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A Single Glycine Residue at the Entrance to the First Membrane-Spanning Domain of the γ-Aminobutyric Acid Type A Receptor β_2 Subunit Affects Allosteric Sensitivity to GABA and Anesthetics

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ABSTRACT

Site-directed mutagenesis of the γ-aminobutyric acid type A ($GABA_A$) receptor β_2 subunit has demonstrated that conversion of a conserved glycine residue located at the entrance to the first transmembrane domain into the homologous ρ_1 residue phenylalanine alters the modulating effects of four different i.v. anesthetics: pentobarbital, alphaxalone, etomidate, and propofol. Using the baculovirus expression system in *Spodoptera frugiperda* 9 cells, anesthetic-induced enhancement of [^{3}H]muscimol and [^{3}H]flunitrazepam binding in receptors containing the β_2(G219F) point mutation displayed a significantly reduced efficacy in modulation by all four i.v. anesthetics tested. Furthermore, $GABA_A$ receptors containing the α_1(G223F) point mutation also significantly decreased the maximal effect of etomidate- and propofol-induced enhancement of ligand binding. Conversely, the homologous point mutation in ρ_1 receptors (F261G) changed the i.v. anesthetic-insensitive receptor to confer anesthetic modulation of [^{3}H]muscimol binding. Consistent with the binding, functional analysis of pentobarbital-enhanced GABA currents recorded with whole-cell patch clamp demonstrated the β_2(G219F) subunit mutation eliminated the potentiating effect of the anesthetic. Similarly, propofol-enhanced GABA currents were potentiated less in $\alpha_1\beta_2$(G219F)γ_2 receptors than in $\alpha_1\beta_2\gamma_2$ receptors. Although ligand binding displayed comparable K_D values for muscimol among wild-type, $\alpha_1\beta_2\gamma_2$, and mutant receptors, patch-clamp recordings showed that $\alpha_1\beta_2$(G219F)γ_2 receptors had a significantly more potent response to GABA than did $\alpha_1\beta_2\gamma_2$ or α_1(G223F)$\beta_2\gamma_2$. The $\alpha_1\beta_2$(G219F)γ_2 receptors also were more sensitive to direct channel activation by pentobarbital and propofol in the absence of GABA. These results suggest that the first transmembrane glycine residue on the β_2 subunit may be important for conformational or allosteric interactions of channel gating by both GABA and anesthetics.

The γ-aminobutyric acid type A ($GABA_A$) receptor has been shown to be a target of numerous depressant drugs, including benzodiazepines and general anesthetics (for review, see Carlson et al., 1997). At clinically relevant concentrations, all general anesthetics except ketamine enhance $GABA_A$ receptor function in a reversible and stereospecific manner (Hales and Olsen, 1994). These findings suggest that the depressant behavioral effects of anesthetics are closely related to their actions on $GABA_A$ receptors. The receptor domains pertinent for the actions of general anesthetics, however, have yet to be fully elucidated.

The $GABA_A$ receptor is a ligand-gated Cl^- ion channel that belongs to a family of subunits (α_{1-6}, β_{1-4}, γ_{1-3}, δ, ϵ, and ρ_{1-3}, in the mammalian central nervous system) that forms pentameric complexes (Amin and Weiss, 1996; Chang et al., 1996; Davies et al., 1997a; Tretter et al., 1997). Beside the ρ-homomeric channels that are located primarily in the retina (Cutting et al., 1991), the proposed stoichiometry of native $GABA_A$ receptors in the brain is believed to contain two α subunits, two β subunits, and one γ or one δ, or one ϵ subunit (Chang et al., 1996; Davies et al., 1997a; Tretter et al., 1997). The positive modulating actions of benzodiazepines on $GABA_A$ receptors have been determined to depend on specific amino acids on both the α and the γ subunits (Pritchett and Seeburg, 1991; Wieland et al., 1992; Buhr et al., 1996; Amin et al., 1997). Whereas the actions of general anesthetics appear not to depend on the presence of the γ subunit (Jones et al., 1995), the β subunit has been shown to play an important role in the allosteric modulation of $GABA_A$ receptors by i.v. anesthetics (Harris et al., 1995; Zezula et al.,

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ABBREVIATIONS: $GABA_A$, γ-aminobutyric acid type A; Sf9, *Spodoptera frugiperda* 9; AcNPV, *Autographa californica* nuclear polyhedrosis virus; TM, transmembrane; ABSS, artificial balanced salt solution; DMSO, dimethyl sulfoxide.

1996). To date, only the ϵ (in certain expression systems) and ρ subunits have been shown to confer insensitivity to i.v. anesthetics (Mihic and Harris, 1996; Davies et al., 1997a). With respect to the ρ_1 subunit, its anesthetic-distinct pharmacology has promoted the identification of specific residues in the transmembrane (TM) regions, specifically TM2 and TM3 of the glycine and $GABA_A$ receptors, that harbor sites necessary for the positive allosteric modulation and direct activation induced by volatile and i.v. anesthetics (Belelli et al., 1997; Mihic et al., 1997; Moody et al., 1997; Amin, 1999).

To date, the body of evidence identifying structural determinants for anesthetic action on the $GABA_A$ receptor has focused solely in the TM2 and TM3 regions. In this study, the emphasis was placed on amino acids from other than the TM2 and TM3 regions, one at the entrance of TM1 and the others in the extracellular region between TM2 and TM3 (Fig. 1). Except for the amino acids indicated, Fig. 1 illustrates that these two areas of interest reflect high homology between anesthetic-sensitive proteins and the anesthetic-insensitive ρ_1 subunit. Furthermore, the recent observation that lipid-water interfaces of membrane ion channels may be important sites of action for anesthetics supports the investigative interest in amino acids located near lipid-water interfaces as shown in Fig. 1 (Xu et al., 1998). With site-directed mutagenesis, a phenylalanine residue located at the entrance to TM1 (position 261, human ρ_1 subunit) has been transformed to a glycine, which is the homologous amino acid on α_{1-6}, β_{1-3}, γ_{1-3}, ϵ, and δ $GABA_A$ receptor subunits and on the α_1 subunit of the glycine receptor. The reciprocal point mutations were also made in the α_1 and β_2 subunits, G223F and G219F, respectively, to test the hypothesis that sensitivity to i.v. anesthetics would be diminished in $GABA_A$ receptors on conversion of the conserved glycine to the ρ_1-residue phenylalanine. Anesthetic-induced enhancement of [^{3}H]muscimol and [^{3}H]flunitrazepam binding has shown that $GABA_A$ receptors containing the β_2(G219F) mutation displayed a reduced efficacy in anesthetic modulation by all four of the i.v. anesthetics tested. Consistent with the binding data, functional analysis of these mutant receptors with whole-cell patch clamp demonstrated that enhancement of GABA currents by pentobarbital and propofol was also hindered in the presence of the β_2(G219F) point mutation. On the contrary, the four amino acids in the TM2/TM3 bridge were determined not to be essential for anesthetic modulation. This study identifies a new region of TM1 involved in channel gating and anesthetic modulation.

Materials and Methods

Site-Directed Mutagenesis and Generation of Recombinant Baculoviruses. The mutations were introduced into the cDNAs of the $GABA_A$ receptors subunits with the Altered Sites II in vitro mutagenesis systems (Promega, Madison, WI). Briefly, the entire coding region of the human ρ_1 subunit was subcloned into pAlter plasmid (the same was performed for the rat α_1 and β_2 subunits), and a mutagenic oligonucleotide was used to incorporate the desired mutation according to the manufacturer's suggestions. The oligonucleotides used were: ρ_1 NASM(311–314)-RNSL, 5′-GTCCACCATCATCACGGGCGTGAGAAACTCCCTGCCGCGCGTCTCCTACATC-3′; ρ_1(F261G), 5′-TTGCGTCGCCACATCGGCTTCTTCTTGCTCCAA-3′; α_1(G223F), 5′-TTGAATAACAAAGTAGAAGATTTTTCTCTTCAA-3′; and β_2(G219F), 5′-CAGGATGAAGTAGAAAATGTTTCTTTTCAG-3′.

Successful mutagenesis was verified by DNA sequencing. The point-mutated $GABA_A$ receptor subunits as well as the ρ_1 wild type were subcloned into the appropriate baculovirus transfer vectors pFastBac [β_2(G219F), ρ_1, ρ_1(F261G), ρ_1(NASM-RNSL); Life Technologies, Rockville, MD] or pAcSG2 [α_1(G223F); PharMingen, San Diego, CA] for generation of recombinant baculovirus with either the BAC-BAC expression system (Life Technologies) or BaculoGold transfection kit (PharMingen), respectively.

A

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                                TM1
                                ===============
  rat α1        216   FHLKRKIGYFVIQTYLPCIMTV   237
  rat α2        216   FHLKRKIGYFVIQTYLPCIMTV   237
  rat α3        241   FHLKRKIGYFVIQTYLPCIMTV   262
  rat α4        215   FHLRRKMGYFMIQTYIPCIMTV   236
  rat α5        220   FHLKRKIGYFVIQTYLPCIMTV   241
  rat α6        215   FHLQRKMGYFMIQIYTPCIMTV   236
  rat β1        212   FRLKRNIGYFILQTYMPSTLIT   233
  rat β2        212   FKLKRNIGYFILQTYMPSILIT   233
  rat β3        212   FRLKRNIGYFILQTYMPSIMIT   233
  rat γ1        229   FDLSRRMGYFTIQTYIPCILTV   250
  rat γ2        227   FDLSRRMGYFTIQTYIPCTLIV   248
  rat γ3        230   FELSRRMGYFTIQTYIPCILTV   251
  rat δ         225   FQLRRNRGVYIIQSYMPSVLLV   246
human ε         271   FNVSRRFGYVAFQNYVPSSVTT   292
  rat glα1      214   FHLERQMGYYLIQMYIPSLLIV   235
                      * . *. #.. .* * *.   .

human ρ1        254   FTLRRHIFFFLLQTYFPATLMV   275
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B

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                TM2                    TM3
                ====                   ====
  rat α1   272        ARNSLPKVAYAT       283
  rat α2   272        ARNSLPKVAYAT       283
  rat α3   297        ARNSLPKVAYAT       308
  rat α4   271        ARHSLPKVSYAT       282
  rat α5   276        ARNSLPKVAYAT       287
  rat α6   271        ARHSLPKVSYAT       282
  rat β1   268        LRETLPKIPYVK       279
  rat β2   268        LRETLPKIPYVK       279
  rat β3   268        LRETLPKIPYVK       279
  rat γ1   285        ARKSLPKVSYVT       296
  rat γ2   283        ARKSLPKVSSVT       294
  rat γ3   286        ARKSLPRVSYVT       297
  rat δ    281        ARSSLPRASAIK       292
human ε    327        SRKNFPRVSYIT       338
rat glα1   270        SRASLPKVSYVK       281
                      .# ..* .  ..

human ρ1   310        VNASMPRVSYIK       321
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Fig. 1. Amino acid sequence alignment of the pre-TM1/TM1 (A) and the inter-TM2/TM3 (B) domains for mammalian $GABA_A$ and ρ_1 receptor subunits and the glycine receptor α_1 subunit (glα_1). A, this alignment shows that the pre-TM1 glycine (**G**) is conserved in all of the subunits aligned except the ρ_1 (#), which has a phenylalanine (***F***). In this domain of 22 residues, there are five amino acids that are conserved in all the subunits listed including ρ_1 (*) and seven amino acids that are conserved within subunit families (●). B, this sequence of amino acids represents the extracellular region that bridges the TM2 and TM3 domains. Within a subunit family, there are six amino acids that are conserved. One amino acid, a proline (P), is conserved among all the subunits listed (*); # indicates the arginine (R), which is present in all of the subunits, except ρ_1, which has an asparagine (N). Bolded residues are highly conserved within a subunit family and are the targeted four amino acids in the ρ_1 subunit (bolded and underlined) that have been mutated to the $GABA_A$ receptor α_1 subunit sequence (RNSL). In both A and B, bolded residues represent potentially relevant sites of action for i.v. anesthetics. Note that the glycine receptor α_1 subunit is primarily insensitive to most i.v. anesthetics, except for propofol (Hales and Lambert, 1991). The source for all sequences was GenBank, apart from the glycine receptor α_1 subunit (Grenningloh et al., 1990), $GABA_A$ receptor ϵ subunit (Davies et al., 1997a), and ρ_1 subunit (Cutting et al., 1991).

