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Methionine 286 in transmembrane domain 3 of the GABA_A receptor β subunit controls a binding cavity for propofol and other alkylphenol general anesthetics

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Abstract

γ -Aminobutyric acid type A (GABA_A) receptors are an important target for general anesthetics in the central nervous system. Site-directed mutagenesis techniques have identified amino acid residues that are important for the positive modulation of GABA_A receptors by general anesthetics. In the present study, we investigate the role of an amino acid residue in transmembrane (TM) domain 3 of the GABA_A receptor β_2 subunit for modulation by the general anesthetic 2,6-diisopropylphenol (propofol). Mutation of methionine 286 to tryptophan (M286W) in the β_2 subunit abolished potentiation of GABA responses by propofol but did not affect direct receptor activation by propofol in the absence of GABA. In contrast, substitution of methionine 286 by alanine, cysteine, glutamate, lysine, phenylalanine, serine, or tyrosine was permissive for potentiation of GABA responses and direct activation by propofol. Using propofol analogs of varying molecular size, we show that the β_2 (M286W) mutation resulted in a decrease in the ‘cut-off’ volume for propofol analog molecules to enhance GABA responses at GABA_A $\alpha_1\beta_2\gamma_2\delta$ receptors. This suggests that mutation of M286 in the GABA_A β_2 subunit alters the dimensions of a ‘binding pocket’ for propofol and related alkylphenol general anesthetics.

1. Introduction

2,6-Diisopropylphenol (propofol) has proven to be a useful intravenous general anesthetic since its introduction into clinical practice in the 1980s. There have been a number of efforts to understand the molecular mechanism of action of this clinically useful drug (Trapani et al., 2000). One hypothesis, supported by substantial experimental evidence, cites the ability of propofol to positively modulate the function of γ -aminobutyric acid type A (GABA_A) receptors, a property shared with other general anesthetics (Franks and Lieb, 1994; Belelli et al., 1999; Krasowski and Harrison, 1999; Trapani et al., 2000). Propofol has been shown in electrophysiological assays to allosterically enhance (potentiate) the actions of GABA at the GABA_A receptor (Hales and Lambert, 1991). In addition, propofol prolongs inhibitory postsynaptic currents mediated by GABA_A receptors (Bai et al., 2001) and alters receptor deactivation and desensitization (Bai et al., 1999). Propofol can also open the GABA_A receptor ion channel in the absence of GABA (termed ‘direct activation’), although this usually occurs

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at higher concentrations of propofol than necessary to potentiate responses to GABA (Hales and Lambert, 1991; Hara et al., 1993).

Molecular pharmacology studies have defined individual amino acid residues within GABA_A receptor molecules that are critical for the allosteric effects of general anesthetics (Belelli et al., 1999; Krasowski and Harrison, 1999). Progress in this effort followed from the observation that receptors related to the GABA_A receptor, such as the strychnine-sensitive glycine receptor (Mascia et al., 1996) and the GABA_C ρ receptor (Mihic and Harris, 1996) differ in their sensitivity to general anesthetics. Amino acid residues in transmembrane (TM) domains 2 and 3 of GABA_A receptor α and β subunits are particularly important for the modulatory actions of ether, alkane, and alcohol anesthetics (Mihic et al., 1997; Krasowski et al., 1998a,b; Koltchine et al., 1999; Ueno et al. 1999, 2000; Krasowski and Harrison, 2000; Jenkins et al., 2001) (Fig. 1), as well as for certain intravenous anesthetics such as propofol, etomidate, and the barbiturates (Amin, 1999; Belelli et al., 1999). In the case of propofol, mutation of methionine 286 to tryptophan (M286W) in TM3 of the GABA_A β_1 receptor subunit abolishes potentiation of GABA responses (Krasowski et al., 1998b).

In the present study we further define the importance of this TM3 residue of the β subunit for the modulatory effects of propofol. We also test the effect of tryptophan substitution within TM2 of the β_2 subunit and within TM2 and TM3 of the α_1 subunit. The final objective was to utilize propofol analogs of different molecular size (Krasowski et al., 2001) to test whether amino acid substitutions for GABA_A β_2 (M286) can alter the 'cut-off' for propofol analogs in potentiating GABA responses at GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptors. The cut-off for a homologous series of molecules at a given receptor is believed to reflect a binding pocket of limited dimensions, such that molecules beyond a certain size cannot fit into the binding pocket (Franks and Lieb, 1994; Krasowski and Harrison, 1999; Dwyer and Bradley, 2000). The classic homologous series to show cut-off is the *n*-alcohols, which exhibit cut-offs specific for different ligand-gated ion channels (Li et al., 1994; Peoples and Weight, 1995; Mascia et al., 1996; Mihic and Harris, 1996). This has been taken to suggest that these receptors contain cavities of discrete size for the alcohols, which would be consistent with the finding that alcohol cut-off for modulation of glycine α_1 and GABA_C ρ receptors can be manipulated by mutagenesis within TM2 and TM3 (Wick et al., 1998). The idea in the present study was to investigate whether amino acid substitutions at GABA_A β_2 (M286) could produce a similar effect for analogs to propofol and thereby test the hypothesis that β_2 (M286) influences the dimensions of a propofol 'binding pocket'.

2. Methods

2.1. Site-directed mutagenesis

The GABA_A α_1 (Schofield et al., 1989) and γ_{2s} (Pritchett et al., 1989) receptor subunit cDNAs are of human origin. The GABA_A β_2 receptor subunit cDNA is from the rat (Ymer et al., 1989). The human GABA_A α_1 and γ_{2s} receptor subunit cDNAs were generously provided by the late Dr Dolan Pritchett (University of Pennsylvania, Philadelphia, PA, USA). The rat GABA_A β_2 subunit cDNA was provided by Dr Dennis Grayson (University of Illinois at Chicago, Chicago, IL, USA).

The β_2 (N265W), β_2 (M286F), and β_2 (M286Y) mutations were introduced by the unique site elimination method using the USE kit (Pharmacia Biotech, Piscataway, NJ, USA) as previously described (Krasowski et al., 1998a,b). For the α_1 (S270W,A291W) double mutation, the S270W and A291W mutations were initially introduced separately. The vector containing the S270W mutation was digested with *Bsu36I* and *NotI* (New England Biolabs) to create a 1 kb fragment, which then replaced the same fragment in the similarly digested vector containing the A291W mutation.

The β_2 (M286A), β_2 (M286C), β_2 (M286E), β_2 (M286K), β_2 (M286S), and β_2 (M286W) mutations in the GABA_A β_2 subunit were introduced with the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) as previously described (Krasowski et al., 1998a,b). Positive clones were confirmed by double-stranded sequencing of the entire cDNA insert.

2.2. Cell culture and transfection of receptor cDNAs

GABA_A receptor cDNAs were expressed via the vector pCIS2 which contains one copy of the strong promoter from cytomegalovirus and a polyadenylation sequence from SV40. Human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Rockville, MD, USA) were maintained in culture and passaged weekly by trypsin treatment for a maximum of 20 times before being discarded and replaced with early passage cells. HEK 293 cells were maintained in Eagle's minimum essential medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), l-glutamine (0.292 μ g/ml; Gibco BRL, Grand Island, NY, USA), penicillin G sulfate (100 units/ml; Gibco BRL), and streptomycin sulfate (100 μ g/ml; Gibco BRL).

