Charged Residues in the $\beta_2$ Subunit Involved in GABA$_A$ Receptor Activation

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Fast synaptic inhibition in the mammalian central nervous system is mediated primarily via activation of the $\gamma$-aminobutyric acid type A receptor (GABA$_A$-R). Upon agonist binding, the receptor undergoes a structural transition from the closed to the open state. This transition, known as gating, is thought to be associated with a sequence of conformational changes originating at the agonist-binding site, ultimately resulting in opening of the channel. Using site-directed mutagenesis and several different GABA$_A$-R agonists, we identified a number of highly conserved charged residues in the GABA$_A$-R $\beta_2$ subunit that appear to be involved in receptor activation. We then used charge reversal double mutants and disulfide trapping to investigate the interactions between these flexible loops within the $\beta_2$ subunit. The results suggest that interactions between an acidic residue in loop 7 (Asp$^{146}$) and a basic residue in pre-transmembrane domain-1 (Lys$^{115}$) are involved in coupling agonist binding to channel gating.

Fast synaptic inhibition is a major determinant of network dynamics in the central nervous system (1). In the mammalian brain, this is mediated primarily via activation of the $\gamma$-aminobutyric acid type A receptor (GABA$_A$-R) (2, 3). Each GABA$_A$-R is composed of five subunits arranged around a central ion-conducting pore (4), with each subunit consisting of a large intracellular domain, four transmembrane domains (TM1–TM4), and a larger N-terminal extracellular domain (2). Experimental evidence suggests that the GABA-binding site lies within an asymmetric pocket formed at the interface between the $\alpha$ and $\beta$ receptor subunits (5–8). The channel “gate” in the GABA$_A$-R is believed to be formed by charged residues in the TM1-TM2 loop (9–11). The binding of agonist triggers a complex structural transition that results in the opening of the gate, allowing ions to flow through the channel. The mechanisms by which this occurs remain poorly defined.

The nature of the coupling between binding and channel opening in this receptor family has been recently investigated in several laboratories. Interactions between charged residues in the flexible loops 2 and 7 in the extracellular domain and those in the short linker between the second and third transmembrane domains (TM2-3L) of the GABA$_A$-R $\alpha_1$ subunit have been implicated in the process of receptor activation (12). In addition, a recent report suggests that the pre-TM1 region is critical for receptor activation in the closely related serotonin (5-HT$_3$) receptor (13). In this study, we used site-directed mutagenesis and a number of GABA$_A$-R agonists of varying efficacies to examine the contribution of the corresponding flexible loops in the GABA$_A$-R $\beta_2$ subunit to receptor activation. Based on previous studies (12–14) and the presence of highly conserved charged residues (see Fig. 1), we hypothesized that interactions between charged residues in these domains are crucial for coupling agonist binding to channel gating. We tested this hypothesis using a charge reversal double mutant approach, by inverting the hypothetical interacting pair of residues (e.g. Asp$^{146}$/Lys$^{115}$ to Lys$^{146}$/Asp$^{115}$), and then assaying receptor function. A strong restoration of function relative to either of the single mutant receptors is consistent with a strong interaction between the charged residues.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis**—Full-length cDNAs encoding the GABA$_A$-R $\alpha_1$ (human), $\gamma_2$ (human), and $\beta_2$ (rat) subunits were expressed via the pCIS2 vector, which contains one copy of the strong promoter from cytomegalovirus and a polyadenylation sequence from simian virus 40. Point mutations in the GABA$_A$-R $\beta_2$ subunit were created using the QuikChange kit (Stratagene) and confirmed as described previously (12). GABA$_A$-Rs were expressed in HEK293 cells, maintained, and transfected as described (15).