Cell Culture and Baculovirus Infection. *Spodoptera frugiperda* 9 (Sf9) cells (Life Technologies, Paisley, Scotland) were grown in serum-free medium (Sf900 II medium; Life Technologies) as a shaking culture (140 rpm) at 27°C. At a density of 2×10^6 cells/ml, Sf9 cells were infected with a single or various combination(s) of the following recombinant *Autographa californica* nuclear polyhedrosis viruses (AcNPV), coding for wild-type and point-mutant $GABA_A$ receptors: AcNPV-α_1, AcNPV-α_1(G223F), AcNPV-β_2, AcNPV-β_2(G219F), AcNPV-γ_2, AcNPV-ρ_1, AcNPV-ρ_1(F261G), and AcNPV-ρ_1(NASM-RNSL). The amount of recombinant baculovirus added for infection was determined by maintaining a cumulative multiplicity of infection ≤15. Titers of recombinant baculovirus ranged from 8×10^7 to 8×10^8 plaque-forming units/ml. Virus titer was determined with a plaque assay according to protocol from the Life Technologies Instruction Manual, "Guide to Baculovirus Expression Vector Systems (BEVS) and Insect Cell Culture Techniques." The wild-type viruses AcNPVα_1, AcNPVβ_2, and AcNPVγ_2 were gifts from Dr. D. Gallager (Neurogen, Branford, CT).

Sf9 Cell Membrane Preparation. At 65 h after infection, Sf9 cells were harvested by centrifugation at 1500*g* for 5 min. The pelleted cells were subsequently resuspended in 20 mM KH_2PO_4/K_2HPO_4 and 50 mM KCl (pH 7.4) buffer and pelleted by centrifugation at 1500*g* for 5 min. The buffer was aspirated, and the pellet was homogenized in 10 mM KH_2PO_4/K_2HPO_4, 100 mM KCl (pH 7.4) binding buffer, using an Ultra-Turrax T-25 homogenizer (Janke & Kunkel, Staufen, Germany) at 12,000 rpm for 20 s. The homogenate was centrifuged for 20 min at 20,000*g* at 4°C. This washing/centrifugation procedure was repeated twice. Sf9 membrane pellets were stored at −80°C until use. Before binding, the pellet was resuspended in 10 mM KH_2PO_4/K_2HPO_4, 100 mM KCl binding buffer, using an Ultra-Turrax T-25 homogenizer (Janke & Kunkel) at 12,000 rpm for 30 s.

Binding Assays. Binding of [^{3}H]muscimol (19.1–20.0 Ci/mmol; New England Nuclear, Boston, MA) or [^{3}H]flunitrazepam (84.5 Ci/mmol; New England Nuclear) was determined in a total volume of 0.25 ml consisting of 0.15 ml of Sf9 membrane-bound proteins (300–800 mg/ml), 0.075 ml of binding buffer, and 0.025 ml of radioactive ligand. Nonspecific binding was measured by adding GABA (final concentration, 100 μM) or diazepam (final concentration, 10 μM) in the presence of radioactive ligand. Anesthetic-induced enhancement of radioligand binding was determined by adding each anesthetic solution in the presence of radioactive ligand. Anesthetic concentration-response assays were performed with either 3 nM [^{3}H]muscimol for $\alpha\beta$-containing Sf9 membranes or 40 nM [^{3}H]muscimol for ρ-containing membranes. For assessing anesthetic-modulated benzodiazepine binding in $\alpha\beta\gamma$-containing Sf9 membranes, a final concentration of 1 nM [^{3}H]flunitrazepam was used. All concentrations used to study anesthetic modulation were below the K_D values calculated from competition assays.

Competition binding assays were performed with either 10 nM [^{3}H]muscimol for $\alpha\beta$-containing Sf9 membranes or 40 nM [^{3}H]muscimol for $\alpha\beta\gamma$- and ρ-containing membranes. For assessing competition curves of benzodiazepine binding in $\alpha\beta\gamma$-containing Sf9 membranes, a final concentration of 4 nM [^{3}H]flunitrazepam was used. All K_I values were calculated from the EC_{50} value using the Cheng-Prusoff equation (GraphPad Software, San Diego, CA).

Specific binding for both concentration-response and competition curves was defined as the difference between total binding (i.e., binding in the absence of anesthetic agent and/or cold ligand) and nonspecific binding. Radioligand incubations were performed on ice at 30 or 60 min for [^{3}H]muscimol or [^{3}H]flunitrazepam binding, respectively, and terminated by vacuum filtration over GF/B glass fiber filters (Whatman, Maidstone, England). The filters were washed three times with 4 ml of cold binding buffer and counted for radioactivity by liquid scintillation (Packard 1900 TR, 55% efficiency; Packard Instrument Co., Inc., Meriden, CT). The amount of protein in the membranes was determined by the use of Cu^{2+} and bicinchoninic acid (Pierce, Rockford, IL).

Experimental Design for Binding Assays. Ten concentrations of an anesthetic or cold ligand were used to construct the concentration-response curves for determining EC_{50} and E_{max} values or competition curves for determining K_I values, respectively. For each curve, total radioligand binding in the absence of anesthetic or cold ligand (control), total radioligand binding in the presence of the anesthetic (concentration-response curves) or cold ligand (competition curves), and nonspecific binding were measured in triplicate. Control, anesthetic-treated (or cold ligand-treated), and nonspecific binding groups were assayed in the same experiment, for a total of 36 tubes per receptor combination. Each anesthetic assay of 36 tubes was repeated three or four times for each of the 11 different receptor combinations. Competition assays were repeated at least twice for each of the receptor combinations.

Electrophysiology. Recordings were made from Sf9 cells that had been incubated with virus for 27 to 29 h. A Petri dish containing the cells was transferred to the recording chamber on the stage of a Zeiss (Oberkochen, Germany) Axiovert-10 inverted phase-contrast microscope, where the individual cells were viewed at 200× magnification. The recording chamber contained 2 to 3 ml of artificial balanced salt solution (ABSS) that was renewed by constant perfusion at 0.5 ml/min^{-1} at room temperature (20–22°C). The composition of ABSS was 162.5 mM NaCl, 3.5 mM KCl, 1.25 mM Na_2HPO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.35, at 22°C. Standard patch clamp techniques (Hamill et al., 1981) were used to record from the infected cells in the whole-cell configuration with an EPC-9 amplifier (HEKA Electronik, Lambrecht, Germany). The patch electrodes were manufactured from 1.5-mm o.d. glass (World Precision Instruments, Sarasota, FL). The patch electrodes were pulled just before use with a BB-CH-PC microelectrode puller (Mecanex, Nyon, Switzerland) and were filled with a solution containing 160 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 2 mM Mg-ATP, and 10 mM HEPES, pH 7.3, at 20°C. Electrodes had resistances of 2 to 5 MΩ. A holding potential of −40 mV was used. Series resistance was 60% compensated. Whole-cell currents were plotted on a low-fidelity chart recorder during the experiment. The signals were stored on computer and also recorded on a video recorder with a VR-100 digital data recorder (Instrutech Corporation, Elmont, NY). Results were analyzed with Pulse (HEKA) and Igor Pro (Wavemetrics, Lake Oswego, OR) software.

Stock solutions of the drugs were prepared by dissolving them in distilled water or dimethyl sulfoxide (DMSO) to give a concentration at least 100× greater or 1000× greater, respectively, than that required for perfusion and premixed by diluting solutions in ABSS. The solutions were applied in the vicinity (~100 μm) of the recorded cell from a multibarrelled perfusion pipette constructed from seven hypodermic needles (Kristiansen and Lambert, 1996). Between drug applications, the infected cell was superfused with normal ABSS from one of the barrels. GABA (or high concentrations of anesthetic in the absence of GABA) was applied for 5 s every minute. When anesthetic was used as a modulator, it was applied together with GABA (as a premixed solution) and in some experiments also for 10 s immediately before the combined application. When diazepam was used as a modulator, it was applied for only 15 s immediately before, but not concurrent with, GABA. Before each modulation experiment, a constant response level was established for GABA. The modulated responses were followed by a series of GABA applications that was continued until a stable level was reached (1–2 min.). Results were used only if this level was within ±15% of the original GABA response level. Responses were quantified by measuring the peak current during application of agonist and the current remaining after 5 s of application. For low GABA concentrations, the current reached a plateau that was maintained throughout the application. For higher GABA concentrations, the current rose quickly to a peak and faded while GABA was still applied.

Data Analysis. Curve fitting via nonlinear regression analyses of binding data was used to determine EC_{50}, E_{max}, and K_I values (GraphPad Software). Statistical analyses of EC_{50}, E_{max}, and K_I

values from the binding data were performed using one-way ANOVA, evaluated at a criterion of $P < .01$. Pairwise multiple comparisons, using the Tukey test, were calculated from the mean and S.E. values generated from one-way ANOVA and evaluated at $P < .05$. Additional pairwise comparisons were determined with Student's t test. Analysis of the electrophysiological data was performed with either Kruskal-Wallis ANOVA or Mann-Whitney test, with follow-up comparisons with Dunn's test. All statistical analyses were conducted with Jandel Statistical Software (SigmaStat version 2; San Rafael, CA).