For electrophysiological experiments, cells were plated on glass coverslips coated with poly-D-lysine (Sigma). Each coverslip of cells was individually transfected with the calcium phosphate precipitation technique as previously described (Krasowski et al., 1998a). Each transfection used 1–5 μ g of each cDNA; the cDNA was in contact with the cells for 24 h under an atmosphere containing 3% CO₂ before being removed and replaced with fresh culture medium in an atmosphere of 5% CO₂.

2.3. Electrophysiology and design of pharmacology experiments

Stock solutions of GABA (Sigma) and the propofol analogs were diluted into extracellular solution daily before use. The propofol analogs were all prepared as stock solutions in dimethyl sulfoxide before being dissolved in the extracellular medium. The maximum final concentration of dimethyl sulfoxide was 0.2% (v/v), which was determined during carrier control experiments to have no significant effect on GABA-induced currents in the GABA_A receptors analyzed in this study.

Electrophysiological recordings were performed at room temperature using the whole-cell patch clamp technique as previously described (Krasowski et al., 1998a,b). The coverslips were transferred 48–96 h after removal of the cDNA to a large chamber that was continuously perfused (2–3 ml/min) with extracellular medium containing (in mM): 145 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgCl₂, 5.5 D-glucose, and 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, osmolarity 320–330 mOsmol. The electrode solution contained (in mM): 145 *N*-methyl-D-glucamine hydrochloride, 5 K₂ATP, 5 HEPES/KOH, 2 MgCl₂, 0.1 CaCl₂, and 1.1 ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), pH 7.2, osmolarity 315 mOsmol. Pipette-to-bath resistance was 4–6 M Ω . Cells were voltage-clamped at -60 mV. Since the intracellular and extracellular solutions contained symmetrical chloride concentrations, the chloride equilibrium potential was approximately 0 mV.

All drugs were applied to the cell by local perfusion (Krasowski et al., 1998a,b) using a motor-driven solution exchange device (Bio Logic Rapid Solution Changer RSC-100; Molecular Kinetics, Pullman, WA, USA). Laminar flow was maintained by applying all solutions at identical flow rates via a multi-channel infusion pump (Stoelting, Wood Dale, IL, USA). The solution changer was driven by protocols in the acquisition program pCLAMP5 (Axon Instruments, Foster City, CA, USA). Responses were low-pass-filtered at 5 kHz and digitized (TL-1-125 interface; Axon Instruments) using pCLAMP5 and stored for off-line analysis.

Potential of GABA responses by propofol and other drugs was always assessed by co-application of the drug with an EC₂₀ GABA concentration (i.e. the concentration of GABA that produces 20% of the maximal response to GABA). Anesthetics were always preapplied prior to co-application with GABA to ensure that the anesthetic had reached equilibrium with the receptor. At the end of each potentiation experiment, a maximal GABA response was elicited by an appropriately high concentration of GABA to verify that the average control GABA response fell between EC₁₀ and EC₃₀. This experimental design permits comparison not only of anesthetic EC₅₀ between various receptor combinations but also of the maximal magnitude (relative efficacy) of agonist potentiation between anesthetics (Krasowski and Harrison, 2000). Anesthetic direct activation currents were expressed as a fraction of the maximal current response to GABA.

2.4. Data analysis

Drug-induced potentiation of a GABA-induced current was defined as the percentage increase of the control GABA response (defined as the average of the pre-drug and post-drug GABA-induced currents). Concentration response data were fitted (Kaleidagraph; Reading, PA, USA) with the equation: $I/I_{\max} = 100[\text{drug}]^{nH}/([\text{drug}]^{nH} + (\text{EC}_{50})^{nH})$, where $I/I_{\max}$ is the percentage of the maximum obtainable response, EC₅₀ the concentration producing a half-maximal response, and nH is the Hill coefficient. Pooled data are presented throughout as mean ± SE. Statistical significance was determined by two-way analysis of variance with Dunnett's post-hoc test, unless otherwise specified.

2.5. Molecular volume of the propofol analogs and amino acid residues

Molecular volumes for the propofol analogs were calculated using the cerius² qsar software version 3.0 (Molecular Simulations, San Diego, CA, USA). The molecular volumes of the propofol analogs are listed in Fig. 7. The molecular volumes of the amino acid residue side chains considered in this study are (Harpaz et al., 1994): alanine (A; 0.026 nm³), asparagine (N; 0.064 nm³), cysteine (C; 0.04 nm³), glutamic acid (E; 0.077 nm³), glycine (G; 0 nm³), lysine (K; 0.106 nm³), methionine (M; 0.104 nm³), phenylalanine (F; 0.13 nm³), serine (S; 0.03 nm³), tryptophan (W; 0.168 nm³), tyrosine (Y; 0.133 nm³), and valine (V; 0.075 nm³).

2.6. Sources of drugs

The sources of the propofol analogs and other anesthetics were as follows: 2,6-dimethylphenol, 2-isopropylphenol (Aldrich Chemical Co., Milwaukee, WI, USA), 2,6-diethylphenyl isothiocyanate (Lancaster Synthesis, Windham, NH, USA), and methohexital sodium (Brevital sodium; Eli Lilly and Co., Indianapolis, IN, USA). Each analog was of the highest purity grade available commercially. Propofol, 2,6-diethylphenol, and 2,6-di-*tert*-butylphenol were generously provided by Drs. J.B. Glen and Roger James of Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK; see James and Glen, 1980). Research-grade etomidate was a generous gift from Professor Alfred Doenicke (Institute of Anesthesiology, Ludwig Maximilians University of Munchen, Germany).

3. Results

3.1. Receptor characteristics and GABA concentration–response relationships for wild-type and mutated GABA_Aα₁β₂γ_{2s} receptors

The wild-type GABA_A receptor and all of the receptors containing point mutations in the α₁ or β₂ subunits responded to GABA in a concentration-dependent manner. Fig. 2(A) and (B) shows responses to GABA for the wild-type GABA_Aα₁β₂γ_{2s} receptor and for the GABA_Aα₁β₂(M286W)γ_{2s} receptor. Fig. 3 displays GABA concentration–response curves for

the wild-type GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptor and for the series of eight $\alpha_1\beta_2$ (M286X) γ_{2s} mutated receptors.

The EC₅₀ values for GABA for the $\alpha_1\beta_2$ (M286X) γ_{2s} series of receptors varied over approximately one order of magnitude, with the greatest absolute difference found in the $\alpha_1\beta_2$ (M286C) γ_{2s} receptor, which had a 4.5-fold higher EC₅₀ to GABA than the $\alpha_1\beta_2\gamma_{2s}$ receptor. GABA EC₅₀, Hill slope, and maximal response data for all receptors are summarized in Table 1. In contrast to the relatively modest effects on GABA sensitivity produced by the series of β_2 (M286X) mutant subunits, the GABA_A α_1 (S270W,A291W) $\beta_2\gamma_{2s}$ receptor had an EC₅₀ value for GABA 25 times lower than that for the wild-type GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptor. This is consistent with the previously described effects of the α_2 (S270W) and α_2 (A291W) mutations, which shift the GABA concentration–response relationship for the GABA_A $\alpha_2\beta_1\gamma_{2s}$ receptor to the left by 7.1- and 3.4-fold, respectively (Krasowski et al., 1998b; Koltchine et al., 1999).

