6-fold, we believe that the overall structures of the mutant receptors are not significantly compromised by the cysteine substitutions. If any ligand for a
particular recognition site binds equally well to both wild-type and mutant receptors, it is unlikely that gross structural rearrangement has occurred (46). Consistent with this conclusion, maximal peak currents of all the accessible mutant receptors were similar to wild-type $\alpha_2\beta_2$ receptors (1–5 $\mu$A).

Evidence that Tyr$^{97}$ participates in formation of part of the GABA-binding site is derived from multiple approaches. Both agonist and antagonist protect Y97C from MTS reaction (Fig. 4). We cannot entirely exclude the possibility that GABA and SR95531 reduce MTS access to this residue due to conformational changes. However, because agonist and antagonist binding likely induce different changes within the binding site, the observation that both ligands protect suggests that they sterically block the reaction and that Tyr$^{97}$ faces into the binding site. Expression of Y97C produced the largest rightward shift in GABA responsiveness (109-fold) and the largest shift in SR95531 sensitivity (81-fold rightward shift). A large shift in agonist EC$_{50}$ with parallel slopes and no reductions in current amplitude is consistent with the mutation disrupting the microscopic binding affinity of GABA; however, one needs to be cautious in this interpretation, because it is difficult to compare current maxima between mutant receptors expressed in different oocytes and a change in gating can cause shifts in agonist sensitivity (47). The parallel rightward shift in the sensitivity for the competitive antagonist, SR95531 (lacking efficacy and therefore incapable of gating the channel), lends additional credence to the idea that mutation of Tyr$^{97}$ disrupts binding affinity and $\beta_2$Tyr$^{97}$ is a GABA-binding site residue (47).

We also have evidence that Leu$^{99}$ lines part of the GABA-binding site. Protection from MTS reaction at L99C was robust. Both GABA and SR95531 reduce access completely in $\alpha_2\beta_2$L99C receptors, even at high MTS concentrations, suggesting that regardless of whether these ligands exert this effect by overlying the binding site or by some allosteric mechanism, the L99C residue is in a position that allows for little or no access by MTS. To test for the possibility that the protctants did not actually impede the MTS reaction but rather caused an undetected conformational change in the mutant receptor, each mutant receptor assayed for protection with agonist or antagonist was further exposed to maximal MTS (Fig. 4, B and C). For example, a protected cysteine-like L99C (Fig. 4B) may have reacted with MTS in the presence of ligand differentially than in the unliganded state and irreversibly altered the receptor such that peak $I_{\text{GABA}}$ was increased (e.g. with a leftward shift in the GABA concentration response, or an increase in efficacy for GABA). Similarly, in mutant receptors that were accessible but did not show protection (e.g. V93C), a shift in GABA responsiveness due to MTS modification may have masked any increase or decrease in accessibility. In all cases, $I_{\text{GABA}}$ in mutant receptors could be further inhibited to a level indistinguishable from maximal MTS exposure in naive cells, indicating that protection merely impeded access by MTS to the substituted cysteine, either by direct steric hindrance, or by blocking a local access route for MTS to the substituted cysteine residue. GABA and SR95531 concentration response characteristics for $\alpha_2\beta_2$L99C receptors differ little from wild-type, suggesting that although Leu$^{99}$ is in the binding site this residue may not be a contact residue for GABA binding.

SR95531 has long been considered a classical competitive antagonist at the GABA$_A$ receptor (48), and little evidence is available suggesting any allosteric effects of the antagonist on the GABA-binding site. However, there have been conflicting reports of allosteric block by SR95531 on compounds, which, at higher concentrations, can directly activate the GABA$_A$ receptor, such as pentobarbital and alphaxalone (49, 50). We speculate that SR95531 binds and causes movements in the agonist-binding site that stabilize a closed channel conformation.

The increase in accessibility of $\alpha_2\beta_2$D95C receptors in the presence of SR95531 was unexpected (Fig. 4). In previous studies (17, 34), we have not observed increases in MTS reactivity using antagonists. The mechanism by which SR95531 binding allows MTS better access to D95C is unclear. We speculate that D95C (or residues nearby) move in response to antagonist binding. This movement causes D95C to be in a different environment (i.e. less sterically hindered or more ionized), which increases MTS reaction. Regardless of the mechanism, these data provide evidence that SR95531 binding causes local conformational rearrangements near the GABA-binding site that are different than the movements induced by GABA. A recent crystallographic study of the agonist binding core domain of the glutamate receptor (GluR2) demonstrated that the antagonist 6,7-dinitroquinoxaline-2,3-dione causes a small contraction of the binding site (51) and provides support for the conclusion that antagonist binding can induce structural changes in a ligand-binding site. An alternative explanation for our results is that, once SR95531 is bound within the site, the hydrophobic ring structures of SR95531 may help stabilize the long carbon chain of MTS and increase MTS modification of D95C. However, the major factor in the reactivity of methanethiosulfonate reagents is the ionization of the sulfhydryl side chain, which more likely occurs in an aqueous environment.

The observation that mutation of Leu$^{99}$ (a binding site residue) produces receptors that are spontaneously open is novel. Several studies of GABA$_A$ receptors have reported mutations that cause the channels to remain in an open state (32, 52–54), but to date, these phenomena have been attributed to placement of the mutations in or very near the channel. It is tempting to speculate that the unusual, large leak currents produced by $\beta_2$L99C homomers, which are blocked by PTX (Fig. 5), reflect an allosteric change in channel gating. In the case of L99C, a change in the GABA binding region may create an “at a distance” change in gating, at least when the apposing interface is another $\beta$ subunit. We speculate that Leu$^{99}$ may play a role in coupling agonist binding to channel gating. Alternatively, the L99C mutation may somehow impinge on subunit aggregation, allowing for the surface expression of loosely aggregated $\beta_2$L99C subunits. This “leaky” conformation may be tightened by PTX closure, allowing the homomers to resemble wild-type $\beta_2$ homomers more closely.

In summary, using SCAM, we have identified two novel residues, $\beta_2$Tyr$^{97}$ and Leu$^{99}$, that contribute to forming the GABA binding pocket. In addition, we provide evidence that the $\beta_2$Val$^{95}$-Leu$^{99}$ region of the receptor forms a $\beta$ strand. A residue in this region, Asp$^{95}$, responds differently to GABA and SR95531 binding, and thus, this region may play a role in distinguishing between agonist and antagonist action. Furthermore, we identify a residue within the GABA-binding site, Leu$^{99}$, that when mutated perturbs channel gating. The availability of the crystal structure of the AChBP provides a useful template to overlay residues of the extracellular N termini of the GABA$_A$R subunits (Fig. 6) and to make predictions about binding site residues and distances and interactions between various amino acid residues of interest. Although our experimental data are consistent with the AChBP structure, determining the exact role residues in the loop A region play in GABA$_A$R function will necessitate not only a high resolution structure of the GABA ligand-binding domain but also knowledge of the relative movements of the binding site when the receptor undergoes microscopic binding and gating transitions.

REFERENCES
