A Single M1 Residue in the β2 Subunit Alters Channel Gating of GABA<sub>A</sub> Receptor in Anesthetic Modulation and Direct Activation*  

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General anesthetics allosterically modulate the activity of neuronal γ-aminobutyric acid, type A (GABA<sub>A</sub>) receptors. Previous mutational studies from our laboratory and others have shown that the regions in transmembrane domain 1 (M1) and pre-M1 of α and β subunits in GABA<sub>A</sub> receptors are essential for positive modulation of GABA binding and function by the intravenous (IV) general anesthetics. Mutation of β2Gly-219 to Phe corresponded in p nearly eliminated the modulatory effect of IV anesthetics in α1/β2/γ2S combination. However, the general anesthetics retained the ability to directly open the channel of mutant G219F, and the apparent affinity for GABA was increased, and desensitization rate was reduced. In this study, we made additional single mutations such as 219 Ser, Cys, Ile, Asp, Arg, Tyr, and Trp. The larger side chains of the replacement residues produced the greatest reduction in enhancement of GABA currents by IV anesthetics at clinical concentrations (Trp > Tyr = Phe > Arg > Asp > Ile > Cys > Ser = wild type). Compared with a 2–3-fold response in wild type, pentobarbital and propofol enhanced less than 0.5-fold; etomidate and alphaxalone, respectively, altered channel gating by 20% and 50%, respectively. The larger side chains of the replacement residues were positively modulated by the IV anesthetics.  

Considerable evidence supports the hypothesis that general anesthesia can be produced, at least in part, by enhancing neuronal inhibition mediated by GABA<sub>A</sub> receptors (GABAR). GABA and most volatile and general intravenous (IV) anesthetics bind to GABAR. These are ligand-gated channels that enhance the flow of chloride ions (CI<sup>–</sup>) into the postsynaptic neuron, thus increasing its resting potential and making it less likely to fire, i.e. the inhibitory effect in GABA synapses. GABAR are modulated positively by a wide variety of structurally diverse general anesthetics (1). In particular, volatile anesthetics (2, 3) and IV anesthetic agents such as the barbiturates (4, 5), propofol (6), etomidate (7), and neuroactive steroid anesthetics, like alphaxalone (8–10), all enhance the function of GABAR at clinically relevant concentrations. In addition, IV and volatile anesthetics can activate GABAR directly in the absence of GABA (11). Anesthetics appear to allosterically modulate ligand-gated ion channel (LGIC) receptor function by binding to the receptor itself, but not at the agonist-binding site, and to affect the conformation of the membrane protein in a functional manner. Exactly how such modulatory sites affect agonist-regulated channel opening is a major contemporary question in pharmacology and neurobiology. Obviously, the structure and function of these integral membrane proteins, including GABAR, are sensitive to the membrane environment and its molecular constituents.  

The mammalian GABAR is a heteropentameric complex formed by 19 different glycoprotein subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π, and ρ1–3) that co-assemble to form a chloride channel (12–14). Most GABAR in vivo are composed of pentameric complexes of 2α, 2γ, and 1γ subunits. GABAR are members of the LGIC receptor superfamily and share significant amino acid sequence homology with other members (15, 16). Each subunit in the superfamily has a similar proposed three-dimensional structure and membrane topology, with four membrane-spanning α-helical domains, M1–M4. The M2 transmembrane domain of all five subunits contributes to the channel wall and determines ion permeability, whereas the N-terminal extracellular domain determines the ligand binding specificity. In the pentameric complex of GABAR, there are two putative GABA-binding pockets at the interfaces of α and β subunits. Amino acids that participate in ligand binding have been identified by photoaffinity labeling and sequencing, as well as by site-directed mutagenesis (17–23). Loops of amino acids that form an acetylcholine-binding pocket were identified in the α subunit of nAChR (24, 25), and homologous residues involved in GABA binding were identified in β subunits of GABAR (26, 27). Additional amino acids involved in the GABAR-binding site in the α subunit were homologous to residues involved in binding acetylcholine in the γ subunit of nAChR (21). The predicted three-dimensional structure and alignments with the molluscan acetylcholine-binding protein (28) provide models of the domains of ligand binding and the activation of channel gating in GABAR (29), possibly relevant to sites of anesthetic action. In addition, cryo-electron microscopy of crystalline membranes of electric organ containing nAChR (30) have obtained structural information at 4-Å resolution that is totally consistent with previous conclusions about LGIC functional domains in-
including ligand-induced channel gating by agonists and modulators.