3.2. Propofol pharmacology of wild-type and mutant GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptors

Propofol potentiated submaximal (EC₂₀) GABA responses at the wild-type GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptor (Figs. 2(C) and 4) but failed to enhance submaximal GABA responses at the GABA_A $\alpha_1\beta_2$ (M286W) γ_{2s} receptor (Figs. 2(D) and 4(A); Table 1). In contrast to the lack of potentiation of GABA responses, propofol directly activated the GABA_A $\alpha_1\beta_2$ (M286W) γ_{2s} receptor, although with a 2.3-fold higher EC₅₀ value than at the wild-type GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptor (Figs. 2(D) and 5(A); Table 1). Complete concentration–response curves for potentiation of GABA responses and direct activation by propofol for the wild-type GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptor and for the series of $\alpha_1\beta_2$ (M286X) γ_{2s} mutated receptors are illustrated in Figs. 4 and 5, with summary of data for all receptors presented in Table 1.

To test the importance of the α subunit for the effects of propofol, potentiation of GABA responses by propofol was studied at the GABA_A α_1 (S270W,A291W) $\beta_2\gamma_{2s}$ receptor. This receptor showed potentiation of GABA responses by propofol similar to that at the wild-type GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptor (Table 1).

3.3. Correlation analysis of the properties of the amino acid residue side-chain at position 286 of the GABA_A β_2 subunit with the GABA and propofol pharmacology

We next investigated the relationship between the physical properties of the amino acid residue at position 286 of the GABA_A β_2 subunit and the responses to GABA and propofol. The amino acid properties chosen were molecular volume (Harpaz et al., 1994), polarity (Zimmerman et al., 1968), hydrophathy (Kyte and Doolittle, 1982), and hydrophilicity (Hopp and Woods, 1981). There was no significant correlation between any of these amino acid properties and the GABA EC₅₀, GABA maximal current, EC₅₀ for potentiation of GABA responses by propofol, potentiation of control GABA response by 2, 5, 10, or 20 μ M propofol, EC₅₀ for propofol direct activation, or maximal direct activation by propofol (Pearson's correlation $r^2=0.002-0.56$, $P<0.05$ for each). In addition, the GABA EC₅₀ value did not correlate with the EC₅₀ values for direct activation or potentiation of GABA responses by propofol ($r^2=0.07$ and 0.13 , respectively, $P<0.05$ for each); similarly, the EC₅₀ value for potentiation of GABA responses by propofol also did not correlate with the EC₅₀ value for propofol direct activation ($r^2=0.01$, $P<0.05$).

3.4. Effects of methohexital and etomidate at wild-type and mutant GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptors

We next determined the effects of methohexital (a barbiturate) and etomidate on submaximal GABA responses at the GABA_A $\alpha_1\beta_2\gamma_{2s}$ and $\alpha_1\beta_2$ (M286W) γ_{2s} receptors, to test whether this mutant receptor was capable of being modulated by anesthetics from different chemical classes than propofol. Methohexital (10 μ M) potentiated EC₂₀ GABA responses at both receptors: 199

$\pm 39\%$ potentiation at the GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptor ($n=5$) and $118\pm 18\%$ at the GABA_A $\alpha_1\beta_2(\text{M286W})\gamma_{2s}$ receptor ($n=6$) ($P<0.05$ compared with response at the wild-type receptor). Etomidate (10 μM) also potentiated EC₂₀ GABA responses at both receptors: $237\pm 45\%$ potentiation at the GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptor ($n=6$) and $162\pm 11\%$ at the GABA_A $\alpha_1\beta_2(\text{M286W})\gamma_{2s}$ receptor ($n=5$) ($P<0.05$ compared with response at the wild-type receptor).

3.5. Alteration of the cut-off for propofol analogs by mutations in the GABA_A β_2 subunit

Two approaches were adopted to test the possibility that substitutions at the $\beta_2(\text{M286})$ position might alter the size of propofol analog that could potentiate GABA responses. One approach was to mutate $\beta_2(\text{M286})$ to a smaller amino acid residue to see if this would allow an analog bulkier than propofol to gain access to the propofol binding site by creating a larger binding cavity (an increase in cut-off). The analog chosen was 2,6-di-*tert*-butylphenol, shown in a separate study to be completely inactive at wild-type GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptors (Krasowski et al., 2001). The second approach investigated whether submaximal GABA responses at the GABA_A $\alpha_1\beta_2(\text{M286W})\gamma_{2s}$ receptor could still be potentiated by analogs smaller than propofol (a decrease in cut-off), even though propofol itself is inactive at this mutant receptor.

In the first set of experiments, the effects of 2,6-di-*tert*-butylphenol were tested at GABA_A $\alpha_1\beta_2(\text{M286A})\gamma_{2s}$ and GABA_A $\alpha_1\beta_2(\text{M286S})\gamma_{2s}$ receptors. The M286A and M286S mutations result in 0.078 and 0.074 nm³ decreases in amino acid molecular volume at the 286 position, respectively, while 2,6-di-*tert*-butylphenol is 0.034 nm³ greater in molecular volume than propofol. 2,6-Di-*tert*-butylphenol did not, however, potentiate GABA responses at the GABA_A $\alpha_1\beta_2(\text{M286A})\gamma_{2s}$ and GABA_A $\alpha_1\beta_2(\text{M286S})\gamma_{2s}$ receptors (Table 2). 2,6-Di-*tert*-butylphenol also produced no direct activation at either receptor at concentrations up to 500 μM ($n=5$). In contrast, four analogs smaller than propofol (molecular volume=0.193 nm³), 2,6-dimethylphenol (0.126 nm³), 2-isopropylphenol (0.143 nm³), 2,6-diethylphenol (0.160 nm³), and 2,6-diethylphenyl isothiocyanate (0.187 nm³), each potentiated GABA responses at the GABA_A $\alpha_1\beta_2(\text{M286A})\gamma_{2s}$ and $\alpha_1\beta_2(\text{M286S})\gamma_{2s}$ receptors (Table 2). Thus, the GABA_A $\alpha_1\beta_2(\text{M286A})\gamma_{2s}$ and GABA_A $\alpha_1\beta_2(\text{M286S})\gamma_{2s}$ receptors have a cut-off for propofol analogs similar to that for the wild-type GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptor, at least for the six propofol analogs tested (Fig. 7).

In the second set of experiments, the actions of propofol and five analogs were tested at the GABA_A $\alpha_1\beta_2(\text{M286W})\gamma_{2s}$ receptor. The M286W mutation results in a 0.064 nm³ increase in molecular volume at position 286. The two analogs with the smallest molecular volumes, 2,6-dimethylphenol (0.126 nm³) and 2-isopropylphenol (0.143 nm³), enhanced submaximal GABA currents at the GABA_A $\alpha_1\beta_2(\text{M286W})\gamma_{2s}$ receptor (Figs. 6 and 7; Table 2). In contrast, 2,6-diethylphenol (0.16 nm³), 2,6-diethylphenyl isothiocyanate (0.187 nm³), propofol (0.193 nm³), and 2,6-di-*tert*-butylphenol (0.227 nm³) all failed to potentiate submaximal GABA currents at the GABA_A $\alpha_1\beta_2(\text{M286W})\gamma_{2s}$ receptor (Figs. 2(D), 6 and 7; Table 2). Thus, the GABA_A $\beta_2(\text{M286W})$ mutation reduces the propofol analog cut-off for potentiation of GABA responses from 0.193 nm³ (propofol) to below 0.16 nm³ (2,6-diethylphenol) (Fig. 7).

4. Discussion

We explain these experimental data by proposing that mutation of M286 in the GABA_A receptor β_2 subunit alters the dimensions of a binding cavity for the general anesthetic propofol. We will first discuss our results in relation to those of other studies employing site-directed mutagenesis to probe sites of anesthetic action on GABA_A receptor subunits and then turn to discussion of the putative propofol binding cavity.