Drugs. Stock solutions of GABA (10 mM; Riedel-deHaen, Seelze, Germany) and diazepam (1 mM; Sigma, St. Louis, MO) were diluted into binding buffer daily before use. The i.v. anesthetics used in this study were sodium pentobarbital (Sigma), alphaxalone (5α-pregnan-3α-ol-11,20-dione; Sigma), etomidate (org 24242; gift from Dr. Niall Hamilton, Akzo Nobel, Organon Labs Ltd., Lanarckshire, UK), and propofol (diprivan, 1% 2,6-diisopropylphenol in soy and animal lecithin as an aqueous emulsion, Stuart Pharmaceuticals, Inc., Wilmington, DE, or 2,6-diisopropylphenol, Tocris, Bristol, UK). Pentobarbital stock solutions were prepared in 50 mM Tris base, 120 mM NaCl, 5 mM KCl, pH 10. Alphaxalone and etomidate stock solutions were prepared in DMSO (Riedel-deHaen), and the maximum final concentration of DMSO was 0.1% (v/v), which was determined not to interfere with [^{3}H]muscimol or [^{3}H]flunitrazepam binding. All i.v. anesthetics were dissolved daily in binding buffer immediately before the experiment. With regard to the intralipid version of propofol in this study, the potentiating effects of propofol in this formulation has been shown not to differ from propofol made from an ethanol stock solution (Hales and Lambert, 1991).

Results

Characterization of Wild-Type and Mutant $GABA_A$ Receptors Expressed in Sf9 Cells. For the purposes of this study, the $\alpha_1\beta_2\gamma_2$ receptor is also identified by the IUPHAR nomenclature name A1a2 (Barnard et al., 1998). $GABA_A$ receptors containing the β_2(G219F) subunit demonstrated a slightly higher affinity for [^{3}H]muscimol binding. The rank order for [^{3}H]muscimol affinity was $\alpha_1\beta_2$(G219F), α_1(G223F)β_2(G219F), $\alpha_1\beta_2$, and α_1(G223F)β_2 (K_I, 5.9 ± 1.83, 6.6 ± 2.26, 8.1 ± 0.71, and 8.8 ± 2.47 nM ± S.D., respectively). For $\alpha\beta\gamma$ receptors, the rank order for [^{3}H]muscimol affinity was different from that for the $\alpha\beta$ receptors: α_1(G223F)β_2(G219F)γ_2, $\alpha_1\beta_2\gamma_2$, $\alpha_1\beta_2$(G219F)γ_2, and α_1(G223F)$\beta_2\gamma_2$ (K_I, 10.4 ± 0.85, 13.1 ± 2.68, 21.0 ± 1.4, and 29.3 ± 0.92 nM ± S.D., respectively). For the ρ_1 receptors, wild type, ρ_1(F261G), and ρ_1(NASM-RNSL) showed similar K_I values in muscimol competition assays (78.0 ± 4.2, 70.0 ± 7.1, and 71.0 ± 12.7 nM ± S.D., respectively). Flunitrazepam affinity was assessed by nonradioactive competition assays, and these assays showed comparable K_I values between the wild-type and mutant $\alpha\beta\gamma$ receptor combinations [K_I (nM ± S.D.) = $\alpha_1\beta_2\gamma_2$, 2.2 ± 0.14; α_1(G223F)$\beta_2\gamma_2$, 2.25 ± 0.21; $\alpha_1\beta_2$(G219F)γ_2, 2.5 ± 0.0 nM; and α_1(G223F)β_2(G219F)γ_2, 2.3 ± 0.14 nM]. One-way ANOVA on ranks determined that all the K_I values within each group tested, except the K_I values for [^{3}H]muscimol between α_1(G223F)β_2(G219F)γ_2 and α_1(G223F)$\beta_2\gamma_2$, were not significantly statistically different from each other ($P > .05$).

Mutation of TM1 Glycine on the β_2 Subunit Alters Pentobarbital-Induced Modulation of Ligand Binding. $GABA_A$ receptors containing the β_2(G219F) subunit displayed a decreased maximal effect in pentobarbital-induced potentiation of [^{3}H]flunitrazepam and [^{3}H]muscimol binding (Fig. 2; Table 1). The double-mutant receptors (i.e., α_1(G223F)β_2(G219F)γ_2) demonstrated an intermediate pentobarbital-induced E_{max} compared with the $GABA_A$ receptors containing the single point mutations. In addition, pentobarbital-induced enhancement of [^{3}H]flunitrazepam binding was statistically greater with the α_1(G223F)$\beta_2\gamma_2$ than with the wild-type $\alpha_1\beta_2\gamma_2$ ($P < .05$; Fig. 2). Although there was no difference in the potency of pentobarbital among the $\alpha\beta\gamma$ receptors (Fig. 2), pentobarbital was significantly more potent in the mutant $\alpha\beta$ receptors than in the wild-type $\alpha_1\beta_2$ receptors ($P < .05$; Table 1).

The Effect of the β_2 Mutant on Alphaxalone-Induced Modulation of Ligand Binding Is Altered in the Presence of the γ_2 Subunit. Compared with $\alpha_1\beta_2\gamma_2$, $GABA_A$ receptors containing the β_2(G219F) mutant subunit did not demonstrate any significant changes in the maximal effect induced by alphaxalone ($P > .05$; Fig. 3). Furthermore, alphaxalone was equipotent for all $\alpha\beta\gamma$ receptor combinations (Fig. 3). In the $\alpha\beta$ combinations, however, alphaxalone induced negative modulation of [^{3}H]muscimol binding in the $\alpha_1\beta_2$(G219F) receptor, and alphaxalone was more potent in the mutant $\alpha\beta$ receptors than in the wild-type $\alpha_1\beta_2$ receptors ($P < .05$; Table 1).

Mutation of TM1 Glycine on the α_1 and β_2 Subunits Alters Etomidate-Induced Modulation of Ligand Binding. $GABA_A$ receptors containing the α_1(G223F) and/or the β_2(G219F) subunit(s) displayed a significant decrease in the maximal effect of etomidate-induced potentiation of [^{3}H]flunitrazepam and [^{3}H]muscimol binding (Fig. 4; Table 1). The efficacies (i.e., E_{max}) of etomidate on α_1(G223F)$\beta_2\gamma_2$, $\alpha_1\beta_2$(G219F)γ_2, and α_1(G223F)β_2(G219F)γ_2 receptors were all approximately half of that measured with wild-type $\alpha_1\beta_2\gamma_2$ receptors (Fig. 4). For the $\alpha\beta\gamma$ receptors, etomidate was the most potent with the α_1(G223F)β_2(G219F)γ_2 combination and the least potent with the $\alpha_1\beta_2$(G219F)γ_2 receptor (Fig. 4). For the $\alpha\beta$ receptors, etomidate was significantly more potent at receptors containing the α_1(G223F) subunit

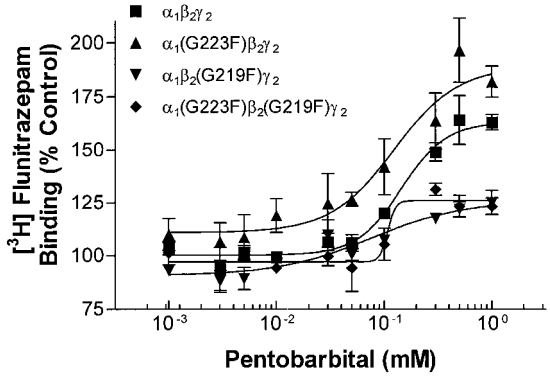

Fig. 2. Concentration-response curves for pentobarbital-induced modulation of ligand binding in wild-type and mutant $GABA_A$ receptors expressed in Sf9 cells. All curves represent the percentage change of specific [^{3}H]flunitrazepam binding (1 nM) in the presence of increasing concentrations of pentobarbital ($n = 3$ experiments performed in triplicate for each $\alpha\beta\gamma$ receptor combination). One-way ANOVA ($P < .001$) determined that there were significant differences in the E_{max} values between the $\alpha\beta\gamma$ receptors. Both $\alpha_1\beta_2$(G219F)γ_2 (▼, 24.7 ± 3.93) and α_1(G223F)β_2(G219F)γ_2 (◆, 28.7 ± 0.67) E_{max} values were statistically less than $\alpha_1\beta_2\gamma_2$ (■, 62.7 ± 3.18) and α_1(G223F)$\beta_2\gamma_2$ (▲, 79.0 ± 4.93) E_{max} values (Tukey test, $P < .05$). The EC_{50} values for pentobarbital in the $\alpha\beta\gamma$ receptors were not statistically different from each other (one-way ANOVA, $P = .054$; $\alpha_1\beta_2\gamma_2$, ■, 120.3 ± 8.65 μM; α_1(G223F)$\beta_2\gamma_2$, ▲, 104.3 ± 5.18 μM; $\alpha_1\beta_2$(G219F)γ_2, ▼, 100.9 ± 6.09 μM; α_1(G223F)β_2(G219F)γ_2, ◆, 152.3 ± 20.51 μM). All values are mean ± S.E.

than the $\alpha_1\beta_2$ or $\alpha_1\beta_2$(G219F) receptors ($P < .05$; Table 1). The double-mutant receptor α_1(G223F)β_2(G219F) demonstrated an intermediate etomidate-induced enhancement of [^{3}H]muscimol binding compared with the α_1(G223F)β_2 and $\alpha_1\beta_2$(G219F) receptor combinations (Table 1).

Mutation of TM1 Glycine on the α_1 and β_2 Subunits Alters Propofol-Induced Modulation of Ligand Binding. Similarly to etomidate, $GABA_A$ receptors containing either or both point mutations, i.e., α_1(G223F) and/or β_2(G219F), demonstrated a diminished maximal effect of propofol-induced enhancement of [^{3}H]flunitrazepam and [^{3}H]muscimol binding (Fig. 5; Table 1). For the $\alpha\beta\gamma$ receptors, propofol was significantly less efficacious for single- and double-mutant $GABA_A$ receptors containing the β_2(G219F) subunit compared with the $\alpha_1\beta_2\gamma_2$ and α_1(G223F)$\beta_2\gamma_2$ receptor combinations ($P < .05$; Fig. 5). In addition, the presence of either or both point mutations in an $\alpha\beta\gamma$ receptor significantly increased the potency of propofol compared with the wild-type complex ($P < .05$; Fig. 5). With $\alpha\beta$ receptors, propofol was the least efficacious and least potent at the double-mutant receptor (Table 1). Although the $\alpha_1\beta_2$(G219F) receptor displayed a 4- to 5-fold decrease in the efficacy of propofol modulation, propofol had a higher potency for this receptor than the wild-type receptor $\alpha_1\beta_2$ ($P < .05$; Table 1).