Pharmaceutical subtypes of GABAR exist due to multiple isoforms of varying subunit composition (10, 31, 32). Heterogeneity in modulation of GABAR [3H]benzodiazepine and [3H]muscimol binding is observed for PB, propofol, etomidate, and steroids (8, 10, 14, 39), and similar differences are seen for receptor subtypes in electrophysiological assays (31, 34, 35). In GABAR, a β subunit is essential for receptor membrane expression and anesthetic modulation (1, 8, 14). Wafford and co-workers (31, 34, 36) identified a β subunit-specific interaction, whereas isoforms containing the β2 and β3 subunits are sensitive to the anxiolytic non-benzodiazepine drug loreclezole, the β1 is not. Furthermore, the same β selectivity applied to the anesthetic etomidate, a structural analog of loreclezole, leading to identification of a residue in the β2 subunit that was subcloned into pBlueScript II SK vector for expression in Xenopus oocytes (19).

EXPERIMENTAL PROCEDURES

Molecular Cloning—Rat α1, β2, and γ2S GABA receptor subunit cDNAs are in the expression vector pBlueScript II SK. For mutants of the β2 subunit in the mature rat protein sequence used in this study, a glycine at position 219 substituted by serine, cysteine, isoleucine, aspartate, arginine, tyrosine, and tryptophan were generated by recombinant PCR using overlapping complementary oligonucleotide methods designed to create a 20-bp overlap at the desired amino acid mutation region. Using a β2 sense oligonucleotide and an antisense oligonucleotide 5'-TTGTTCTTCTAGCTTAAATCTGGA-3' with β2 cDNA as a template, upstream PCR fragments with deletion of a HindIII site without changing the amino acid residue at position 219 were generated. Simultaneously, by using a sense oligonucleotide with a single mutation 5'-TCCTAAAGGGCTTAAAGGAGCAACTTTTCTTCTGATCGTAAATCTGGA-3' (for example, G219F) and an antisense oligonucleotide after the termination codon with β2 cDNA as template, downstream PCR fragments with a single mutation were also prepared in separate reactions. Upstream and downstream PCR fragments were then combined and amplified to create the full-length β2 cDNA fragments that were subcloned into pBlueScript II SK vector for expression in Xenopus oocytes. All mutants were verified by restriction digest (HindIII used for the first mutation screen, XhoI and NotI used for the orientation of ligation) and double-stranded DNA sequencing using standard techniques.

Expression of Rat GABA Receptors in Xenopus Oocytes—Capped mRNA (cRNA) transcribing for the wild type and mutant subunits was synthesized by in vitro transcription from ApoI-linearized cDNA construct in pBlueScript II SK using the mMessage mMachine kit (Ambion). cRNA concentrations were calculated by UV absorption and corroborated by comparison to RNA standards on 1% agarose RNA HEPS gels. Oocytes from Xenopus laevis were prepared as described previously (46) and injected with a total volume of 50 nl of cRNA (100–200 pg/nl/subunit) mixed in a ratio of 1:1:2 (c1:c2:γ2S). Oocytes were maintained in 6-well plates at 17–19 °C in SOS solution (in mM, 100 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2, and 5 HEPS), supplemented with 50 μg/ml gentamycin and 100 μg/ml streptomycin and penicillin, and were used for electrophysiological experiments 3–7 days (5–10 days for the better expression of G219R mutant) following injection. The total amount of cRNA was scaled to yield maximal GABA-induced currents of 1.5–2 μA for wild type α1β2γ2S.