4.1. Contrasting effects of mutations within GABA_A α or β subunit isoforms

The effects of the GABA_A β_2 mutations described in this study contrast with previously described effects of mutations within the GABA_A α_2 subunit or the glycine α_1 subunits. The GABA_A β_2 (M286W) mutation, which abolishes potentiation of GABA responses by propofol, has only small effects on the potency of GABA or for direct activation by propofol at the GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptor (1.2 to 2.6-fold shifts of the respective concentration–response curves; see Table 1). In contrast, site-directed substitutions of GABA_A α_2 (S270) or α_2 (A291), or of glycine α_1 (S267) or α_1 (A288) (see Fig. 1), markedly affect *both* the ability of general anesthetics to potentiate receptor function *and* receptor gating by agonists or anesthetics (Ye et al., 1998; Krasowski et al., 1998a,b; Koltchine et al., 1999; Ueno et al., 1999, 2000; Yamakura et al., 1999).

More specifically, the molecular volume of the amino acid side chain at the GABA_A α_2 (S270), glycine α_1 (S267), or glycine α_1 (A288) positions correlates negatively with agonist EC₅₀ and with anesthetic potentiation of agonist responses (Ye et al., 1998; Koltchine et al., 1999; Yamakura et al., 1999). In contrast, the present study and others show that site-directed substitutions of the 265 or 286 positions of GABA_A β_1 or β_2 subunits do not produce any consistent alterations in agonist EC₅₀ (Krasowski et al., 1998a,b; Amin, 1999; Ueno et al., 1999). This may relate to the dominant role of GABA_A α subunits in determining agonist potency and efficacy (Levitan et al., 1988; Sigel et al., 1990; Ebert et al., 1994; O’Shea and Harrison, 2000).

4.2. Interpretation of the effects of site-directed mutations

There are a number of ways in which amino acid substitutions at β_2 (M286) could potentially disrupt the modulatory effects of propofol. First, the mutations could result in receptors that are already ‘maximally sensitive’ to GABA, such that propofol cannot shift the concentration–response relationship to GABA further to the left. This mechanism, which may apply to GABA_A receptors containing the β_2 (L259S) mutation (Thompson et al., 1999), is improbable in the GABA_A $\alpha_1\beta_2$ (M286W) γ_{2s} receptor due to the minimal alterations in EC₅₀ for GABA and the observation that etomidate and methohexital potentiate submaximal responses at this receptor (albeit with responses significantly smaller than at the wild-type GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptor).

A second possibility is that the mutated receptor is more easily gated by propofol, which might obscure the measurement of potentiation of GABA-evoked currents. This problem has been encountered in other studies of anesthetic effects on mutated GABA_A α_2 and β_2 receptor subunits (Krasowski et al., 1998b; Thompson et al., 1999). However, the GABA_A $\alpha_1\beta_2$ (M286W) γ_{2s} receptor analyzed in this study actually shows *less* direct activation by propofol concentrations below 20 μ M than the wild-type GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptor, discounting the possibility that direct activation obscures potentiation of GABA responses by propofol at this mutated receptor.

The remaining possibilities to explain the effects of the β_2 mutations are that the mutations disrupt the binding site for propofol or, alternatively, disrupt conformational change(s) of the GABA_A receptor that occur following the binding of propofol to the receptor. Distinguishing between these two possibilities is difficult because fine-resolution, three-dimensional structural data for the ligand-gated ion channels are not yet available. We therefore pursued an alternate approach, namely that of considering whether site-directed mutagenesis of β_2 (M286) altered the characteristics of a putative propofol binding cavity.

4.3. Probable characteristics of a propofol binding cavity in the GABA_A receptor

A number of investigators have suggested that general anesthetic molecules may exert their effects by binding fortuitously to cavities naturally present on proteins, thereby stabilizing certain conformational states such as the open and closed states of the GABA_A receptor ion channel (LaBella, 1981; Franks and Lieb, 1985; Williams and Akabas, 1999). Although GABA_A receptors and other ligand-gated ion channels have so far resisted attempts to determine high-resolution structures, there have been a number of studies which demonstrate general anesthetic binding to cavities in soluble proteins that serve as models for true anesthetic targets. The high resolution structures of firefly luciferase complexed with bromoform (Franks et al., 1998) and human serum albumin complexed with halothane and propofol (Bhattacharya et al., 2000) demonstrate striking examples of anesthetic binding to pre-existing amphiphilic protein cavities.

In addition, alkylphenol binding cavities have been demonstrated on a variety of proteins, including the enzymes phenol hydroxylase (Neujahr, 1990; Enroth et al., 1998), vanillyl-alcohol oxidase (Mattevi et al., 1997; van den Heuvel et al., 1998), and *p*-cresol methylhydroxylase (McIntire et al., 1985; Cunane et al., 2000). In these three enzymes, structural determination by X-ray crystallography has revealed hydrophobic cavities that selectively bind alkylphenols of specific shapes and sizes. For instance, the 0.2 nm³ catalytic cavity of vanillyl-alcohol oxidase is completely filled by the competitive inhibitor *p*-(1-heptenyl)phenol, which explains why alkylphenols bearing aliphatic substituents longer than seven carbon atoms at the *para* position do not bind to the enzyme (Mattevi et al., 1997; van den Heuvel et al., 1998). The substrate binding cavity of phenol hydroxylase has a shape and size that can accommodate 4-*tert*-butylphenol but not 2-isopropylphenol (Enroth et al., 1998; Neujahr, 1990).

Much like the structure–affinity relationships for alkylphenols at the three enzymes described above, the structure–activity relationships for propofol analogs for modulating GABA_A receptors or producing loss of righting reflex in *Xenopus laevis* tadpoles exhibit distinct ligand requirements, for which steric factors seem to play a major role (Krasowski et al., 2001). For example, propofol (molecular volume=0.193 nm³) and 2,6-diethylphenyl isothiocyanate (0.187 nm³) are ‘active’, whereas 2,6-dicyclopentylphenol (0.24 nm³) and 2,6-diisopropylphenyl isocyanate (0.211 nm³) are completely inactive. These structure–activity relationships are consistent with the hypothesis that propofol analogs interact with a binding cavity of limited dimensions on the GABA_A receptor, similar perhaps to the alkylphenol binding cavities on phenol hydroxylase, vanillyl-alcohol oxidase, or *p*-cresol methylhydroxylase.

4.4. Alteration of the cut-off for propofol analogs by mutation of the GABA_A β₂ subunit

If β₂(M286) is indeed part of a binding cavity for propofol, then mutagenesis of this residue might alter the specificity of propofol analogs that can modulate GABA_A receptor function. This is precisely what happened, with the propofol analog cut-off for potentiation of GABA responses reduced from 0.193 nm³ (propofol) in the wild-type GABA_A α₁β₂γ_{2s} receptor to below 0.16 nm³ (2,6-diethylphenol) in the GABA_A α₁β₂(M286W)γ_{2s} receptor (Fig. 6). In this context, it is of interest to consider studies of the catalytic pockets of alcohol dehydrogenase (Weinhold and Benner, 1995) and *L*-hydroxyisocaproate dehydrogenase (Feil et al., 1997). In both of these enzymes, it is possible to alter substrate specificity by site-directed mutagenesis of amino acid residues lining the substrate binding pocket.

Future studies will continue to elucidate the role of the amino acid residues involved in the actions of anesthetics. Even more exciting, perhaps, is the use of targeted gene manipulations in mice to test the relevance of certain ligand-gated ion channels in mediating the behavioral

actions of general anesthetics (Belelli et al., 1999; Krasowski and Harrison, 1999). The GABA_A receptor β_2 (M286W) mutation described in this study may be an ideal candidate for the creation of knock-in mice, in which the endogenous β_2 subunit is replaced by the mutated, anesthetic-insensitive subunit.