TABLE 1
Anesthetic-induced modulation of [^{3}H]muscimol binding in wild-type and mutant $GABA_A$ receptors

Subunit Combination	EC_{50}	E_{max}
	μM	*% control*
Pentobarbital		
$\alpha_1\beta_2$	23.40 ± 2.62	52.20 ± 5.69
α_1(G223F)β_2	14.70 ± 2.14[a]	56.20 ± 6.55
$\alpha_1\beta_2$(G219F)	9.60 ± 1.77[a]	21.20 ± 4.99[a,b]
α_1(G223F)β_2(G219F)	5.60 ± 1.21[a,b]	41.80 ± 4.97
ρ_1	NR[g]	NR[g]
ρ_1(F261G)	306.30 ± 49.70[a,e,f]	−29.80 ± 6.51[a,e,f]
ρ_1(NASM-RNSL)	NR[g]	NR[g]
Alphaxalone		
$\alpha_1\beta_2$	0.44 ± 0.07	60.80 ± 4.13
α_1(G223F)β_2	0.17 ± 0.03[a]	71.80 ± 9.47
$\alpha_1\beta_2$(G219F)	0.12 ± 0.03[a]	−11.80 ± 1.49[a,b,d]
α_1(G223F)β_2(G219F)	0.08 ± 0.03[a]	35.50 ± 3.93[a,b]
ρ_1	NR[g]	NR[g]
ρ_1(F261G)	0.27 ± 0.11	−28.00 ± 3.70[a,b,d,e]
ρ_1(NASM-RNSL)	0.70 ± 0.19[e]	−15.0 ± 3.16[a,b,d]
Etomidate		
$\alpha_1\beta_2$	1.83 ± 0.13	300.30 ± 39.40
α_1(G223F)β_2	0.48 ± 0.04[a,c]	117.70 ± 6.17[a]
$\alpha_1\beta_2$(G219F)	2.92 ± 0.56	19.70 ± 5.70[a,b]
α_1(G223F)β_2(G219F)	0.94 ± 0.20[a,c]	59.30 ± 0.88[a]
ρ_1	NR[g]	NR[g]
ρ_1(F261G)	0.40 ± 0.15[a,c]	19.70 ± 1.45[a,b,e,f]
ρ_1(NASM-RNSL)	NR[g]	NR[g]
Propofol		
$\alpha_1\beta_2$	6.20 ± 0.85	166.30 ± 16.33
α_1(G223F)β_2	4.09 ± 0.99[d]	77.30 ± 1.20[a]
$\alpha_1\beta_2$(G219F)	1.61 ± 0.47[a,d]	35.30 ± 5.04[a,b]
α_1(G223F)β_2(G219F)	8.06 ± 1.00	17.30 ± 4.48[a,b]
ρ_1	NR[g]	NR[g]
ρ_1(F261G)	6.70 ± 0.49	21.30 ± 7.31[a,b]
ρ_1(NASM-RNSL)	NR[g]	NR[g]

From the curve fit of concentration-response data, values (n = 3–4 experiments performed in triplicate, mean ± S.E.) indicate the EC_{50} and E_{max} for modulation by each i.v. anesthetic. Control values were evaluated in the absence of anesthetic.
[a] $P < .05$ compared with $\alpha_1\beta_2$.
[b] $P < .05$ compared with α_1(G223F)β_2.
[c] $P < .05$ compared with $\alpha_1\beta_2$(G219F).
[d] $P < .05$ compared with α_1(G223F)β_2(G219F).
[e] $P < .05$ compared with ρ_1.
[f] $P < .05$ compared with ρ_1(NASM-RNSL).
[g] NR, tested with no statistically significant response, $P > .05$.

Point Mutations on ρ_1 receptors Alter Anesthetic Insensitivity. The TM1 mutation in ρ_1(F261G) receptors manifested a pentobarbital-induced and alphaxalone-induced inhibition of [^{3}H]muscimol binding (Table 1). This inhibition was found to be significantly different from wild-type $GABA_A$

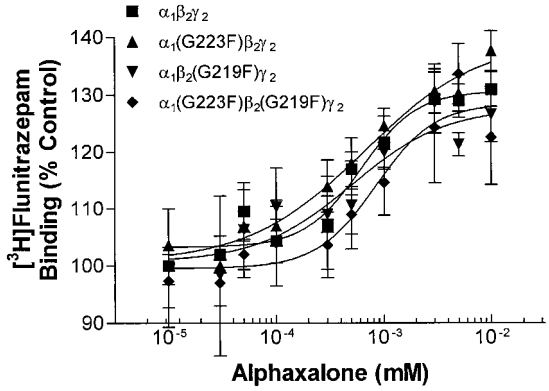

Fig. 3. Concentration-response curves for alphaxalone-induced modulation of ligand binding in wild-type and mutant $GABA_A$ receptors expressed in Sf9 cells. All curves represent the percentage change of specific [^{3}H]flunitrazepam binding (1 nM) in the presence of increasing concentrations of alphaxalone (n = 3 experiments performed in triplicate for each $\alpha\beta\gamma$ receptor combination). The rank order for E_{max} values of $\alpha\beta\gamma$ combinations is as follows: α_1(G223F)$\beta_2\gamma_2$, 37.7 ± 4.41 (▲); $\alpha_1\beta_2\gamma_2$, 34.3 ± 1.20 (■); α_1(G223F)β_2(G219F)γ_2, 30.0 ± 3.22 (♦); and $\alpha_1\beta_2$(G219F)γ_2, 22.7 ± 1.85 (▼). One-way ANOVA and follow-up Tukey test determined that there was a significant difference ($P = .033$ and $P < .05$, respectively) between the mean E_{max} value of $\alpha_1\beta_2$(G219F)γ_2 and the mean E_{max} value calculated from the α_1(G223F)$\beta_2\gamma_2$ curve. The EC_{50} values for alphaxalone in the $\alpha\beta\gamma$ receptors were not statistically different from each other (one-way ANOVA, $P = .80$; $\alpha_1\beta_2\gamma_2$, ■, 0.97 ± 0.37 μM; α_1(G223F)$\beta_2\gamma_2$, ▲, 0.67 ± 0.19 μM; $\alpha_1\beta_2$(G219F)γ_2, ▼, 0.76 ± 0.14 μM; α_1(G223F)β_2(G219F)γ_2, ♦, 0.83 ± 0.03 μM). All values are mean ± S.E.

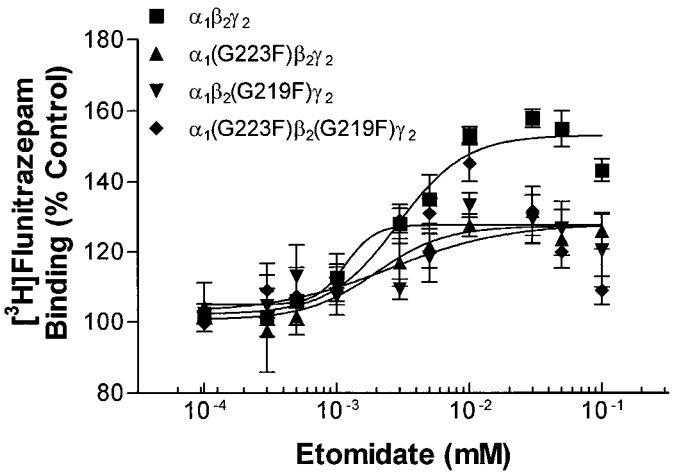

Fig. 4. Concentration-response curves for etomidate-induced modulation of ligand binding in wild-type and mutant $GABA_A$ receptors expressed in Sf9 cells. All curves represent the percentage change of [^{3}H]flunitrazepam binding (1 nM) in presence of increasing concentrations of etomidate (n = 3 experiments performed in triplicate for each $\alpha\beta\gamma$ receptor combination). One-way ANOVA and the follow-up Tukey test ($P < .001$ and $P < .05$, respectively) determined that there were significant differences between the E_{max} values of α_1(G223F)$\beta_2\gamma_2$ (▲, 22.7 ± 1.20), $\alpha_1\beta_2$(G219F)γ_2 (▼, 22.3 ± 1.20), and α_1(G223F)β_2(G219F)γ_2 (♦, 27.3 ± 2.91) and the E_{max} value of $\alpha_1\beta_2\gamma_2$ (■, 55.0 ± 1.53). One-way ANOVA ($P < .001$) determined that there were significant differences in the EC_{50} values between the $\alpha\beta\gamma$ receptors. Both $\alpha_1\beta_2$(G219F)γ_2 (▼, 4.63 ± 0.37 μM) and α_1(G223F)β_2(G219F)γ_2 (♦, 0.48 ± 0.22 μM) EC_{50} values were statistically different from each other as well as from $\alpha_1\beta_2\gamma_2$ (■, 2.69 ± 0.37 μM) and α_1(G223F)$\beta_2\gamma_2$ (▲, 2.00 ± 0.20 μM) EC_{50} values (Tukey test, $P < .05$). All values are mean ± S.E.

receptors (i.e., ρ_1, $\alpha_1\beta_2$, and $\alpha_1\beta_2\gamma_2$; $P < .05$). Pentobarbital was significantly less potent in modulating ρ_1(F261G) receptors than wild-type receptors $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_2$ ($P < .05$; Table 1); however, alphaxalone was equally potent in modulating ρ_1(F261G) receptors as seen with $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_2$ receptors. In addition to the ρ_1(F261G) mutant, ρ_1(NASM-RNSL) homomers demonstrated a significant alpaxalone-induced inhibition of [^{3}H]muscimol binding compared with ρ_1, $\alpha_1\beta_2$, and $\alpha_1\beta_2\gamma_2$ receptor combinations ($P < .05$; Table 1). The potency of alphaxalone in modulating ρ_1(NASM-RNSL) receptors also was not statistically different from $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_2$ receptors ($P > .05$). In the presence of alphaxalone, wild-type ρ_1 receptors did not show any statistically significant changes in specific [^{3}H]muscimol binding ($P > .05$; Table 1).

In contrast to pentobarbital and alphaxalone, ρ_1(F261G) receptors manifested etomidate- and propofol-induced enhancement of [^{3}H]muscimol binding. Etomidate and propofol were significantly more potent yet less effective in enhancing ligand binding in ρ_1(F261G) receptors compared with wild-type receptors $\alpha_1\beta_2$ and/or $\alpha_1\beta_2\gamma_2$ ($P < .05$; Table 1). Note that etomidate and propofol displayed a potency and efficacy at ρ_1(F261G) homomers that was not different from those determined for the double-mutant receptors α_1(G223F)β_2(G219F) and α_1(G223F)β_2(G219F)γ_2. In the presence of pentobarbital, etomidate, or propofol, ρ_1 and ρ_1(NASM-RNSL) receptors did not show any statistically significant modulation of specific [^{3}H]muscimol binding ($P > .05$; Table 1).

Functional Characterization of Wild-Type and Mutant Heteromeric and Homomeric $GABA_A$ Receptors. From the GABA concentration-response curves (Fig. 6B), mutation of the α_1 subunit (G223F) did not significantly affect the concentration-response relation for GABA-induced peak currents. The corresponding mutation in the β_2 subunit (G219F), however, significantly decreased the EC_{50} of the receptor for GABA ($P < .001$), suggesting an increase in affinity for GABA. The $\alpha_1\beta_2$(G219F)γ_2 combination had an EC_{50} comparable with the homomeric ρ_1 receptor (not significantly different). The Hill coefficients determined for the four subunit combinations were not significantly different. To confirm the presence of the γ_2 subunit in the receptor combinations tested, the effect of 1 μM diazepam on a GABA-induced current (20 μM GABA) was tested. The diazepam modulated peak current was 165 ± 27% greater than without applied benzodiazepine ($n = 3$, $\alpha_1\beta_2\gamma_2$). The effect of diazepam disappeared within 1 to 2 min. The $\alpha_1\beta_2$(G219F)γ_2 combination was also observed to be positively modulated by benzodiazepines (data not shown).