Two-electrode Voltage Clamp Analysis—Oocytes under a two-electrode voltage clamp (47) (voltage hold at −70 mV) were gravity-perfused continuously with ND96 recording solution containing (in mM) 96 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2, and 5 HEPS, pH 7.4, at an approximate rate of 5 ml/min. In general, drugs and reagents (GABA, Sigma) were dissolved in ND96. Stock drug solutions (50–100 μM) were made in 10% dimethyl sulfoxide and further diluted in the same medium to the desired final concentrations. Methyl 1,2-diacylglycerol (1.5–2 μM) for all receptors was elicited by a 30-s application of a saturation concentration of 1 mM GABA, which was measured to be a lower concentration (100 μM) in some mutant receptor combinations. A standard two-electrode voltage clamp recording was carried out using an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA) interfaced to a computer with a DigData 1522-A device (Axon Instruments). Electrodes were filled with 3 M KCl and had a resistance of 0.5–1.5 MΩ. By using a standard similar Hill equation to model the GABA dose responses, data acquisition and analyses were performed using pClamp 8.2, Axo-Dta, Axo-Graph (Axon Instruments), Excel (Microsoft), and Prism software (GraphPad). Statistical comparisons of potentiation data employed non-linear least square Excel for multiple independent samples and sigmoid curve fitting of dose-response curves using Equation 1,

\[ I = I_{max}/(1 + EC_{50}/[A])^{n} \]  

(1)

where \( I \) is the peak amplitude of the current elicited by a given concentration of agonist \([A]\); \( I_{max} \) is the maximum amplitude of the current; \( EC_{50} \) is the concentration required for half-maximal inhibition, and \( n \) is the Hill coefficient. The normalized data are presented as % control to the control EC10 GABA-induced current.

RESULTS

GABA-sensitive Point-mutated Recombinant GABAR—We introduced point mutations Gly-219 located at the entrance of transmembrane domain 1 (M1) into the rat GABAR β2 subunit cDNA (Fig. 1a), including the residue Phe found in the anesthetic-insensitive homomeric μ1 subunit (GABA\(_{μ1}\) receptor) (Fig. 1b).

The Concentration-dependent Responses of Wild Type and Mutant Receptors—The wild type and point-mutated β2 subunit capped mRNAs (cRNAs) were mixed and co-injected into Xenopus oocytes with rat α1 and γ2S (short form) subunit cRNAs. The GABA-induced current could be measured after a 30-s perfusion with GABA (1 μM), and washing out the applied drugs for 1.5–2.5 min (a longer washout time is required for etomidate and alphaxalone application). \( I_{GABA} \) is the control EC10 GABA-induced current. The correlation between anesthetic modulation and residue volume were fitted to the one-phase exponential Equation 2,

\[ EF = E_{0} + E_{0} \exp^{(-V/\text{residue volume})} \]  

(2)

where \( EF \) is enhancement fold; \( E_{0} \) represents the enhancement for residue-occupied "zero" volume; \( E_{0} \) is the correlation \( V \) vs residue volume; \( V \) is the residue volume; and \( t \) is rate of enhancement.
The wild type receptor α1β2γ2S was 45.8 μM (Fig. 2b), which is similar to the value previously obtained in our laboratory on oocyte expression (46). The GABA dose-response curve for the α1β2(G219F)γ2 receptor, which has larger aromatic side chain volume, was shifted to the left, with a decreased EC50 value of 15.9 μM, significantly different (a 3-fold shift) from wild type (**, p < 0.001) (Fig. 2b). The Hill slopes of the GABA concentration-response relationships for the point-mutated β2 containing receptors were assumed similarly and obtained the value of 0.72 under running the non-linear least square curve fitting by Excel software. The maximal GABA-induced currents for mutant receptor combinations were less. Theoretically, the fully open probability for maximal channel activation could not be elicited by GABA application alone, so we let the concentration-response curves float to a higher value of 100% to generate the full open probability for maximal channel activation could not be elicited by GABA application alone, so we let the concentration-response curve fitting by Excel software. The maximal GABA-induced currents in oocytes expressing the wild type α1β2γ2S receptor (calculated by the following equation: \( I_{\text{Aneut.}} + I_{\text{GABA}} - I_{\text{GABA}} \times I_{\text{control}} \), as the enhancement fold), GABA responses were greatly enhanced by co-application of either PB (1.9 ± 0.6-fold, n = 32 versus control) or propofol (2.3 ± 0.4-fold, n = 29). When β2Gly-219 was substituted by a large amino acid such as Trp, the mutations at position Gly-219 of β2 subunit nearly eliminated positive modulation (less than 0.5 enhancement fold) by PB and propofol (0.4 ± 0.03- and 0.28 ± 0.08-fold of control value, respectively, Fig. 4b), suggesting that β2Gly-219 is one of the key residues for the modulation of GABAR by IV general anesthetics. On the other hand, greater potentiation by the anesthetics was observed in some β2Gly-219 mutated receptors. For example, α1β2(G219W)γ2S, in which glycine was substituted by the amino acid isoleucine that is smaller than an aromatic group Phe or Trp, both PB and propofol elicited a greater and significant potentiation of EC10 GABA currents (1.2 ± 0.25- and 1.1 ± 0.31-fold of control value, respectively, Fig. 4b).