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References

- Amin J. A single hydrophobic residue confers barbiturate sensitivity to γ -aminobutyric acid type C receptor. *Molecular Pharmacology* 1999;55:411–423. [PubMed: 10051524]
- Bai D, Pennefather PS, MacDonald JF, Orser BA. The general anesthetic propofol slows deactivation and desensitization of GABA_A receptors. *Journal of Neuroscience* 1999;19:10635–10646. [PubMed: 10594047]
- Bai D, Zhu G, Pennefather PS, Jackson MF, MacDonald JF, Orser BA. Distinct functional and pharmacological properties of tonic and quantal inhibitory postsynaptic currents mediated by γ -aminobutyric acid_A receptors in hippocampal neurons. *Molecular Pharmacology* 2001;59:814–824. [PubMed: 11259626]
- Belelli D, Pistis M, Peters JA, Lambert JJ. General anaesthetic action at transmitter-gated inhibitory amino acid receptors. *Trends in Pharmacological Sciences* 1999;20:496–502. [PubMed: 10603492]
- Bhattacharya AA, Curry S, Franks NP. Binding of the general anesthetics propofol and halothane to human serum albumin: high-resolution crystal structures. *Journal of Biological Chemistry* 2000;275:38731–38738. [PubMed: 10940303]
- Cunane LM, Chen ZW, Shamala N, Mathews FS, Cronin CN, McIntire WS. Structures of the flavocytochrome *p*-cresol methylhydroxylase and its enzyme substrate complex: gated substrate entry and proton relays support the proposed catalytic mechanism. *Journal of Molecular Biology* 2000;295:357–374. [PubMed: 10623531]
- Cutting GR, Lu L, O'Hara BF, Kasch LM, Montrose-Rafizadeh C, Donovan DM, Shimada S, Antonarakis SE, Guggino WB, Uhl GR, Kazazian HH. Cloning of the γ -aminobutyric acid (GABA) $\rho 1$ cDNA: a GABA receptor subunit highly expressed in the retina. *Proceedings of the National Academy of Sciences of the United States of America* 1991;88:2673–2677. [PubMed: 1849271]
- Dwyer DS, Bradley RJ. Chemical properties of alcohols and their protein binding sites. *Cellular and Molecular Life Sciences* 2000;57:265–275. [PubMed: 10766022]
- Ebert B, Wafford KA, Whiting PJ, Krosgaard-Larsen P, Kemp JA. Molecular pharmacology of γ -aminobutyric acid type A receptor agonists and partial agonists in oocytes injected with different α , β , and γ receptor subunit combinations. *Molecular Pharmacology* 1994;46:957–963. [PubMed: 7969086]
- Enroth C, Neujahr H, Schneider G, Lindqvist Y. The crystal structure of phenol hydroxylase in complex with FAD and phenol provides evidence for a concerted conformational change in the enzyme and its cofactor during catalysis. *Structure* 1998;6:605–617. [PubMed: 9634698]
- Feil IK, Hendle J, Schomburg D. Modified substrate specificity of *L*-hydroxyisocaproate dehydrogenase derived from structure-based protein engineering. *Protein Engineering* 1997;10:255–262. [PubMed: 9153075]
- Franks NP, Jenkins A, Conti E, Lieb WR, Brick P. Structural basis for the inhibition of firefly luciferase by a general anesthetic. *Biophysical Journal* 1998;75:2205–2211. [PubMed: 9788915]
- Franks NP, Lieb WR. The firefly throws light on anesthesia. *Chemistry in Britain* 1985;21:919–921.
- Franks NP, Lieb WR. Molecular and cellular mechanisms of general anaesthesia. *Nature* 1994;367:607–614. [PubMed: 7509043]

- Grenningloh G, Rienitz A, Schmitt B, Methfessel C, Zensen M, Beyreuther K, Gundelfinger ED, Betz H. The strychnine binding subunit of the glycine receptor shows homology with nicotinic acetylcholine receptors. *Nature* 1987;328:215–220. [PubMed: 3037383]
- Hadingham KL, Wingrove P, Le Bourdelles B, Palmer KJ, Ragan CI, Whiting PJ. Cloning of cDNA sequences encoding human α_2 and α_3 γ -aminobutyric acid_A receptor subunits and characterization of the benzodiazepine pharmacology of recombinant α_1 -, α_2 -, α_3 -, and α_5 -containing human γ -aminobutyric acid_A receptors. *Molecular Pharmacology* 1993a;43:970–975. [PubMed: 8391122]
- Hadingham KL, Wingrove PB, Wafford KA, Bain C, Kemp JA, Palmer KJ, Wilson AW, Wilcox AS, Sikela JM, Ragan CI, Whiting PJ. Role of the β subunit in determining the pharmacology of human γ -aminobutyric acid type A receptors. *Molecular Pharmacology* 1993b;44:1211–1218. [PubMed: 8264558]
- Hales TG, Lambert JJ. The actions of propofol on inhibitory amino acid receptors of bovine adrenomedullary chromaffin cells and rodent central neurones. *British Journal of Pharmacology* 1991;104:619–628. [PubMed: 1665745]
- Hara M, Kai Y, Ikemoto Y. Propofol activates GABA_A receptor-chloride ionophore complex in dissociated hippocampal pyramidal neurons of the rat. *Anesthesiology* 1993;79:781–788. [PubMed: 8214758]
- Harpaz Y, Gerstein M, Chothia C. Volume changes on protein folding. *Structure* 1994;2:641–649. [PubMed: 7922041]
- Hopp TP, Woods KR. Prediction of protein antigenic determinants from amino acid sequences. *Proceedings of the National Academy of Sciences of the United States of America* 1981;78:3824–3828. [PubMed: 6167991]
- James R, Glen JB. Synthesis, biological evaluation, and preliminary structure-activity considerations of a series of alkylphenols as intravenous anesthetic agents. *Journal of Medicinal Chemistry* 1980;23:1350–1357. [PubMed: 7452689]
- Jenkins A, Greenblatt EP, Faulkner HJ, Bertaccini E, Light A, Lin A, Andreassen A, Viner A, Trudell JR, Harrison NL. Evidence for a common binding cavity for three general anesthetics within the GABA_A receptor. *Journal of Neuroscience* 2001;21 (RC-136):1–4.
- Koltchine VV, Finn SE, Jenkins A, Nikolaeva N, Lin A, Harrison NL. Agonist gating and isoflurane potentiation in the human γ -aminobutyric acid type A receptor determined by the volume of a TM2 residue. *Molecular Pharmacology* 1999;56:1087–1093. [PubMed: 10531417]
- Krasowski MD, Finn SE, Ye Q, Harrison NL. Trichloroethanol modulation of recombinant GABA_A, glycine, and GABA ρ_1 receptors. *Journal of Pharmacology and Experimental Therapeutics* 1998a;284:934–942. [PubMed: 9495852]
- Krasowski MD, Harrison NL. General anaesthetic actions on ligand-gated ion channels. *Cellular and Molecular Life Sciences* 1999;55:1278–1303. [PubMed: 10487207]
- Krasowski MD, Harrison NL. The actions of ether, alcohol, and alkane general anaesthetics on GABA_A and glycine receptors and the effects of TM2 and TM3 mutations. *British Journal of Pharmacology* 2000;129:731–743. [PubMed: 10683198]
- Krasowski MD, Jenkins A, Flood P, Kung AY, Hopfinger AJ, Harrison NL. The general anesthetic potencies of a series of propofol analogs correlate with potency for potentiation of GABA current at the GABA_A receptor but not with lipid solubility. *Journal of Pharmacology and Experimental Therapeutics* 2001;297:338–351. [PubMed: 11259561]
- Krasowski MD, Koltchine VV, Rick CE, Ye Q, Finn SE, Harrison NL. Propofol and other intravenous anesthetics have sites of action on the γ -aminobutyric acid_A receptor distinct from that for isoflurane. *Molecular Pharmacology* 1998b;53:530–538. [PubMed: 9495821]
- Kyte J, Doolittle RF. A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* 1982;157:105–132. [PubMed: 7108955]
- LaBella FS. Is there a general anesthesia receptor? *Canadian Journal of Physiology and Pharmacology* 1981;59:432–442. [PubMed: 6263435]
- Leviton ES, Blair LA, Dionne VE, Barnard EA. Biophysical and pharmacological properties of cloned GABA_A receptor subunits expressed in *Xenopus* oocytes. *Neuron* 1988;1:773–781. [PubMed: 2856103]