To estimate possible differences in desensitization between the wild-type and mutant receptors, the fading of the responses was calculated from the maximum GABA-induced responses [$\alpha_1\beta_2\gamma_2$, 2 mM GABA; α_1(G223F)$\beta_2\gamma_2$, 2 mM GABA; $\alpha_1\beta_2$(G219F)γ_2, 200 μM GABA; ρ_1, 2 mM GABA]. The amount of current remaining after 5 s of GABA application was expressed relative to the peak current (mean ± S.E.): $\alpha_1\beta_2\gamma_2$, 17.7 ± 2.3%, $n = 9$; α_1(G223F)$\beta_2\gamma_2$, 14.8 ± 1.8%, $n = 10$; $\alpha_1\beta_2$(G219F)γ_2, 11.0 ± 2.0%, $n = 16$. These values were not significantly different from each other, indicating that desensitization kinetics induced by GABA were not grossly affected by these point mutations. The GABA-induced currents from the nondesensitizing ρ_1 receptor showed only a small fade at high GABA concentrations, and after 5 s, the current remaining was 82.7 ± 1.7% (mean ± S.E., $n = 11$), which is probably because of a depletion of cell chloride content, resulting in a decrease in the electrochemical driving force during the response (refer to Fig. 6A, ρ_1-current tracing). This current remaining after 5 s from the ρ_1 receptor was significantly larger ($P < .001$) than that with the other three receptor combinations.

As for the ρ_1(F261G) mutation, these homomeric channels were tested ($n = 150$ Sf9 cells infected) with the application of 2 mM GABA, but none of the cells tested displayed a current. The high number of cells tested renders it highly likely that the ρ_1(F261G) subunit fails to express functional receptors in the cell membranes. Assuming a proportion of infection to be 10%, which is probably low considering that only one virus needs to infect the cells to produce a homomeric receptor, the possibility that all of the cells tested by chance were uninfected is 1.4×10^{-7} (i.e., 0.9^{150}). Therefore, it was concluded that either the receptors consisting of the mutant subunit ρ_1(F261G) do not reach the cell membrane or the receptors are in the membrane but are not functional.

Mutation of TM1 Glycine on β_2 Subunit Eliminates Pentobarbital-Induced Enhancement of GABA Currents. The effect of pentobarbital was tested both with and without a 10-s pretreatment of pentobarbital (Fig. 7). There was no statistically significant effect of pretreatment compared with no pretreatment (Fig. 7, filled columns). For the wild-type $\alpha_1\beta_2\gamma_2$ and α_1(G223F)$\beta_2\gamma_2$ combinations, pentobarbital concentrations of 10 and 50 μM resulted in statistically significant concentration-dependent increases in the GABA-induced peak current, compared with control (10 μM: $P < .05$ with or without pretreatment for both receptor combinations; 50 μM: $\alpha_1\beta_2\gamma_2$, $P < .01$ with and $P < .001$ without pretreatment; α_1(G223F)$\beta_2\gamma_2$, $P < .01$ with or without pretreatment; Kruskal-Wallis ANOVA and Dunn's tests). No significant

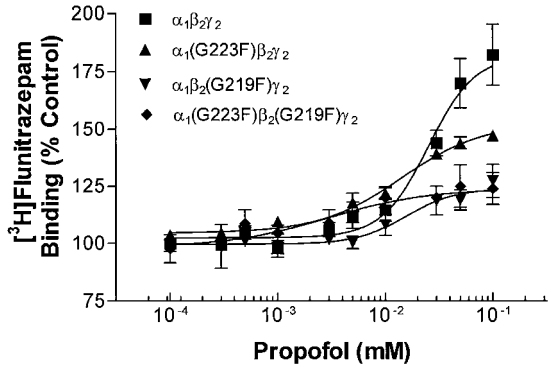

Fig. 5. Concentration-response curves for propofol-induced modulation of ligand binding in wild-type and mutant $GABA_A$ receptors expressed in Sf9 cells. All curves represent the percentage change of specific [^{3}H]flunitrazepam binding (1 nM) in the presence of increasing concentrations of propofol ($n = 3$ experiments performed in triplicate for each $\alpha\beta\gamma$ receptor combination). One-way ANOVA and the follow-up Tukey test ($P < .001$ and $P < .05$, respectively) determined that there were significant differences between the E_{max} values of α_1(G223F)$\beta_2\gamma_2$ (▲, 51.0 ± 5.20), $\alpha_1\beta_2$(G219F)γ_2 (▼, 21.0 ± 3.79), and α_1(G223F)β_2(G219F)γ_2 (◆, 22.0 ± 3.61) and the E_{max} value of $\alpha_1\beta_2\gamma_2$ (■, 78.7 ± 8.17). In addition, the E_{max} value for α_1(G223F)$\beta_2\gamma_2$ was evaluated to be significantly different from the E_{max} values of $\alpha_1\beta_2$(G219F)γ_2 and α_1(G223F)β_2(G219F)γ_2 ($P < .05$). One-way ANOVA and the follow-up Tukey test ($P < .001$ and $P < .05$, respectively) determined that there were significant differences in the EC_{50} values of α_1(G223F)$\beta_2\gamma_2$ (▲, 14.3 ± 2.38 μM), $\alpha_1\beta_2$(G219F)γ_2 (▼, 13.0 ± 1.95 μM), and α_1(G223F)β_2(G219F)γ_2 (◆, 6.8 ± 1.40 μM) compared with the EC_{50} value of $\alpha_1\beta_2\gamma_2$ (■, 26.9 ± 2.13 μM). All values are mean ± S.E.

differences were found between the wild-type receptor $\alpha_1\beta_2\gamma_2$ and the mutant α_1(G223F)$\beta_2\gamma_2$ at either 10 or 50 μM pentobarbital. For the $\alpha_1\beta_2$(G219F)γ_2 combination, only a low concentration of 5 μM was tested, because higher concentrations of pentobarbital gave rise to relatively large currents (refer to direct activation curves, Fig. 8), making it impossible to determine the modulating effect on GABA currents. In the presence of the β_2(G219F) mutant, 5 μM pentobarbital did not enhance GABA currents. In addition, the wild-type ρ_1 receptor was not modulated by 50 μM pentobarbital.

Mutation of TM1 Glycine on β_2 Subunit Diminishes Propofol-Induced Enhancement of GABA Currents. The effect of propofol was tested both with and without a 10-s pretreatment of propofol (Fig. 8). There was no statistically significant effect of pretreatment compared with no pretreatment (Fig. 8, solid columns). For the wild-type $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2$(G219F)γ_2 combinations, propofol concentrations of 1 and 5 μM resulted in statistically significant concentration-dependent increases in the GABA-induced peak current compared with control (1 μM: $P < .05$ with pretreatment for both receptor combinations; 5 μM: $P < .01$ with pretreatment for both receptor combinations). In all cases, however, the mean modulation was smaller for the $\alpha_1\beta_2$(G219F)γ_2 combination, and the modulating effect of 5 μM propofol (without pretreatment) was statistically significantly smaller for the $\alpha_1\beta_2$(G219F)γ_2 combination compared with the $\alpha_1\beta_2\gamma_2$ combination (Mann-Whitney, $P = .030$). Note that concentrations of 1 and 5 μM propofol did not elicit any direct activation of $GABA_A$ chloride currents in either receptor combination.

Mutation of TM1 Glycine on β_2 Subunit Alters Pentobarbital- and Propofol-Induced Direct Activation in the Absence of GABA. The $\alpha_1\beta_2$(G219F)γ_2 combination demonstrated a biphasic concentration-response curve and was significantly more sensitive than the $\alpha_1\beta_2\gamma_2$ combination to the direct effect of pentobarbital in the lower concentration range of 20 to 50 μM. No difference was detected between the direct effect of pentobarbital on the $\alpha_1\beta_2\gamma_2$ and the α_1(G223F)$\beta_2\gamma_2$ combinations (Fig. 9). The concentration-response relation for the α_1(G223F)$\beta_2\gamma_2$ combination could be fitted with a logistic equation of the form: $E = E_{max} \times [\text{pentobarbital}]^n/[EC_{50}{}^n + (\text{pentobarbital})^n]$, where n is the Hill coefficient (Fig. 9). The results for pentobarbital were as follows (95% CI in parentheses): E_{max} = 26% (20–32%), EC_{50} = 47 μM (22–73 μM), n = 1.68 (0.39–2.97). For the $\alpha_1\beta_2\gamma_2$ combination, a Hill coefficient could not be estimated because of the lack of points in the middle (increasing) range of the curve. Instead, using a Hill coefficient of the same value as estimated for the α_1(G223F)$\beta_2\gamma_2$ combination (n = 1.68), the corresponding E_{max} and EC_{50} values were estimated as follows (95% CI in parentheses): E_{max} = 38% (27–50%) and EC_{50} = 84 μM (24–145 μM). These values were not significantly different from the corresponding values for the α_1(G223F)$\beta_2\gamma_2$ combination. For the $\alpha_1\beta_2$(G219F)γ_2 combination, the concentration-response curve was clearly biphasic, and at 500 μM pentobarbital, the peak current increased significantly relative to 200 μM ($P < .001$). Because of the lack of an asymptote for the second phase of the curve, the data were impossible to fit into the logistic equation. Rather, for display purposes, a splined curve is shown in Fig. 9.

The fading of direct pentobarbital-induced currents was analyzed from the largest concentrations of pentobarbital used (i.e., 500 μM) with the wild-type $\alpha_1\beta_2\gamma_2$ receptor and the mutant receptors α_1(G223F)$\beta_2\gamma_2$ and $\alpha_1\beta_2$(G219F)γ_2. The percentages of the peak current remaining after 5 s of pentobarbital application were (mean ± SEM) $\alpha_1\beta_2\gamma_2$: 14.4% ± 6.1%, n = 6; α_1(G223F)$\beta_2\gamma_2$: 15.8% ± 3.7%, n = 4; and $\alpha_1\beta_2$(G219F)γ_2: 32.4% ± 5.8%, n = 13. Because of the variance in elicited pentobarbital direct channel activation for