The Effects of Point Mutations on Potentiation of GABA Responses by Etomidate and Alphaxalone—To determine whether the mutated amino acid residues influenced the ability of IV general anesthetics to potentiate GABAR function, the effects of etomidate and alphaxalone on potentiation of EC10 GABA responses were examined. Fig. 5a, a–d shows the potentiation by etomidate (5 μM) and alphaxalone (0.5 μM) of EC10 GABA-induced currents in oocytes expressing the wild type α1β2γ2S receptor. GABA responses were greatly enhanced by co-application of either etomidate (3.1 ± 0.35-fold, n = 21 versus control) or alphaxalone (3.3 ± 0.4-fold, n = 18). When β2Gly-219 was substituted by a large amino acid such as Trp, the mutations at position Gly-219 of β2 subunit decreased the positive modulation down to less than 1-fold enhancement by etomidate and alphaxalone (0.75 ± 0.09- and 0.7 ± 0.11-fold of control value, respectively, Fig. 5b). On the other hand, greater potentiation by etomidate and alphaxalone such as by PB and propofol was also observed in some β2Gly-219 mutated receptors. Noteworthy, α1β2(G219R)γ2S, in which glycine was substituted by a positively charged Arg, both etomidate and alphaxalone elicited a significantly lower potentiation of EC10 GABA currents (1.1 ± 0.13- and 1.3 ± 0.06-fold of control value, respectively, Fig. 5b), compared with wild type receptor and other non-aromatic group substituted residue combinations.

The Correlation of Anesthetic Modulation Relative to the Substituted Side Chain Residue Volume at Position 219 of β2 Subunit—When the α1β2γ2S GABAR α1β2γ2S mutants were tested for modulation by the IV general anesthetics PB and propofol, there appeared to be a limiting volume for the side chain, such that amino acid residues with small side chains such as Gly and Ile demonstrated greater anesthetic potentiation, whereas very large residues such as Trp dramatically decreased the anesthetic potentiation by pentobarbital and...
propofol (10 and 5 μM), respectively, Fig. 4, a and b. Similar results were obtained in the modulation of etomidate and alphaxalone (5 and 0.5 μM) respectively, Fig. 5, a and b. The potentiation of EC_{50} GABA responses by anesthetics in α1β2(Gly-219)γ2S mutant GABA_{A} receptors declines with increasing side chain volume of the amino acid at position Gly-219 using the one-phase exponential equation (Equation 2) (Fig. 6), suggesting that Gly-219 of the β2 subunit is a key residue for anesthetic potentiation of GABA_{A} receptors. There was a strong correlation between the substituted side chain volume and the degree of anesthetic potentiation.