- Li C, Peoples RW, Weight FF. Alcohol action on a neuronal membrane receptor: evidence for a direct interaction with the receptor protein. *Proceedings of the National Academy of Sciences of the United States of America* 1994;91:8200–8204. [PubMed: 8058780]
- Mascia MP, Machu TK, Harris RA. Enhancement of homomeric glycine receptor function by long-chain alcohols and anaesthetics. *British Journal of Pharmacology* 1996;119:1331–1336. [PubMed: 8968539]
- Mattevi A, Fraaije MW, Mozzarelli A, Olivi L, Coda A, van Berkel WJ. Crystal structures and inhibitor binding in the octameric flavoenzyme vanillyl-alcohol oxidase: the shape of the active-site cavity controls substrate specificity. *Structure* 1997;5:907–920. [PubMed: 9261083]
- McIntire W, Hopper DJ, Singer TP. *p*-Cresol methylhydroxylase: assay and general properties. *Biochemical Journal* 1985;228:325–335. [PubMed: 2990444]
- Mihic SJ, Harris RA. Inhibition of $\rho 1$ receptor GABAergic currents by alcohols and volatile anesthetics. *Journal of Pharmacology and Experimental Therapeutics* 1996;277:411–416. [PubMed: 8613949]
- Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, Mascia MP, Valenzuela CF, Hanson KK, Greenblatt EP, Harris RA, Harrison NL. Molecular sites of alcohol and volatile anaesthetic action on GABA_A and glycine receptors. *Nature* 1997;389:385–389. [PubMed: 9311780]
- Neujahr, HY. Phenol hydroxylase. In: Nuller, F., editor. *Chemistry and Biochemistry of Flavoenzymes*. 2. CRC Press; Boca Raton, FL, USA: 1990. p. 65-85.
- O'Shea SM, Harrison NL. Arg 274 and Leu 277 of the GABA_A receptor $\alpha 2$ subunit define agonist efficacy and potency. *Journal of Biological Chemistry* 2000;275:22764–22768. [PubMed: 10801806]
- Peoples RW, Weight FF. Cutoff in potency implicates alcohol inhibition of *N*-methyl-D-aspartate receptors in alcohol intoxication. *Proceedings of the National Academy of Sciences of the United States of America* 1995;92:2825–2829. [PubMed: 7708732]
- Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR, Seeburg PH. Importance of a novel GABA_A receptor subunit for benzodiazepine pharmacology. *Nature* 1989;338:582–585. [PubMed: 2538761]
- Schofield PR, Pritchett DB, Sontheimer H, Kettenmann H, Seeburg PH. Sequence and expression of human GABA_A $\alpha 1$ and $\beta 1$ subunits. *FEBS Letters* 1989;244:361–364. [PubMed: 2465923]
- Sigel E, Baur R, Trube G, Mohler H, Malherbe P. The effect of subunit composition of rat brain GABA_A receptors on channel function. *Neuron* 1990;5:703–711. [PubMed: 1699569]
- Thompson SA, Smith MZ, Wingrove PB, Whiting PJ, Wafford KA. Mutation at the putative GABA_A ion-channel gate reveals changes in allosteric modulation. *British Journal of Pharmacology* 1999;127:1349–1358. [PubMed: 10455284]
- Trapani G, Altomare C, Sanna E, Biggio G, Liso G. Propofol in anesthesia. Mechanism of action, structure-activity relationships, and drug delivery. *Current Medicinal Chemistry* 2000;7:249–271. [PubMed: 10637364]
- Ueno S, Lin A, Nikolaeva N, Trudell JR, Mihic SJ, Harris RA, Harrison NL. Tryptophan scanning mutagenesis in TM2 of the GABA_A receptor α subunit: effects on channel gating and regulation by ethanol. *British Journal of Pharmacology* 2000;131:296–302. [PubMed: 10991923]
- Ueno S, Wick MJ, Ye Q, Harrison NL, Harris RA. Subunit mutations affect ethanol actions on GABA_A receptors expressed in *Xenopus* oocytes. *British Journal of Pharmacology* 1999;127:377–382. [PubMed: 10385236]
- van den Heuvel RHH, Fraaije MW, Laane C, van Berkel WJH. Regio- and stereospecific conversion of 4-alkylphenols by the covalent flavoprotein vanillyl-alcohol oxidase. *Journal of Bacteriology* 1998;180:5646–5651. [PubMed: 9791114]
- Weinhold EG, Benner SA. Engineering yeast alcohol dehydrogenase. Replacing Trp54 by Leu broadens substrate specificity. *Protein Engineering* 1995;8:457–461. [PubMed: 8532667]
- Wick MJ, Mihic SJ, Ueno S, Mascia MP, Trudell JR, Brozowski SJ, Ye Q, Harrison NL, Harris RA. Mutations of γ -aminobutyric acid and glycine receptors change alcohol cutoff: evidence for an alcohol receptor? *Proceedings of the National Academy of Sciences of the United States of America* 1998;95:6504–6509. [PubMed: 9600996]
- Williams DB, Akabas MH. γ -aminobutyric acid increases the water accessibility of M3 membrane-spanning segment residues in γ -aminobutyric acid type A receptor. *Biophysical Journal* 1999;77:2563–2574. [PubMed: 10545357]

- Yamakura T, Mihic SJ, Harris RA. Amino acid volume and hydropathy of a transmembrane site determine glycine and anesthetic sensitivity of glycine receptors. *Journal of Biological Chemistry* 1999;274:23006–23012. [PubMed: 10438467]
- Ye Q, Koltchine VV, Mihic SJ, Mascia MP, Wick M, Finn SE, Harrison NL, Harris RA. Enhancement of glycine receptor function by ethanol is inversely correlated with molecular volume at position $\alpha 267$. *Journal of Biological Chemistry* 1998;273:3314–3319. [PubMed: 9452448]
- Ymer S, Schofield PR, Draguhn A, Werner P, Kohler M, Seeburg PH. GABA_A receptor β subunit heterogeneity: functional expression of cloned cDNAs. *EMBO Journal* 1989;8:1665–1670. [PubMed: 2548852]
- Zimmerman JM, Eliezer N, Simha R. The characterization of amino acid sequences in proteins by statistical methods. *Journal of Theoretical Biology* 1968;21:170–201. [PubMed: 5700434]

		TM2		TM3
Gly α_1	250	PARVGLGITT VLTMTTQ SSG ²⁶⁹ . . .	282	AIDIWM AVC LLFVFSALLE ³⁰⁰
GABA _A α_1	253	PARTVFGVTT VLTMTTL SIS ²⁷² . . .	285	AMDWFI AVC YAFVFSALIE ³⁰³
GABA _A α_2	253	PARTVFGVTT VLTMTTL SIS ²⁷² . . .	285	AMDWFI AVC YAFVFSALIE ³⁰³
GABA _A β_1	248	AARVALGITT VLTMTTI STH ²⁶⁷ . . .	280	AIDIYLMGC FVFVFLALLE ²⁹⁸
GABA _A β_2	248	AARVALGITT VLTMTTI NTH ²⁶⁷ . . .	280	AIDMYLMGC FVFVFMALLE ²⁹⁸
GABA _C ρ_1	290	PARVPLGITT VLTMTSTI ITG ³⁰⁹ . . .	322	AVDIYLVWS FVFVFLSVLE ³⁴⁰

Fig. 1.