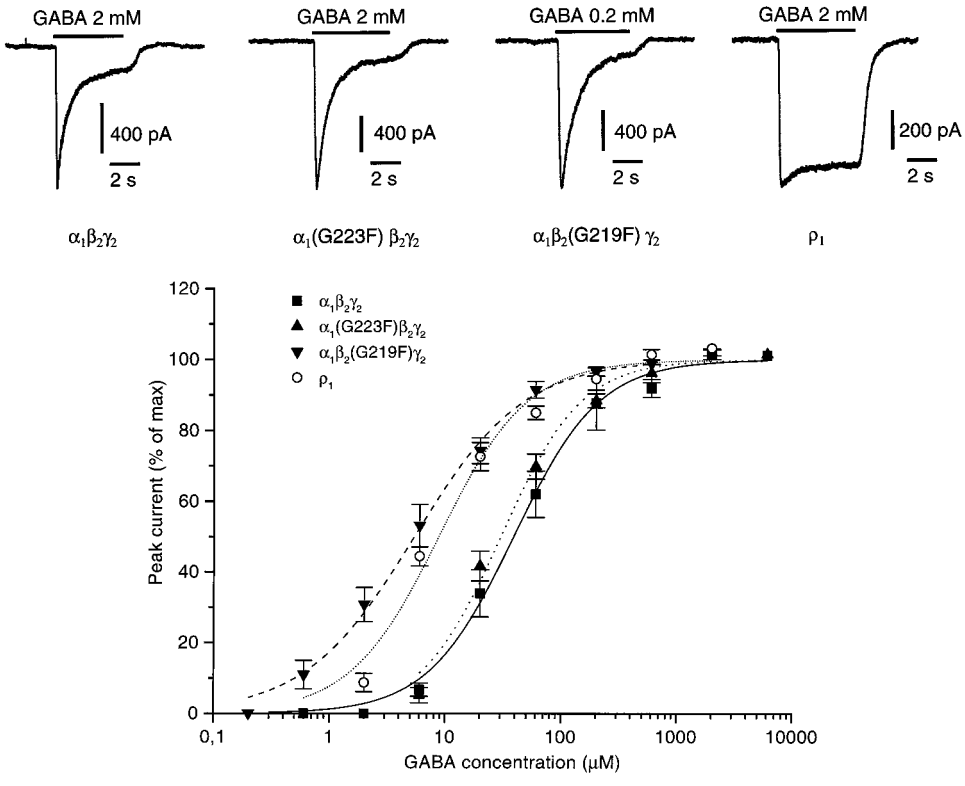

Fig. 6. Concentration-response curves for GABA-induced peak currents. Top, representative current traces from the maximum GABA-induced response for each combination tested. The line above each current trace represents duration of GABA application. Desensitization in the presence of GABA is prominent in all currents, except for the ρ_1 receptor. Bottom, results are shown as mean ± S.E. (n = 4–14 Sf9 cells tested/combination). The currents for each cell were normalized relative to the GABA-induced peak current of the highest concentration used for each subunit combination. The concentration-response relation for all four combinations shown were estimated by nonlinear regression with a logistic equation of the form: $E = E_{max} \times [GABA]^n/(EC_{50}{}^n + [GABA]^n)$, where n is the Hill coefficient. Each curve was normalized to make E_{max} = 100%. The following values (EC_{50}, μM; Hill coefficient, n) represent the nonlinear regression analyses of GABA-induced peak currents for wild-type and mutant $GABA_A$ receptors ($\alpha_1\beta_2\gamma_2$: 39 μM (29–49), 1.19 (0.98–1.40); α_1(G223F)$\beta_2\gamma_2$: 30 μM (23–37), 1.27 (0.91–1.62); $\alpha_1\beta_2$(G219F)γ_2: 5.4 μM (4.3–6.5)*, 0.93 (0.78–2.01); ρ_1: 8.8 μM (5.9–11.6), 1.14 (0.76–1.53). Numbers in brackets represent 95% CIs. *$P < .001$, significant difference between the EC_{50} of $\alpha_1\beta_2$(G219F)γ_2 compared with $\alpha_1\beta_2\gamma_2$ and α_1(G223F)$\beta_2\gamma_2$.

the $\alpha_1\beta_2$(G219F)γ_2 combination, the percentage of peak current remaining (i.e., 32.4%) appears to be qualitatively different; however, it is not statistically different from wild-type or the α mutant receptor. In addition, the $\alpha_1\beta_2$(G219F)γ_2-current that remained after a 5-s application of 50 μM pentobarbital was also analyzed, because it represented the plateau of the concentration-response curve. The fade displayed by 50 μM pentobarbital was (mean ± S.E.) 19.7 ± 7.9%, n = 13. This percentage was not significantly different from the percentages calculated for the combinations $\alpha_1\beta_2\gamma_2$ and α_1(G223F)$\beta_2\gamma_2$.

For propofol, the wild-type $\alpha_1\beta_2\gamma_2$ combination displayed no significant direct activation at various concentrations, up to and including 1 mM propofol (n = 6–10 Sf9 cells/concentration tested). This lack of direct activation in the presence of a β_2 subunit is consistent with the literature (Sanna et al., 1995) and might be why most studies investigating the actions of propofol have used β_1-containing $GABA_A$ receptors. The β_2(G219F) mutant receptors, however, demonstrated a concentration-dependent activation of chloride currents in the absence of GABA. The peak currents (normalized to a maximum GABA-activated peak current, i.e., 200 μM GABA) induced by propofol alone were as follows (mean ± S.E., n = 5–18 Sf9 cells/concentration of propofol tested): 30 μM, 4.2 ± 1.1%; 100 μM, 9.1 ± 1.5%; 300 μM, 24.3 ± 5.5%; 1 mM, 51.1 ± 8.6%.

Discussion

Mutation of TM1 glycine diminishes anesthetic efficacy in $GABA_A$ receptor binding. Our data suggest that G219 on the rat β_2 subunit may be an important component for allosteric changes induced by i.v. anesthetics. Finding an essential residue on the β_2 subunit is consistent with other studies implicating the β subunit as an important subunit for anesthetic modulation. For example, β-homomeric channels have been shown to be insensitive to GABA but can be directly activated by propofol and pentobarbital (Cestari et al., 1996; Davies et al., 1997b). More specifically, key amino acids in the TM2 domain, i.e., β_1(S265), β_2(N289), and β_3(N290), and in the TM3 domain, i.e., M286, of the β_1 subunit have been shown to be essential for the positive potentiating effects of volatile and i.v. anesthetics (Belelli et al., 1997; Mihic et al., 1997; Moody et al., 1997; Amin, 1999). However, the same point mutations, which were critical for the positive modulation of $GABA_A$ and glycine receptors by volatile anesthetics

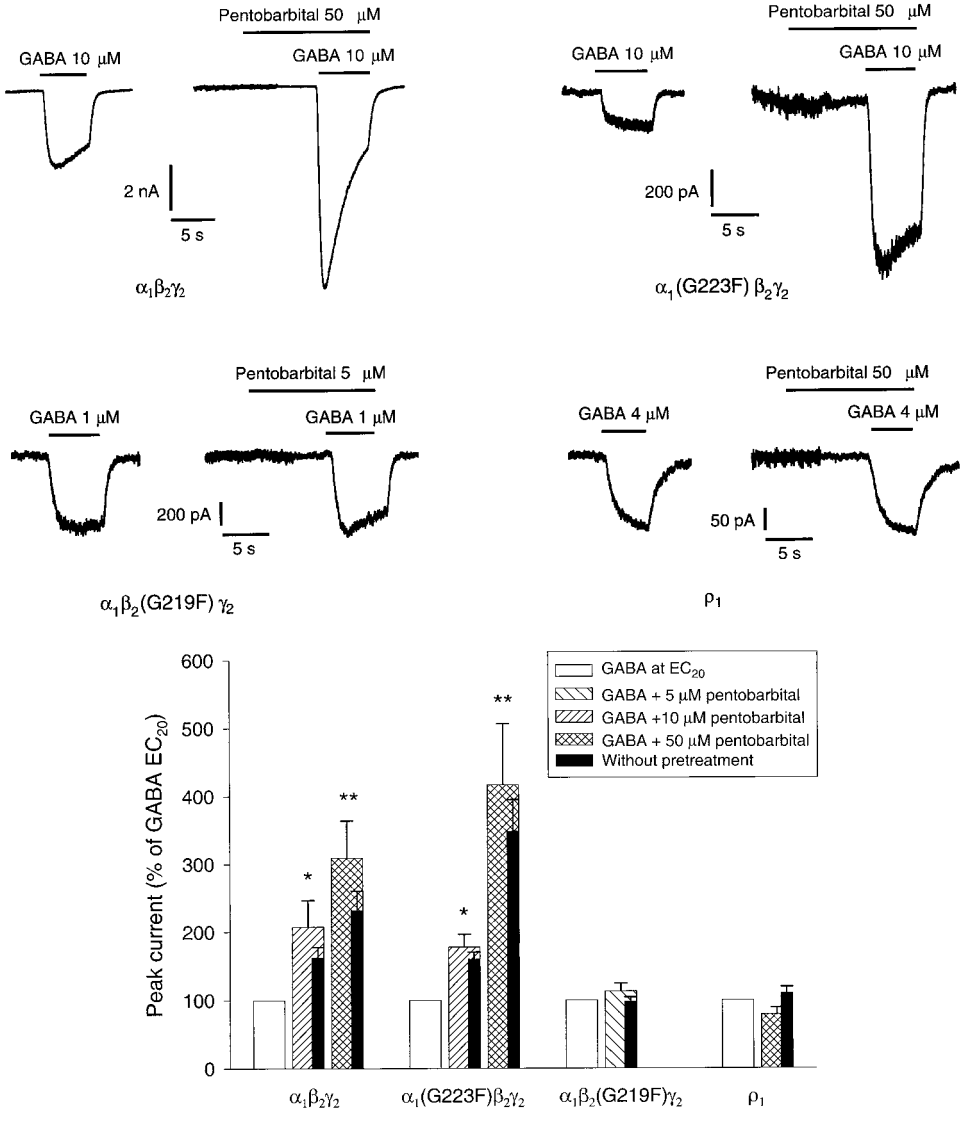

Fig. 7. Modulating effect of pentobarbital on GABA-induced peak currents. Top, representative current traces from each combination depicting the modulating effect of pentobarbital on currents induced by GABA (EC_{20}). The increased current variation in the beginning of the pentobarbital-modulated traces is caused by transient voltage pulses used to monitor cell membrane conductance and capacitance. These transient pulses are suspended 5 s before agonist application. Bottom, peak currents were normalized for each cell to the response of GABA at approximately the EC_{20} for each receptor combination ($\alpha_1\beta_2\gamma_2$, 10 μM GABA; α_1(G223F)$\beta_2\gamma_2$, 10 μM GABA; $\alpha_1\beta_2$(G219F)γ_2, 1 μM GABA; ρ_1, 4 μM GABA) and are shown as a mean ± S.E. (n = 3–9 Sf9 cells tested/combination). *P < .05; **P < .01, significant increase in pretreated responses compared with control.

(Mihic et al., 1997), appeared not to affect the potentiating actions of several i.v. anesthetics, such as methohexital (a barbiturate), alphaxalone, etomidate, and propofol (Krasowski et al., 1998). These findings indicate that the structural determinants for volatile and i.v. anesthetics are not necessarily the same.