Effects of Mutations in β2 Subunit at Gly-219 on Direct Activation of GABA_{A} Receptors by Intravenous Anesthetics

The characteristics of wild type and mutant GABA_{A} receptor from GABA-induced current data

From curve fitting of concentration response data, values indicated the EC_{50}, calculated EC_{10} for the experiments of anesthetic positive modulation, and E_{max} of control wild type α1β2γ2S was at the same injection day (n = 20-30 experiments). In general, the expression of mutants were less than that of wild type except for G219S and G219I.

| Subunit combination | EC_{50} μM | EC_{10} μM | E_{max} % |
|---------------------|------------|------------|-----------|
| α1β2γ2S            | 45.8 ± 0.94| 5.03       | 100       |
| α1β2(G219I)γ2S     | 39 ± 1.5   | 5.01       | 95 ± 12   |
| α1β2(G219C)γ2S     | 41 ± 1.5   | 5.22       | 81 ± 5    |
| α1β2(G219F)γ2S     | 31.9 ± 0.9 | 4.52       | 106 ± 8   |
| α1β2(G219D)γ2S     | 36 ± 1.2   | 4.1        | 73 ± 16   |
| α1β2(G219R)γ2S     | 24.7 ± 0.7 | 3.54       | 74 ± 24   |
| α1β2(G219F)γ2S     | 15.9 ± 3.9 | 0.98       | 63 ± 13   |
| α1β2(G219Y)γ2S     | 18.3 ± 0.5 | 1.01       | 55 ± 7    |
| α1β2(G219W)γ2S     | 21.8 ± 0.78| 1.15       | 71 ± 5    |

less evident in mutant α1β2(G219F)γ2S and α1β2(G219W)γ2S receptors (Fig. 7 and Table II). Direct activation of wild type GABA_{A} α1β2γ2S receptors by IV anesthetics was described previously (35). High concentrations of IV anesthetics can produce a profound inhibition on the effect of GABA responses (such as propofol >50 μM) (6), and there is an immediate “rebound” current upon washout, i.e. the current did not immediately return to the control level. In these experiments, inhibitory and rebound effects were most evident at concentrations more than PB (1 mM), propofol (50 μM), etomidate (100 μM), and alphaxalone (50 μM), respectively, although small rebound currents immediately after washout of applied anesthetics were occasionally observable at the concentrations listed above (Fig. 7a). An IV anesthetic concentration chosen in our experiment thus was suitable to compare near-maximal direct activation in different receptor combinations with mini-
Modulation of \( \text{GABA}_A \) \( \beta_2 \) Mutants by Anesthetics

**Fig. 4.** a, a–e, enhancement of \( \text{EC}_{10} \) GABA responses by pentobarbital (PB, 10 \( \mu \)M) and propofol (Pro, 5 \( \mu \)M). \( \text{EC}_{10} \) GABA currents in wild type \( \text{GABA}_A \), \( \alpha_1\beta_2\gamma_2\)S receptors are strongly enhanced by pre-perfusion for 15 s before co-application with \( \text{EC}_{10} \) GABA (wild type \( \alpha_1\beta_2\gamma_2\)S, \( \text{EC}_{10} \), 5.03 \( \mu \)M; \( \alpha_1\beta_2\gamma_2\)S, EC10, 4.52 \( \mu \)M; \( \alpha_1\beta_2\gamma_2\)S, EC10, 3.55 \( \mu \)M; \( \alpha_1\beta_2\gamma_2\)S, EC10, 0.98 \( \mu \)M; \( \alpha_1\beta_2\gamma_2\)S, EC10, 1.15 \( \mu \)M) at clinically relevant concentrations. Note that the constant concentrations of all anesthetics that were examined would not elicit more than \( \text{EC}_5 \) of maximal GABA current in all receptor combinations of pentobarbital (PB, 10 \( \mu \)M) and propofol (Pro, 5 \( \mu \)M). In contrast, \( \text{EC}_{10} \) GABA-induced currents in \( \alpha_1\beta_2\gamma_2\)S or \( \alpha_1\beta_2\gamma_2\)S mutant with substituted aromatic group receptors are less enhanced by co-application of PB (10 \( \mu \)M) or propofol (5 \( \mu \)M). The statistics panels of \( \text{EC}_{10} \) GABA modulation by \( \square \) PB or \( \square \) Pro are shown as the enhancement fold in b. Individual recordings are from oocytes injected with cRNAs encoding the indicated subunit combination. GW means, for example, \( \alpha_1\beta_2\gamma_2\)S receptor combination.