**Electrophysiology and Data Analysis**—Recordings were made 48–72 h after transfection at room temperature (20–22 °C) using the whole-cell patch-clamp technique (15). Concentration-response curves were determined for GABA in wild-type and mutant GABA$_A$-Rs as described (12). The relative efficacies (e) of piperidine 4-sulfonate (P4S) and taurine were defined as $e = I_{\text{max(partial)}}/I_{\text{max(GABA)}}$, where $I_{\text{max(partial)}}$ is the maximal current elicited by a saturating concentration of either P4S or taurine, and $I_{\text{max(GABA)}}$ is the maximal current elicited by a saturating concentration of GABA. All data are reported as means ± S.E. Statistical significance was assessed using either Student’s two-tailed unpaired t test or, where appropriate, one-way analysis of variance (ANOVA) with the appropriate post-test. Cross-linking experiments were performed essentially as described previously (16). Cu$^{2+}$ solutions were prepared by diluting stock solutions of CuSO$_4$ and 1,10-phenanthroline (Sigma) to 100 and 400 μM, respectively, in buffer. In experiments in which dithiothreitol (DTT) was used, a 10 mM solution was made fresh daily in buffer. When GABA was co-applied with Cu$^{2+}$, a saturating GABA concentration was used to ensure that all of the channels were in the activated state during the period of reagent application. The percent modulation was $(I_{\text{GABA/DTT}} - I_{\text{GABA/DTT before}})/I_{\text{GABA/DTT before}}$ × 100, where $I_{\text{GABA/DTT before}}$ is the peak current of the initial GABA applications, and $I_{\text{GABA/DTT}}$ is the peak current of the GABA test pulses after the application of reagent.

**Molecular Modeling**—The molecular model of a GABA$_A$-R $\beta_2$ subunit

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§ The abbreviations used are: GABA$_A$-R, $\gamma$-aminobutyric acid type A receptor; TM, transmembrane domain; TM2-3L, TM2-TM3 linker; P4S, piperidine 4-sulfonate; ANOVA, analysis of variance; Cu$^{2+}$, CuSO$_4$; 1,10-phenanthroline; DTT, dithiothreitol; AChBP, acetylcholine-binding protein; nAChR, nicotinic acetylcholine receptor.

*Mutagenic primer sequences are available upon request.
of the agonist (measured here as the EC_{50} for GABA) can be influenced at either step in the simplified receptor activation pathway: agonist binding or isomerization from the closed to open state (18, 19). With high efficacy agonists (E > 10), slight reductions in the ability of the agonist-bound receptor to isomerize from the closed to open state typically produce a reduction in sensitivity to agonist, but little reduction in maximal response. This can create a problem in the interpretation of the effects of receptor mutations, as the effects of modest changes in efficacy can closely resemble the effects of mutations that alter agonist binding affinity (20). The use of low efficacy agonists (“partial agonists”) circumvents this problem. Mutations that alter agonist efficacy will reduce the maximal current elicited by a saturating concentration of partial agonist (20). We used the low efficacy GABA_A-R agonist P4S (7, 11, 17) to investigate the effects of these mutations. A comparison of the saturating P4S responses of the wild-type \( \alpha_{1}\beta_2\gamma_2 \) GABA_A-R (Fig. 2D) and the mutant \( \alpha_{1}\beta_2(R269A)\gamma_2 \) GABA_A-R (Fig. 2E) in HEK293 cells demonstrates the reduction in the relative efficacy (\( \epsilon \)) of P4S for the mutant receptor. The \( \beta_2(R269A) \) and \( \beta_2(K274D) \) mutations caused a significant reduction in P4S relative efficacy, whereas the relative efficacy of P4S for the \( \beta_2(L272A), \beta_2(R269D), \) and \( \beta_2(K279D) \) mutations was indistinguishable from that for the wild-type receptor (Fig. 2F).