Compared with wild-type receptors (i.e., $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_2$), our binding data demonstrated that inclusion of the β_2(G219F) subunit in a recombinant $\alpha\beta$ or $\alpha\beta\gamma$ $GABA_A$ receptor hindered positive modulation by all four anesthetics tested. However, as determined by the K_I values, binding affinities for [^{3}H]muscimol (on $\alpha\beta$ receptors) or [^{3}H]flunitrazepam (on $\alpha\beta\gamma$ receptors) were not altered. This result indicates that this point mutation most likely did not modify the binding sites for GABA/muscimol or benzodiazepines. Furthermore, on studying the effect of the β_2 mutant on anesthetic modulation of ligand binding between $\alpha\beta$ and $\alpha\beta\gamma$ receptors, it was concluded that the presence of γ_2 subunit did not change the modulation induced by all the anesthetics tested, except for alphaxalone. In the presence of the steroid anesthetic, a reduced efficacy was not apparent for $\alpha_1\beta_2$(G219F)γ_2 receptors; however, alphaxalone was the only anesthetic to induce negative modulation of ligand binding in $\alpha_1\beta_2$(G219F) recombinant receptors. Perhaps the presence of the γ_2 subunit in an $\alpha_1\beta_2$(G219F)γ_2 complex may provide additional structural determinants that are sufficient for preserving alphaxalone-induced enhancement of [^{3}H]flunitrazepam binding, therefore masking the deleterious effect of the point mutation on the β_2 subunit, as seen in the $\alpha\beta$ combination. Sanna et al. (1997) have shown that $\alpha\gamma$ receptors can be directly activated by alphaxalone but not etomidate, underlining the importance of the γ_2 subunit for the allosteric changes induced by alphaxalone on $GABA_A$ receptors.

With regard to the TM1 point mutation on the α_1 subunit, α_1(G223F), the potentiating effects of pentobarbital and alphaxalone on ligand binding were not altered by this mutation. However, the efficacy of etomidate- and propofol-induced enhancement of both [^{3}H]muscimol and [^{3}H]flunitrazepam binding was significantly reduced in receptors containing the α_1(G223F) subunit. This finding, first, indicates that structural criteria for allosteric modulation is different among these i.v. anesthetics, and second, in addition to the TM1 glycine on the β_2 subunit, this residue on the α_1 subunit is also essential for the allosteric effects of etomidate and propofol. This observation is consistent with a functional study demonstrating the importance of the α_1 subunit for the potentiating effects of both etomidate and propofol (Uchida et al., 1997). Furthermore, it

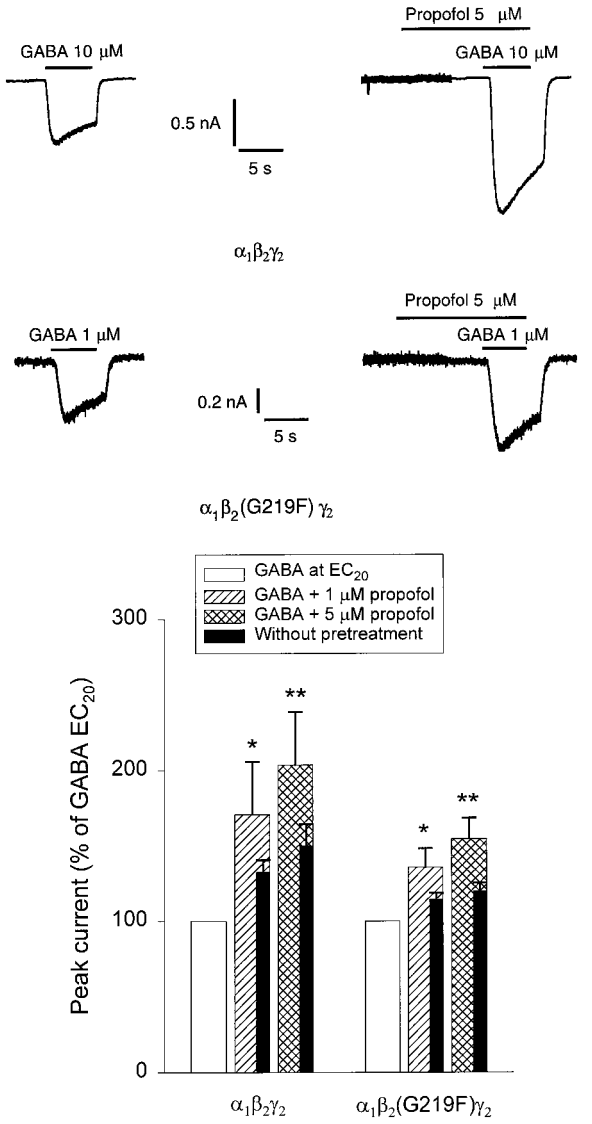

Fig. 8. Modulating effect of propofol on GABA-induced peak currents. Top, representative current traces from the $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2$(G219F)γ_2 combinations depicting the modulating effect of propofol on currents induced by GABA (EC_{20}: $\alpha_1\beta_2\gamma_2$, 10 μM GABA; $\alpha_1\beta_2$(G219F)γ_2, 1 μM GABA). Bottom, peak currents were normalized for each cell to the response of GABA at approximately the EC_{20} for each combination and are shown as mean ± S.E. (n = 6–10 Sf9 cells/combination). In all cases, the mean modulation was smaller for the $\alpha_1\beta_2$(G219F)γ_2 combination. *P < .05; **P < .01, significant concentration-dependent increases in pretreated responses compared with control.

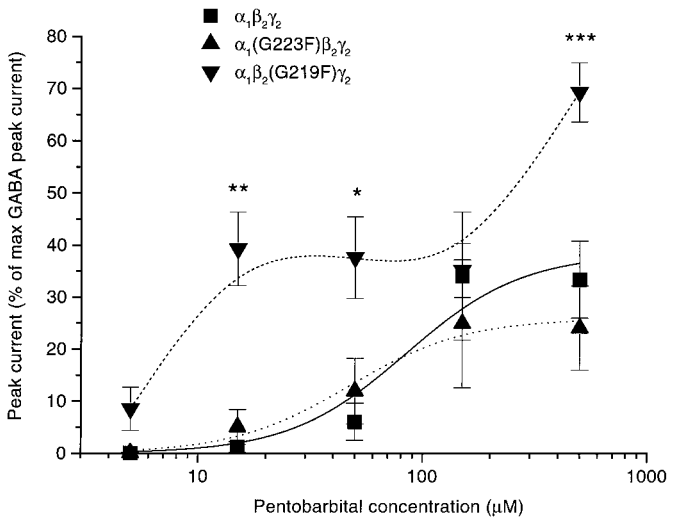

Fig. 9. Concentration-response curves for pentobarbital-induced direct activation. Peak currents were normalized to the peak current of a maximum GABA response in each Sf9 cell and are shown as the mean ± S.E. (n = 5–15 Sf9 cells tested/combination). The concentrations used to elicit maximum GABA responses were: $\alpha_1\beta_2\gamma_2$, 2 mM; α_1(G223F)$\beta_2\gamma_2$, 2 mM; and $\alpha_1\beta_2$(G219F)γ_2, 200 μM. No significant differences were found between the $\alpha_1\beta_2\gamma_2$ combination (■) and the α_1(G223F)$\beta_2\gamma_2$ combination (▲) at any of the concentrations of pentobarbital tested, whereas the peak currents induced by pentobarbital differed significantly between the $\alpha_1\beta_2$(G219F)γ_2 combination (▼) and the $\alpha_1\beta_2\gamma_2$ combination at some concentrations. *P < .05; **P < .01; ***P < .001).

has been shown that amino acids from the TM2 and TM3 domains of the α subunit, which were homologous to essential residues on the β subunit, were critical for positive modulation by volatile anesthetics (Mihic et al., 1997). Taken together, the α subunit can be important for the allosteric modulation induced by both i.v. and volatile anesthetics. Note there was not an additive effect in reducing the efficacy of etomidate and propofol in $\alpha\beta\gamma$ receptors containing the double mutant (i.e., point mutations in both the α_1 and β_2 subunits). This result may indicate that one of the point mutations is sufficient to disrupt the conformational changes needed for anesthetic-induced modulation of ligand binding.

The β_2(G219F) Point Mutation Alters Functional Aspects of GABA-, Pentobarbital-, and Propofol-Induced $GABA_A$ Chloride Channel Gating. In this study, pentobarbital was not able to enhance GABA currents in $\alpha_1\beta_2$(G219F)γ_2 receptors at concentrations that were not confounded by the direct activation effects of pentobarbital. Note, however, that at higher concentrations of pentobarbital, the potentiating effect could possibly be present, although it would be impossible to quantitate in the presence of the direct activation effect. Furthermore, propofol-induced GABA currents were less efficacious in the $\alpha_1\beta_2$(G219F)γ_2 receptors at the same anesthetic concentrations tested in $\alpha_1\beta_2\gamma_2$ receptors. These data, first, provide functional correlates for the receptor binding data, demonstrating reduced efficacies in anesthetic-induced enhancement of ligand binding with β_2(G219F) mutant receptors; and second, strongly support the hypothesis that this glycine residue at the entrance to TM1 is important for the allosteric actions of anesthetics. Considering that this TM1 point mutation had minimal or no effect on anesthetic potency in binding experiments or on high-affinity ligand binding, the phenylalanine on the β_2(G219F) subunit may not impair the actual anesthetic or GABA binding site but perhaps alters conformational changes involved in channel gating that are allosterically regulated by GABA and anesthetics. Our findings that the sensitivity of GABA, pentobarbital, and propofol in direct channel-gating function were increased support this conclusion. Note that essential residues in the TM2 domain of the β_2 subunit also appear to be critical for the conformational changes induced by GABA as well as pentobarbital (Birnir et al., 1997; Tierney et al., 1998). Thus, it appears that anesthetics and GABA allosterically induce a potentially similar transduction mechanism for direct channel gating. Because the TM1 point mutation on the β subunit altered the agonistic and modulatory actions of pentobarbital and propofol in a diametrically opposed fashion, this observation supports the working hypothesis that there are distinct structural requirements for this duality of anesthetic action (Jones et al., 1995).

TM1 Point Mutation on the ρ_1 Subunit Inhibits the Expression of Functional Channels. As discussed, the mutation of the polar glycine residue to the hydrophobic phenylalanine residue appeared to alter conformational flexibility of the $\alpha\beta\gamma$ receptors. Because glycine is known to confer conformational freedom to peptide chains (Renard et al., 1999), it was surprising to find that the ρ_1(F261G) receptors did not produce any functional channels. This finding is difficult to resolve with the binding data, which show that the ρ_1(F261G) subunit produced a small change in anesthetic sensitivity for modulating [^{3}H]muscimol binding as well as demonstrated comparable K_I values for [^{3}H]muscimol, as seen in wild-type ρ_1 receptors. It is possible that the binding data for the ρ_1(F261G) receptors could reflect intracellular homomeric receptors that have yet to be expressed on the cell surface. A recent study has shown that mutagenesis of certain extracellular residues in $GABA_A$ receptor α_1 subunit, expressed in Sf9 cells, resulted in muscimol binding activity without cell surface expression (Srinivasan et al., 1999). On the other hand, because all receptor combinations demonstrating binding also conferred channels that were gated by GABA, including the ρ_1(NASM-RNSL) homomer (data not shown), the mutant, ρ_1(F261G), homomeric receptors may have been expressed on the cell surface yet were not capable of being gated by GABA (or pentobarbital). Our findings may indicate that this point mutation, which is expressed on every subunit of the ρ_1(F261G) homomer, obstructed the proper subunit-subunit interactions needed for channel gating. Because the functional wild-type and bridge mutant ρ_1(NASM-RNSL) receptors have three consecutive phenylalanines at the entrance to the TM1 domain on each subunit, it would be of interest to pursue the importance of the adjacent phenylalanine (F262) in ρ_1 with regard to channel gating and allosteric events induced by anesthetics.