**Fig. 5.** a, a–d, enhancement of \( \text{EC}_{10} \) GABA responses by etomidate (5 \( \mu \)M) and alphaxalone (0.5 \( \mu \)M). \( \text{EC}_{10} \) GABA currents in wild type \( \text{GABA}_A \), \( \alpha_1\beta_2\gamma_2\)S receptors are strongly enhanced by pre-perfusion for 15 s before co-application with \( \text{EC}_{10} \) GABA (wild type \( \alpha_1\beta_2\gamma_2\)S, \( \text{EC}_{10} \), 5.03 \( \mu \)M; \( \alpha_1\beta_2\gamma_2\)S, EC10, 4.52 \( \mu \)M; \( \alpha_1\beta_2\gamma_2\)S, EC10, 3.55 \( \mu \)M; \( \alpha_1\beta_2\gamma_2\)S, EC10, 0.98 \( \mu \)M; \( \alpha_1\beta_2\gamma_2\)S, EC10, 1.15 \( \mu \)M) at clinically relevant concentrations of etomidate (ETO, 5 \( \mu \)M) and alphaxalone (Alpha, 0.5 \( \mu \)M). In contrast, \( \text{EC}_{10} \) GABA-induced currents in \( \alpha_1\beta_2\gamma_2\)S or \( \alpha_1\beta_2\gamma_2\)S mutant with substituted aromatic group receptors are less enhanced by co-application of etomidate (5 \( \mu \)M) and alphaxalone (0.5 \( \mu \)M). The statistics panels of \( \text{EC}_{10} \) GABA modulation by \( \square \) ETO or alphaxalone (\( \square \) Alpha) are shown as the enhancement fold in b. Individual recordings are from oocytes injected with cRNAs encoding the indicated subunit combination. GW means, for example, \( \alpha_1\beta_2\gamma_2\)S receptor combination.

**Direct Activation Curve of GABAR by PB or Etomidate**—Further examination of the direct activation by PB revealed that the \( \alpha_1\beta_2\gamma_2\)S or \( \alpha_1\beta_2\gamma_2\)S mutant receptor has a 3–5-fold higher apparent affinity (from 140 to 30 and 51 \( \mu \)M, for G219F and G219W, respectively) than the wild type \( \alpha_1\beta_2\gamma_2\)S receptor, as shown by the leftward shift in the concentration-response relationship for direct activation by PB (Fig. 7b). The GABA concentration-response curve for the \( \alpha_1\beta_2\gamma_2\)S or \( \alpha_1\beta_2\gamma_2\)S mutant receptor is shifted to the left in a similar manner. PB (1 \( \mu \)M) activated the \( \alpha_1\beta_2\gamma_2\)S or \( \alpha_1\beta_2\gamma_2\)S mutant receptor directly but elicited significantly smaller maximal currents compared with the wild type \( \alpha_1\beta_2\gamma_2\)S receptors (\( p < 0.05 \) compared with wild...
type; Table II). As with PB, etomidate has an increased apparent affinity in the /H9251//H9252//H92532(G219F) or /H9251//H9252//H92532(G219W)2S mutant receptor, as indicated by a leftward shift in the etomidate direct activation concentration-response curve (Fig. 7c). Direct activation by 50 μM etomidate of the /H9251//H9252//H92532(G219F)2S or /H9251//H9252//H92532(G219W)2S receptor was reduced slightly compared with the wild type /H9251//H9252//H92532S receptor (Table II). Although high concentrations of etomidate can produce channel inhibition and re-
bound currents at GABAR (49), these were not seen during our experiments at etomidate concentrations up to 100 μM. Similarly, the direct activation of GABAR by propofol and alphaxalone was more potent, however, with slightly reduced maximal response currents of both at 50 μM (Table II).

**DISCUSSION**

Little is known to date about the domains within GABAR that participate in the action of anesthetics. Mutation of certain amino acid residues within the M2 region reduced anesthetic sensitivity of GABAR (50), i.e. reduced enhancement of GABAR but not direct agonism (51).