Characterization of Mutations in Loops 2 and 7 of the GABA_A-R \( \beta_2 \) Subunit—To determine the role of charged residues in these domains, we created the following \( \beta_2 \) subunit mutations: E52K, D56K, D139K, R216D, D146K, and D147K. The \( \beta_2(D56K) \), \( \beta_2(D139K) \), and \( \beta_2(D146K) \) mutations caused a significant reduction in GABA sensitivity, whereas the \( \beta_2(E52K) \), \( \beta_2(E147K) \), \( \beta_2(R216D) \), and \( \beta_2(R142E) \) mutations had no effect on GABA sensitivity (Table I). In addition, the \( \beta_2(D56K) \), \( \beta_2(D139K) \), and \( \beta_2(D146K) \) mutations caused a significant reduction in the relative efficacy of P4S, whereas this was unchanged in the \( \beta_2(E147K) \) and \( \beta_2(E52K) \) mutations (Fig. 3A). We also examined receptor sensitivity to another partial agonist, taurine, and found that the mutations reduced the relative efficacy in a similar manner (Fig. 3B).

Characterization of Mutations in the Pre-TM1 Region of the GABA_A-R \( \beta_2 \) Subunit—A previous report has suggested that a cluster of conserved basic residues in the pre-TM1 region of the 5-HT_3 receptor are involved in receptor activation (13). To determine whether these residues have a similar role in the GABA_A-R \( \beta_2 \) subunit, we created the following \( \beta_2 \) subunit mutations: R215D, R216D, and N217D. GABA sensitivity was significantly reduced by the \( \beta_2(R215D) \) mutation and was unchanged by the \( \beta_2(R216D) \) and \( \beta_2(N217D) \) mutations (Table I). The \( \beta_2(R215D) \) and \( \beta_2(R216D) \) mutations caused a significant reduction in P4S relative efficacy, whereas the \( \beta_2(N217D) \) mutation had no effect (Fig. 3A). Taurine relative efficacy was significantly reduced in the \( \beta_2(R215D) \) mutation, but was unchanged in the \( \beta_2(R216D) \) and \( \beta_2(N217D) \) mutations (Fig. 3B).

Characterization of Charge Reversal Double Mutants—Using charge reversal double mutants, we previously demonstrated that specific residues in loops 2 and 7 interact with TM2-3L of the GABA_A-R \( \alpha \) subunit (12). To determine whether similar interactions might occur in the GABA_A-R \( \beta_2 \) subunit, we created and characterized the following double mutants: E52K,D274D, D56K,D274D, D139K,D274D, D146K,D274D, and E147K,D274D. All of these double mutant receptors were less sensitive to GABA compared with the wild-type receptor (Table II). We also created and characterized a second series of double mutants in which charges were exchanged between loops 2 and 7 and the pre-TM1 region in the GABA_A-R \( \beta_2 \) subunit. In several of these double mutant receptors, notably \( \beta_2(D146K,K215D) \), the sensitivity to GABA was substantially
restored, approaching the EC_{50} for the wild-type receptor, and significantly improved relative to the corresponding single mutants (Table II). In addition, the relative efficacy of P4S for the β2(D146K,K215D) double mutant receptor was enhanced relative to that for β2(D146K) (Fig. 4B).

Disulfide Trapping Experiments—To examine the relative proximity and mobility of the domains investigated in this study, a disulfide bond trapping technique was utilized (16, 21, 22). We analyzed the interaction between cysteine residues inserted at potential contact points by examining the effects of oxidation and reduction on the function of wild-type and double mutant receptors. The ability to induce disulfide bond formation was assayed by studying the effect of the oxidizing reagent Cu:phen on receptor function. In the wild-type receptor, application of Cu:phen or the reducing agent DTT had no effect on receptor function in the absence or presence of GABA (Fig. 5A). Similarly, the single mutant receptors incorporating D56C, D139C, D146C, K215C, or K274C were also unaffected by