Concluding Remarks. There is strong evidence in support of anesthetics binding directly to protein targets as opposed to indirectly affecting protein targets via disrupting the lipid bilayer for their mechanism of action (Franks and Lieb, 1991; Eckenhoff, 1998). However, until specific radiolabeled anesthetics are developed, it will remain difficult to determine whether the residues studied here, as well as others (Belelli et al., 1997; Mihic et al., 1997; Moody et al., 1997; Amin, 1999), are involved specifically in forming the binding sites for anesthetics. From our data, first, anesthetic modulation of binding appears to be a predictable indicator of its functional correlate. This conclusion is not novel, and the allosteric modulation of GABA, benzodiazepine, and picrotoxin sites by positive and negative modulators of $GABA_A$ receptors has been found to correlate extremely well for a series of compounds, including stereoisomers, with modulation of $GABA_A$ currents in cultured cells, and with animal behavior (reviewed in Olsen et al., 1991; Carlson et al., 1997). Second, it is concluded that the TM1 glycine residue, which is located at the membrane interface of the protein with the extracellular fluid, is more likely involved in the conformational or allosteric control of channel gating by anesthetics (or GABA) rather than a specific anesthetic binding site. Consistent with this idea are studies on interactions of anesthetics with model membrane ion channels indicating the importance of amphiphilic channel residues at the lipid-water interface (Xu et al.,1998). Furthermore, in muscle-type acetylcholine ligand-gated ion channels and $GABA_A$ receptors, it has been demonstrated that the N-terminal region of TM1 domain may work in concert with the TM2 domain for channel gating (Akabas and Karlin, 1995; Thompson et al., 1999). Thus, the TM1 domain is potentially a link in the chain of conformational events elicited by GABA and anesthetics.

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References

Akabas MH and Karlin A (1995) Identification of acetylcholine receptor channel-lining residues in the M1 segment of the α-subunit. *Biochemistry* **34:**12496–12500.

Amin J (1999) A single hydrophobic residue confers barbiturate sensitivity to γ-aminobutyric acid type C receptor. *Mol Pharmacol* **55:**411–423.

Amin J, Brooks-Kayal A and Weiss DS (1997) Two tyrosine residues on the α subunit are crucial for benzodiazepine binding and allosteric modulation of γ-aminobutyric acid$_A$ receptors. *Mol Pharmacol* **51:**833–841.

Amin J and Weiss DS (1996) Insights into the activation mechanism of ρ_1 GABA receptors obtained by coexpression of wild type and activation-impaired subunits. *Proc R Soc Lond B* **263:**273–282.

Barnard EA, Skolnick P, Olsen RW, Möhler H, Sieghart W, Biggio G, Braestrup C, Bateson AN and Langer SZ (1998) International Union of Pharmacology. XV. Subtypes of γ-aminobutyric acid$_A$ receptors: Classification on the basis of subunit structure and receptor function. *Pharmacol Rev* **50:**291–313.

Belelli D, Lambert JJ, Peters JA, Wafford K and Whiting PJ (1997) The interaction of the general anesthetic etomidate with the γ-aminobutyric acid type A receptor is influenced by a single amino acid. *Proc Natl Acad Sci USA* **94:**11031–11036.

Birnir B, Tierney ML, Dalziel JE, Cox GB and Gage PW (1997) A structural determinant of densensitization and allosteric regulation by pentobarbitone of the GABA$_A$ receptor. *J Membr Biol* **155:**157–166.

Buhr A, Baur R, Malherbe P and Sigel E (1996) Point mutations of the α1β2γ2 γ-aminobutyric acid$_A$ receptor affecting modulation of the channel ligands of the benzodiazepine binding site. *Mol Pharmacol* **49:**1080–1084.

Carlson BX, Hales TG and Olsen RW (1997) GABA$_A$ receptors and anesthesia, in *Anesthesia, Biologic Foundations* (Yaksh TL et al. eds) pp 259–275, Lippincott-Raven, Philadelphia, PA.

Cestari IN, Uchida I, Li L, Burt D and Yang J (1996) The agonistic action of pentobarbital on GABA$_A$ β-subunit homomeric receptors. *Neuroreport* **7:**943–947.

Chang Y, Wang R, Barot S and Weiss DS (1996) Stoichiometry of a recombinant GABA$_A$ receptor. *J Neurosci* **16:**5415–5424.

Cutting GR, Lu L, O'Hara BF, Kasch LM, Montrose-Rafizadeh C, Donovan DM, Shimada S, Antonarakis SE, Guggino WB, Uhl GR and Kazazian HH (1991) Cloning of the γ-aminobutyric acid (GABA) ρ_1 cDNA: A GABA receptor subunit highly expressed in the retina. *Proc Natl Acad Sci USA* **88:**2673–2677.

Davies PA, Hanna MC, Hales TG and Kirkness EF (1997a) Insensitivity to anaesthetic agents conferred by a class of GABA$_A$ receptor subunit. *Nature (Lond)* **385:**820–823.

Davies PA, Kirkness EF and Hales TG (1997b) Modulation by general anaesthetics of rat GABA$_A$ receptors comprised of α1β3 and β3 subunits expressed in human embryonic kidney 293 cells. *Br J Pharmacol* **120:**899–909.

Eckenhoff RG (1998) Do specific or nonspecific interactions with proteins underlie inhalational anesthetic action? *Mol Pharmacol* **54:**610–615.

Franks NP and Lieb WR (1991) Stereospecific effects of inhalational general anesthetic optical isomers on nerve ion channels. *Science (Wash DC)* **254:**427–430.

Grenningloh G, Schmieden V, Schofield PR, Seeburg PH, Siddique T, Mohandas TK, Becker C-M and Betz H (1990) Alpha subunit variants of the human glycine receptor: Primary structures, functional expression and chromosomal localization of the corresponding genes. *EMBO J* **9:**771–776.

Hales TG and Lambert JJ (1991) The actions of propofol on inhibitory amino acid receptors of bovine adrenomedullary chromaffin cells and rodent central neurones. *Br J Pharmacol* **104:**619–628.

Hales TG and Olsen RW (1994) Basic pharmacology of intravenous induction agents, in *The Pharmacological Basis of Anesthesiology: Basic Science and Practical Applications* (Bowdle TA, Horita A and Kharasch ED eds) pp 295–306, Churchill Livingstone, New York.

Hamill OP, Marty A, Neher E, Sakmann B and Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch* **391:**85–100.

Harris BD, Wong G, Moody EJ and Skolnick P (1995) Different subunit requirements for volatile and nonvolatile anesthetics at γ-aminobutyric acid type A receptors. *Mol Pharmacol* **47:**363–367.

Jones MV, Harrison NL, Pritchett DB and Hales TG (1995) Modulation of the GABA$_A$ receptor by propofol is independent of the γ subunit. *J Pharmacol Exp Ther* **274:**962–968.

Krasowski MD, Koltchine VV, Rick CE, Ye Q, Finn SE and Harrison NL (1998) Propofol and other intravenous anesthetics have sites of action on the γ-aminobutyric acid type A receptor distinct from that for isoflurane. *Mol Pharmacol* **53:**530–538.

Kristiansen U and Lambert JDC (1996) Benzodiazepine and barbiturate ligands modulate responses of cultured hippocampal neurones to the GABA$_A$ receptor partial agonist 4-PIOL. *Neuropharmacology* **35:**1181–1191.

Mihic SJ and Harris RA (1996) Inhibition of ρ_1 receptor GABAergic currents by alcohols and volatile anesthetics. *J Pharmacol Exp Ther* **277:**411–416.

Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, Mascia MP, Valenzuela CF, Hanson KK, Greenblatt EP, Harris RA and Harrison NL (1997) Sites of alcohol and volatile anaesthetic action on GABA$_A$ and glycine receptors. *Nature (Lond)* **389:**385–389.

Moody EJ, Knauer C, Granja R, Strakhova M and Skolnick P (1997) Distinct loci mediate the direct and indirect actions of the anesthetic etomidate at GABA$_A$ receptors. *J Neurochem* **69:**1310–1313.

Olsen RW, Sapp DW, Bureau MH, Turner DM and Kokka N (1991) Allosteric actions of CNS depressants including anesthetics on subtypes of the inhibitory GABA$_A$ receptor-chloride channel complex. *Ann NY Acad Sci* **625:**145–154.

Pritchett DB and Seeburg PH (1991) GABA$_A$ receptor point mutations increases the affinity of compounds for the benzodiazepine site. *Proc Natl Acad Sci USA* **88:**1421–1425.

Renard S, Olivier A, Granger P, Avenet P, Graham D, Sevrin M, George P and Besnard F (1999) Structural elements of the γ-aminobutyric acid type A receptor conferring subtype selectivity for benzodiazepine site ligands. *J Biol Chem* **274:**13370–13374.

Sanna E, Mascia MP, Klein RL, Whiting PJ, Biggio G and Harris RA (1995) Actions of the general anesthetic propofol on recombinant human GABA$_A$ receptors: Influence of receptor subunits. *J Pharmacol Exp Ther* **274:**353–360.

Sanna E, Murgia A, Casula A and Biggio G (1997) Differential subunit dependence of the actions of the general anesthetics alphaxalone and etomidate at γ-aminobutyric type A receptors expressed in *Xenopus laevis* oocytes. *Mol Pharmacol* **51:**484–490.

Srinivasan S, Nichols CJ, Olsen RW and Tobin AJ (1999) Two invariant tryptophans define domains necessary for GABA$_A$ receptor assembly. *J Biol Chem* **274:** 26633–26638.

Thompson SA, Arden SA, Marshall G, Wingrove PB, Whiting PJ and Wafford KA (1999) Residues in transmembrane domains I and II determine γ-aminobutyric type A$_A$ receptor subtype-selective antagonism by furosemide. *Mol Pharmacol* **55:**993–999.

Tierney ML, Birnir B, Cromer B, Howitt SM, Gage PW and Cox GB (1998) Two threonine residues in the M2 segment of the $\alpha_1\beta_1$ GABA$_A$ receptor are critical for ion channel function. *Receptors Channels* **5:**113–124.

Tretter V, Ehya N, Fuchs K and Sieghart W (1997) Stoichiometry and assembly of a recombinant GABA$_A$ receptor subtype. *J Neurosci* **17:**2728–2737

Uchida I, Li L and Yang J (1997) The role of the GABA$_A$ receptor α1 subunit N-terminal extracellular domain in propofol potentiation of chloride current. *Neuropharmacology* **36:**1611–1621.

Wieland HA, Lüddens H and Seeburg PH (1992) A single histidine in GABA$_A$ receptors is essential for benzodiazepine agonist binding. *J Biol Chem* **267:**1426–1429.

Xu Y, Tang P and Liachenko S (1998) Unifying characteristics of sites of anesthetic action. *Toxicol Lett* **100–101:**347–352.

Zezula J, Slany A and Sieghart W (1996) Interaction of allosteric ligands with GABA$_A$ receptors containing one, two, or three different subunits. *Eur J Pharmacol* **301:**207–214.

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