Channel residues can affect the apparent EC₅₀ and binding of agonists and modulators by allosteric coupling (29, 40). Although the residue in M2 that forms a cavity with residues within M3 for the modulation of volatile anesthetics and α-alcohols (40) is the most implicated in anesthetic action to date, nevertheless, it may not be the anesthetic-binding site but merely conformationally coupled to anesthetic action. However, modeling of the α-helix of M2 suggests that the residue actually is not within the channel pore but may be on the opposite side of the helix, related to the rotation and movement in an activated state of channel opening (52) and thus in contact with lipids or other membrane-spanning domains of the same or other subunits that may represent a pocket, a site for attachment.

By using sequence scanning of ρ versus non-ρ, by the approach of mutating β2Gly-219 to Phe found in ρ subunit, we identified a point mutation in this region that is required for IV anesthetic modulation. Glycine and proline are conformationally important amino acids in that they appear to influence the conformation of the polypeptide (53). Glycine essentially lacks a side chain and therefore can adopt conformations that are sterically forbidden for other amino acids. This confers a high degree of local flexibility on the polypeptide. Accordingly, glycine residues are frequently found in turn regions of proteins where the backbone has to make a sharp turn. Glycine occurs abundantly in certain fibrous proteins due to its flexibility and because its small size allows adjacent polypeptide chains to pack together closely. These studies confirm our previous findings that mutation β2G219F in α1β2γ2S can ablate potentiation by the IV anesthetics (43), suggesting that Gly-219 of the β subunit is a key residue for anesthetic potentiation of GABAR.

We further characterized this site located at the entrance of M1 in the β2 subunit, as to whether it works as a modulation site or a binding site, by replacing Gly with residues of various sizes and hydrophobicity. GABA-induced currents of receptors mutated at β2Gly-219 were somewhat potentiated by IV general anesthetics at constant concentration, and the degree of potentiation was decreased in a manner correlated with the molecular volume of substituted amino acids (Fig. 6). By comparing the substitutions, the largest potentiation by anesthetics was observed with wild type α1β2γ2S, which showed the largest EC₅₀ value for GABA in the absence of modulator, whereas the smallest potentiation by anesthetics was seen in aromatic residues, β2 (G219F, G219Y, and G219W), which also showed the biggest left-shift for GABA EC₅₀ values. There was a strong negative correlation between the left-shift for GABA EC₅₀ value and the degree of anesthetic potentiation.

Similar results, i.e. a correlation between left-shift either for GABA or for anesthetics and for anesthetic modulation with volume of residue, were also found in mutation at the residues within M2 and M3, Ser-270 and Ala-291, of GABA α subunit and were interpreted as evidence for an actual site of contact with ligand (40, 54). The authors suggested (42, 54) that large amino acid residues already increased the probability of channel opening resulting in a correspondingly lower opportunity for additional enhancement by binding of an anesthetic ligand in the same space.

The correlation with the volume of the residue at position β2Gly-219 and IV anesthetic enhancement of GABAR observed here also is consistent with this residue being in the binding pocket for anesthetics. However, mutation at β2Gly-219 failed to prevent direct gating by the same IV anesthetics, and thus this residue is not in the binding site involved in this action of the ligands. The two actions of the IV anesthetics could involve two separate sites of binding, although several models suggest a single binding site could mediate both (55). Whether this position is or is not a contact point for anesthetics needs further investigation, e.g. on ligand “cut-off” analysis (42) or using irreversible cysteine-modifying reagents to covalently label this site by selectively mutating to β2G219C (56, 57).

The observation that the size of residue at β2Gly-219 affects the apparent affinity (left-shift) for direct gating by both GABA agonists and IV anesthetics suggests increased probability of opening, a finding more consistent with involvement of this residue in an allosteric coupling domain rather than an anesthetic contact site. This possibility will require examination of channel kinetics using a system other than oocyte expression. Furthermore, a report (30) using cryo-electron microscopy to examine the structure of the nAChR, which like GABAR is a member of the LGIC family, showed features of possible relevance to these results on GABAR β2Gly-219 position. Whereas M2 forms the channel pore wall, M1, M3, and M4 are bundled together near M2. Hydrophobic residues near Gly-219 at the extracellular end of M1 were found to come in contact with the back side of M2 and thus potentially interact. Thus the latest structural information supports the importance of the top of M1 including Gly-219 in possible interactions with the pore-lining M2 and an effect on channel gating plus modulation by allosteric ligands.

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