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Table 1

| GABA_{A,R} | EC_{50} for GABA | n for GABA | I_{max} | n |
|------------|----------------|-----------|--------|---|
| Wild-type  | 17 ± 2         | 1.2 ± 0.1 | 680 ± 200 | 49 |
| β2(R269A) | 57 ± 17        | 1.4 ± 0.1 | 880 ± 210 | 8  |
| β2(R269D) | 16 ± 2         | 1.3 ± 0.2 | 590 ± 80  | 6  |
| β2(L272A) | 22 ± 5         | 1.3 ± 0.2 | 1400 ± 240 | 8  |
| β2(K274D) | 111 ± 32       | 1.5 ± 0.1 | 700 ± 90  | 12 |
| β2(K279D) | 11 ± 2         | 1.0 ± 0.1 | 450 ± 30  | 8  |
| β2(R269K) | 26 ± 9         | 1.1 ± 0.2 | 700 ± 100 | 5  |
| β2(D56K)  | 57 ± 11        | 1.3 ± 0.1 | 145 ± 30  | 7  |
| β2(D139K) | 163 ± 25       | 1.3 ± 0.1 | 420 ± 80  | 9  |
| β2(D146K) | 177 ± 33       | 1.7 ± 0.1 | 470 ± 70  | 13 |
| β2(E147K) | 23 ± 9         | 1.0 ± 0.1 | 720 ± 150 | 9  |
| β2(R141E) | 31 ± 6         | 1.3 ± 0.2 | 670 ± 220 | 8  |
| β2(R142E) | 19 ± 3         | 1.1 ± 0.2 | 760 ± 200 | 9  |
| β2(K215D) | 104 ± 17       | 1.4 ± 0.1 | 1380 ± 420 | 8  |
| β2(R216D) | 27 ± 4         | 1.0 ± 0.1 | 1100 ± 190 | 8  |
| β2(N217D) | 18 ± 2         | 1.2 ± 0.1 | 740 ± 230 | 8  |

* p < 0.05 (values that are significantly different compared with the wild-type receptor value using Student’s unpaired t test).

p < 0.01.

p < 0.001.
Cuphens (Cu:phen) or DTT (data not shown). Application of Cu:phen alone and in the presence of GABA significantly inhibited receptor function in all of the loop 2 and 7 and TM2-3L double cysteine mutants (Fig. 5D). This effect was reversed following incubation with 10 mM DTT (Fig. 5B). In double cysteine mutants between loops 2 and 7 and the pre-TM1 region, Cu:phen alone had no effect, whereas Cu:phen plus GABA significantly inhibited receptor function (Fig. 5, C and E). All of the mutants investigated in this study were unaffected by treatment with the reducing agent DTT (10 mM), indicating there were no spontaneously formed disulfide bonds (data not shown).

**DISCUSSION**

**TM2-TM3 Linker Mutations**—Recent work has begun to illuminate the mechanisms of receptor activation in the Cys loop ligand-gated ion channel superfamily. In particular, studies of inherited mutations in the glycine receptor (23) and the nicotinic acetylcholine receptor (nAChR) (24) first revealed the importance of the TM2-3L region. Naturally occurring mutations in TM2-3L have been reported to have strong inhibitory (23) or facilitatory (24) effects on receptor function that are unrelated to agonist binding, leading to the idea that this loop is intimately involved in the coupling of ligand binding to ion channel opening. In this study, we have identified two mutations in TM2-3L of the β2 subunit (R269A and K274D) that interfere with receptor function by reducing agonist efficacy. Furthermore, we investigated the role of other basic residues located in TM2-3L of the β2 subunit (Arg269 and Lys279) and found that only the basic residue at position 274 is sensitive to a charge reversal mutation, suggesting that it is uniquely involved in electrostatic interactions important for receptor activation. This study also confirms the importance of TM2-3L of the GABA_A-R β2 subunit receptor in receptor activation, as previously reported for the α1, α2, β1, and β2 subunits (12, 14, 18, 25). An interesting difference between the α1 and β2 subunits was observed here because β2/L272A failed to alter receptor function, whereas the α1 ortholog (L277A) reduces GABA sensitivity dramatically (EC_{50} = 278 μM) (26).