Amino acid sequence alignment of TM2 and TM3 from human glycine α_1 (Grenningloh et al., 1987), GABA_A α_1 (Schofield et al., 1989), α_2 (Hadingham et al., 1993a), β_1 (Schofield et al., 1989), rat or human GABA_A β_2 (Ymer et al., 1989; Hadingham et al., 1993b), and human GABA_A ρ_1 receptor subunits (Cutting et al., 1991). Residue positions in **bold type** within TM2 and TM3 of glycine α_1 (S267 and A288), GABA_A α_1 and α_2 (S270 and A291), GABA_A β_1 (S265 and M286), and GABA_A β_2 (N265 and M286) receptor subunits are critical for potentiation of agonist responses by alcohol, alkane, and ether anesthetics (Mihic et al., 1997; Ye et al., 1998; Krasowski et al., 1998a; Koltchine et al., 1999; Ueno et al. 1999, 2000; Yamakura et al., 1999; Krasowski and Harrison, 2000; Jenkins et al., 2001). GABA_A β_1 (M286) is necessary for potentiation of GABA responses by propofol (Krasowski et al., 1998b). GABA_A β_2 (M286) is the main residue position of interest for the current study. GABA_A α_1 (S270), α_1 (A291), and β_2 (N265) are also considered in this paper.

Fig. 2.

GABA_A $\alpha_1\beta_2\gamma_{2s}$ and $\alpha_1\beta_2(\text{M286W})\gamma_{2s}$ receptors have similar sensitivity to GABA, but the $\alpha_1\beta_2(\text{M286W})\gamma_{2s}$ receptor is insensitive to the modulatory effects of propofol. (A) and (B) Traces from individual HEK 293 cells expressing either GABA_A $\alpha_1\beta_2\gamma_{2s}$ or GABA_A $\alpha_1\beta_2(\text{M286W})\gamma_{2s}$ receptors in response to application of 2, 5, 20, 500, and 1000 μM GABA. Time of application is indicated by the bars above the traces. (C) Submaximal (EC_{20}) GABA current responses at the wild-type GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptor are enhanced by co-application of 0.2, 1, or 5 μM propofol. Note also the direct activation by 1 and 5 μM propofol, evident during the pre-application of propofol. (D) In contrast, propofol does not potentiate submaximal GABA currents at the GABA_A $\alpha_1\beta_2(\text{M286W})\gamma_{2s}$ receptor, but does produce direct receptor

activation at a concentration of 10 μ M. Traces shown in (A)–(D) are recordings from individual HEK 293 cells transfected with cDNAs encoding the indicated receptor subunit combinations.

Fig. 3. Concentration–response curves for GABA at the wild-type $\text{GABA}_A \alpha_1\beta_2\gamma_{2s}$ receptor and the $\alpha_1\beta_2(\text{M286X})\gamma_{2s}$ series of mutant receptors (see Table 1 for parameters derived by curve fitting to the pooled data).

Fig. 4.

Concentration–response relationships for potentiation of GABA responses by propofol at the wild-type $\text{GABA}_A \alpha_1\beta_2\gamma_{2s}$ receptor and the $\alpha_1\beta_2(\text{M286X})\gamma_{2s}$ series of mutant receptors. (A) Propofol potentiates GABA responses at $\text{GABA}_A \alpha_1\beta_2\gamma_{2s}$, $\alpha_1\beta_2(\text{M286A})\gamma_{2s}$, $\alpha_1\beta_2(\text{M286F})\gamma_{2s}$, and $\alpha_1\beta_2(\text{M286Y})\gamma_{2s}$ receptors but fails to enhance GABA responses at $\text{GABA}_A \alpha_1\beta_2(\text{M286W})\gamma_{2s}$ receptors. (B) Propofol potentiates GABA responses at $\text{GABA}_A \alpha_1\beta_2\gamma_{2s}$, $\alpha_1\beta_2(\text{M286C})\gamma_{2s}$, $\alpha_1\beta_2(\text{M286E})\gamma_{2s}$, $\alpha_1\beta_2(\text{M286K})\gamma_{2s}$, and $\alpha_1\beta_2(\text{M286S})\gamma_{2s}$ receptors (see Table 1 for parameters derived by curve fitting to the pooled data).

Fig. 5. Concentration–response relationships for direct activation by propofol at the wild-type GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptor and the $\alpha_1\beta_2(\text{M286X})\gamma_{2s}$ series of mutant receptors (see Table 1 for parameters derived by curve fitting to the pooled data).

Fig. 6.

The GABA_A β_2 (M286W) mutation alters the cut-off for potentiation of GABA responses by propofol analogs at the GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptor. (A) The ‘small’ propofol analogs 2,6-dimethylphenol and 2-isopropylphenol (molecular volumes=0.126 and 0.143 nm³, respectively) potentiate submaximal (EC₂₀) GABA responses at the GABA_A $\alpha_1\beta_2$ (M286W) γ_{2s} receptor. (B) In contrast, the larger propofol analogs 2,6-diethylphenol (0.16 nm³) and 2,6-diethylphenyl isothiocyanate (0.187 nm³) fail to enhance submaximal GABA responses at the GABA_A $\alpha_1\beta_2$ (M286W) γ_{2s} receptor. Traces shown in (A) and (B) are recordings from individual HEK 293 cells transfected with cDNAs encoding the GABA_A α_1 , β_2 (M286W), and γ_{2s} receptor subunits.

	Molecular Volume (nm ³)	GABA _A $\alpha_1\beta_2\gamma_{2s}$ Wild-type	β_2 (M286A) and β_2 (M286S)	β_2 (M286W)
2,6-dimethylphenol	0.126	+++	+++	+++
2-isopropylphenol	0.143	+++	+++	+++
<div style="display: flex; align-items: center;"> <div style="margin-right: 10px;"> </div> Altered cut-off </div>
2,6-diethylphenol	0.16	+++	+++	no effect
2,6-diethylphenyl isothiocyanate	0.187	+++	+++	no effect
Propofol	0.193	+++	+++	no effect
<div style="display: flex; align-items: center;"> <div style="margin-right: 10px;"> </div> Wild-type cut-off </div>
2,6-di- <i>tert</i> -butylphenol	0.227	no effect	no effect	no effect

Fig. 7.