**Pre-TM1 Mutations**—Of the three mutations in the pre-TM1 cluster of basic residues in the β2 subunit, only K215D had a significant effect on receptor function, producing a large decrease in GABA sensitivity. This mutation also reduced the relative efficacy of both taurine and P4S, suggesting that the major effect is a reduction in agonist efficacy. It is also worth noting that, although there are residues homologous to the pre-TM1 region in AChBP, they are not included in the structure because they are disordered (27). Our model (Fig. 5A) predicts that those residues should be in a β strand, as an α helix would be less than half as long and would not reach the top of TM1.

**Loop 2 and 7 Mutations**—Three charge reversal mutations in loops 2 (D56K) and 7 (D139K and D146K) inhibited receptor function, apparently by reducing agonist efficacy. These data confirm an early report suggesting that mutations in loop 7 of the β2 subunit interfere with gating/coupling rather than with the GABA-binding site (28). The role of these residues appears to be conserved across the receptor superfamily, as homologous mutations in the glycine receptor α1 subunit have similar ef-

**Fig. 3.** Specific charge reversal mutations in loops 2 and 7 and the pre-TM1 region of the GABA_A-R β2 subunit reduce the relative efficacy of both P4S and taurine compared with the wild-type receptor. A, bar graph denoting P4S relative efficacy for wild-type (WT) and mutant receptors as I_{max(P4S)}/I_{max(GABA)}. Values represent means of multiple cells (n = 5–25). *p < 0.01 (values that are significantly different from the wild-type receptor value calculated using one-way ANOVA with Dunnett’s post hoc test). B, bar graph denoting taurine relative efficacy for wild-type and mutant receptors as I_{max(Taurine)}/I_{max(GABA)}. Values represent means of multiple cells (n = 5–25). *p < 0.01 (values that are significantly different from the wild-type receptor value calculated using one-way ANOVA with Dunnett’s post hoc test).

**Table II**

| GABA_A-R | EC_{50} for GABA | n | I_{max} | pA |
|----------|-----------------|---|--------|-----|
| WT       | 17 ± 2          | 1.2 ± 0.1 | 680 ± 200 | 49 |
| β2(E52K,K274D) | 70 ± 13" | 1.3 ± 0.2 | 360 ± 60 | 9 |
| β2(D56K,K274D) | 101 ± 8" | 1.2 ± 0.1 | 360 ± 40 | 11 |
| β2(D139K,K274D) | 109 ± 29" | 1.3 ± 0.1 | 402 ± 85 | 9 |
| β2(D146K,K274D) | 234 ± 52" | 1.5 ± 0.1" | 250 ± 70 | 11 |
| β2(E147K,K274D) | 88 ± 23" | 1.4 ± 0.1 | 750 ± 150 | 11 |
| β2(E52K,K215D) | 66 ± 3" | 1.1 ± 0.1 | 480 ± 40 | 6 |
| β2(D56K,K215D) | 43 ± 9" | 1.2 ± 0.1 | 1000 ± 80 | 12 |
| β2(D139K,K215D) | 33 ± 4" | 1.0 ± 0.1 | 950 ± 160 | 8 |
| β2(D146K,K215D) | 26 ± 4" | 1.2 ± 0.1 | 1200 ± 140 | 10 |
| β2(E147K,K215D) | 62 ± 11" | 1.3 ± 0.3 | 910 ± 230 | 9 |
| β2(D56K,K216D) | 73 ± 18" | 0.9 ± 0.1" | 990 ± 180 | 9 |
| β2(D139K,R216D) | 63 ± 13" | 1.1 ± 0.1 | 790 ± 120 | 10 |
| β2(D146K,R216D) | 68 ± 16" | 1.0 ± 0.1" | 1204 ± 190 | 14 |
| β2(E147K,R216D) | 65 ± 16" | 1.0 ± 0.1 | 1060 ± 120 | 13 |

*p < 0.01 (values that are significantly different compared with the wild-type receptor value using Student’s unpaired t test).

*p < 0.001.

*p < 0.05.
Double Mutant Experiments Reveal Asp<sup>146</sup>-Lys<sup>215</sup> Interactions—After establishing that the charged residues located in these domains appeared to be involved in receptor activation, we used a charge reversal double mutant approach to examine the interactions between these domains. This approach has been used successfully to identify intramolecular interactions in transmembrane (30) and soluble (31) proteins. Our results with the D146K,K215D double mutant strongly suggested that an interaction between these residues is critical for optimal receptor activation. Although we did observe small differences in function between the D146K,K215D mutant and the wild-type GABA<sub>A</sub>-R, this is not surprising, as the reversal of a charged pair may alter the energetics of the interaction (30) and hence perturb the functional properties of the protein (31).
To determine the proximity of the domains investigated in this study, we performed engineered disulfide trapping experiments, the results of which indicate that loops 2 and 7 are located in close proximity to TM2-3L, independent of the presence of agonist. One potential explanation is that interactions between loops 2 and 7 and TM2-3L are involved in stabilizing the closed state of the receptor. By contrast, loops 2 and 7 appear to be located within close proximity of the pre-TM1 region only in the presence of GABA, suggesting that these domains move relative to one another during the open or desensitized state. It is of interest to note that a recent study that examined state-selective incorporation of hydrophobic probes in the nAChR identified loop 7 of the nAChR α1 subunit as a region that undergoes a conformational change during receptor activation (32). Based on these results, we propose that there are intramolecular interactions between loop 7 and the pre-TM1 region that are important for receptor activation. This idea is structurally reasonable based on our molecular model (Fig. 6A), which incorporates both the extracellular and transmembrane domains of the GABA$_A$-R. We also note with interest the high degree of similarity between the molecular model we present here and the recently published structure of the nAChR (33).

**Functional Asymmetry between α and β Subunits**—An intriguing result was the failure of charge reversal double mutants in the β2 subunit between loops 2 and 7 and TM2-3L to restore receptor function. This finding contrasts with our previous data from studies of the α1 subunit, where we found evidence for coupling between orthologous residues in loops 2 and 7 (Asp$^{57}$ and Asp$^{149}$) and Lys$^{279}$ in TM2-3L (12). These differences in intradomain interactions between α and β subunits may reflect local dissimilarities in structure or an asymmetry in the propagation of conformational change within the individual subunits. A possible origin for such differences could be the asymmetric nature of the agonist-binding site itself, which is believed to constrict upon binding GABA (7). The constriction of this cleft between adjacent subunit polypeptides implies that the adjacent domains of the α and β subunits are essentially pulled in opposite directions, thereby resulting in an asymmetric change in subunit structure. This would be consistent with reports of asymmetric motion within the extracellular domains of nAChR subunits following receptor activation (34). Our latest results suggest that there may also be asymmetric conformational change in the extracellular domain following agonist binding in the GABA$_A$-R and that, within the β subunit, the structural changes initiated upon agonist binding are coupled to the transmembrane domain via interactions between loop 7 and the pre-TM1 region.

**Fig. 6.** A, this molecular model shows the proximity of the domains examined in this study. The domains of interest are labeled. The view is from the center of the ion channel pore toward the outside, with the amino acid backbone shown as a green tube, β strands as yellow ribbons, and α helices as red cylinders. The cylinders are labeled 1–4 corresponding to the transmembrane domains they represent. Breaks in the cylinder representations of TM1 and TM4 are caused by the occurrence of proline residues. The model was prepared as an αβ heteromer to establish more realistic structural constraints; for simplicity, only a single β subunit is shown B, several key residues have also been rendered with space-filling or ball-and-stick surfaces to show their positions. Residues rendered with side chains have carbon, nitrogen, oxygen, and hydrogen atoms colored green, blue, red, and white, respectively, and are labeled.

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