Summary of the effects of six propofol analogs on the wild-type GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptor and the GABA_A $\alpha_1\beta_2$ (M286A) γ_{2s} , $\alpha_1\beta_2$ (M286S) γ_{2s} , and $\alpha_1\beta_2$ (M286W) γ_{2s} receptors. The wild-type GABA_A $\alpha_1\beta_2\gamma_{2s}$ receptor supports potentiation of GABA responses by 2,6-dimethylphenol, 2-isopropylphenol, 2,6-diethylphenol, 2,6-diethylphenyl isothiocyanate, and propofol, but not the bulkier analog 2,6-di-*tert*-butylphenol. The mutation of methionine 286 to the smaller alanine or serine residues does not change this profile. On the other hand, the β_2 (M286W) mutation results in a receptor now insensitive to 2,6-diethylphenol, 2,6-diethylphenyl isothiocyanate, and propofol, as well as 2,6-di-*tert*-butylphenol. Thus, the molecular volume cut-off for propofol potentiation of GABA responses is reduced from 0.193 nm³ in the GABA_A $\alpha_1\beta_2\gamma_{2s}$, $\alpha_1\beta_2$ (M286A) γ_{2s} , and $\alpha_1\beta_2$ (M286S) γ_{2s} receptors ('Wildtype cut-off', single arrow) to less than 0.16 nm³ in the GABA_A $\alpha_1\beta_2$ (M286W) γ_{2s} receptor ('Altered cut-off', double arrow).

Table 1

Summary data of concentration-response relationships for GABA activation and for potentiation of GABA responses and direct activation by propofol at wild-type and mutant GABA_A receptors expressed in HEK 293 cells^a

GABA activation				Propofol potentiation of GABA responses					Propofol direct activation				
GABA _A receptor combination	EC ₅₀ (μM)	Hill slope	n _{cells}	Maximal GABA response (pA)	n _{cells}	EC ₅₀ (μM)	Hill slope	E _{max}	n _{cells}	EC ₅₀ (μM)	Hill slope	E _{max}	n _{cells}
α ₁ β ₂ γ _{2s}	29.5±2.4	1.4±0.1	7	631±54	300	1.5±0.2	1.8±0.5	241±16	16	10.4±0.8	1.4±0.1	59.8±1.6	10–16
α ₁ β ₂ (N265W)γ _{2s}	9.1±1.1***	1.4±0.2	8	468±172	8	2.3±1.1	1.2±0.5	205±39	5	12.4±1.8	1.3±0.2	32.9±1.5***	5
α ₁ β ₂ (M286A)γ _{2s}	10.6±0.8***	1.2±0.1	7	447±172	25	2.3±0.4	2.0±0.5	149±10**	6	11.7±1.0	2.7±0.6	51.2±3.9	4
α ₁ β ₂ (M286C)γ _{2s}	132±12***	1.1±0.1	6	346±51	19	1.5±0.1	3.7±0.7	126±5***	6	41.8±10.8*	2.2±0.9	41.0±5.9	6
α ₁ β ₂ (M286E)γ _{2s}	13.6±1.1***	1.1±0.1	9	519±95	22	4.2±0.7*	2.4±0.8	77±7.7***	6	49.3±2.2***	1.8±0.1	49.3±3.8	6
α ₁ β ₂ (M286F)γ _{2s}	11.9±0.6***	1.7±0.1	9	315±58*	9	2.6±0.2*	1.7±0.2	285±9.0	7	27.7±1.8***	1.4±0.1	53.5±1.3	5
α ₁ β ₂ (M286K)γ _{2s}	98.9±3.8***	1.6±0.1	7	371±36*	17	2.6±0.6	2.8±1.3	98±10***	6	51.7±4.9***	3.0±0.8	68.0±4.7	5
α ₁ β ₂ (M286S)γ _{2s}	24.7±2.4	1.0±0.1	7	570±79	20	6.8±0.6***	2.3±0.4	239±16	6	9.0±1.4	2.0±0.6	50.6±3.0	5
α ₁ β ₂ (M286W)γ _{2s}	24.8±2.6	1.3±0.2	7	508±118	41	none***	N/A	N/A	7	24.1±3.5*	2.7±0.9	51.5±5.5	11
α ₁ β ₂ (M286Y)γ _{2s}	60.0±4.8***	1.5±0.1	10	441±62	10	3.9±0.5***	2.4±0.5	102±7***	9	14.8±2.2	1.7±0.3	49.3±2.2*	5
α ₁ (S270L, A291W)β ₂ γ _{2s}	1.2±0.1***	1.1±0.1	5	250±82**	5	2.0±0.2***	2.3±0.4	141±5***	5	N/A	N/A	N/A	N/A

^aThe EC₅₀, Hill slope, and maximal effect values are presented as mean±SE. n_{cells} indicates number of cells contributing to mean values.

* P<0.05,

** P<0.01,

*** P<0.0001 compared with response in wild-type GABA_A α1β2γ2s receptor combination.

Table 2

Molecular volume of propofol and related alkylphenol analogs and their effects on wild-type and mutated GABA_A $\alpha_1\beta_2\gamma_{2S}$ receptors ^a

Propofol analog	Molecular volume (nm ³)	Concentration (μ M)	GABA _A $\alpha_1\beta_2\gamma_{2S}$	GABA _A $\alpha_1\beta_2$ (M286A) γ_{2S}	GABA _A $\alpha_1\beta_2$ (M286S) γ_{2S}	GABA _A $\alpha_1\beta_2$ (M286W) γ_{2S}
2,6-Dimethylphenol	0.447	200	131 $\pm$ 12	78 $\pm$ 13	90 $\pm$ 15	49 $\pm$ 11 ^{**}
		500	199 $\pm$ 49	170 $\pm$ 29	166 $\pm$ 33	47 $\pm$ 10
2-Isopropylphenol	0.495	100	53 $\pm$ 13	49 $\pm$ 11	32 $\pm$ 10	80 $\pm$ 21
		200	78 $\pm$ 26	80 $\pm$ 16	67 $\pm$ 19	45 $\pm$ 22
2,6-Diethylphenol	0.548	20	175 $\pm$ 56	168 $\pm$ 47	133 $\pm$ 21	-3 $\pm$ 6 ^{b**}
		50	210 $\pm$ 58	222 $\pm$ 51	201 $\pm$ 29	2 $\pm$ 5 ^{b**}
2,6-Diethylphenyl isothiocyanate	0.638	50	172 $\pm$ 41	129 $\pm$ 25	157 $\pm$ 35	-2 $\pm$ 4 ^{b**}
		100	184 $\pm$ 27	190 $\pm$ 30	200 $\pm$ 31	2 $\pm$ 5 ^{b**}
Propofol	0.641	2	133 $\pm$ 28	52 $\pm$ 14	75 $\pm$ 34	-3 $\pm$ 3 ^{b**}
		5	179 $\pm$ 31	123 $\pm$ 31	172 $\pm$ 33	-4 $\pm$ 2 ^{b**}
2,6-Di-<i>tert</i>-butylphenol	0.711	100	-2 $\pm$ 3 ^b	0 $\pm$ 3 ^b	2 $\pm$ 4 ^b	-2 $\pm$ 2 ^b
		200	1 $\pm$ 4 ^b	-3 $\pm$ 3 ^b	0 $\pm$ 2 ^b	-1 $\pm$ 5 ^b

^a Molecular volume is a calculated parameter for each of the analogs (see Section 2). The other values indicate mean $\pm$ SE for percent potentiation of an EC₂₀ test concentration of GABA by the compound, with data from 5 to 12 cells contributing to each mean value.

^b Compound did not produce potentiation of GABA responses at the indicated concentration (i.e. effect did not differ significantly from no alteration of control GABA response).

* P<0.05,

** P<0.001 compared with response in wild-type GABA_A $\alpha_1\beta_2\gamma_{2S}$ receptor combination.