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A Conserved Tyrosine in the β_2 Subunit M4 Segment Is a Determinant of γ-Aminobutyric Acid Type A Receptor Sensitivity to Propofol

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Background: **The γ-aminobutyric acid type A receptor ($GABA_A$-R) β subunits are critical targets for the actions for several intravenous general anesthetics, but the precise nature of the anesthetic binding sites are unknown. In addition, little is known about the role the fourth transmembrane (M4) segment of the receptor plays in receptor function. The aim of this study was to better define the propofol binding site on the $GABA_A$-R by conducting a tryptophan scan in the M4 segment of the β_2 subunit.**

Methods: **Seven tryptophan mutations were introduced into the C-terminal end of the M4 segment of the $GABA_A$-R β_2 subunit. $GABA_A$-R subunit complementary DNAs were transfected into human embryonic kidney 293 cells grown on glass coverslips. After transfection (36–72 h), coverslips were transferred to a perfusion chamber to assay receptor function. Cells were whole cell patch clamped and exposed to GABA, propofol, etomidate, and pregnenolone. Chemicals were delivered to the cells using two 10-channel infusion pumps and a rapid solution exchanger.**

Results: **All tryptophan mutations were well tolerated, and with one exception, all resulted in minimal changes in receptor activation by GABA. One mutation, β_2(Y444W), selectively suppressed the ability of propofol to enhance receptor function while retaining normal sensitivity to etomidate and pregnenolone.**

Conclusions: **This is the first report of a mutation that selectively reduces propofol sensitivity without altering the action of etomidate. The reduction in propofol sensitivity is consistent with the loss of a hydrogen bond within the propofol binding site. These results also suggest a possible orientation of the propofol molecule within its binding site.**

SINCE its discovery more than a century ago, general anesthesia has expanded the scope of medical intervention. It continues to broaden therapeutic possibilities as technologic and chemical advances improve both efficacy and safety. Despite its impact on medicine and the world, how general anesthetics work has been a mystery and has only recently begun to be understood. Studies over the past 30 yr have pointed to the γ-aminobutyric acid type A receptor ($GABA_A$-R) as one of the important proteins in producing the effect we describe as general anesthesia.[1] $GABA_A$-Rs subserve fast synaptic inhibition mediated by γ-aminobutyric acid (GABA), the most common inhibitory neurotransmitter in the central nervous system. $GABA_A$-Rs are ligand-gated ion channels, constructed from five subunits. Although there are more than half a million possible subunit combinations, 20 subunit combinations account for approximately 99% of the $GABA_A$-Rs in the central nervous system. The most prevalent receptor subtype in synapses of the adult mammalian central nervous system (accounting for approximately 40% of the total complement of $GABA_A$-Rs) is $\alpha_1\beta_2\gamma_2$.[2–4]

$GABA_A$-R function is modulated by most general anesthetics.[5] Using chimeric subunits and site-directed mutagenesis, four amino acid residues have been identified within the α subunit, one in each of the transmembrane segments: M1 (leucine 232), M2 (serine 270), M3 (alanine 291), and M4 (tyrosine 411).[6–8] All have been shown to be critical for inhaled anesthetic sensitivity because at all four positions, the substitution of a small amino acid with a larger residue, such as tryptophan, blocks the action of the halogenated general anesthetics isoflurane and halothane.[6–9] Similarly, amino acid residues critical for receptor modulation by furosemide and intravenous anesthetics[10–13] have been identified within the transmembrane segments of the β subunit: M1 (glycine 219), M2 (asparagine 265), and M3 (methionine 286). Guided by the high degree of homology between the α and β subunits, along with positive findings in previous studies using a similar technique,[8] we elected to perform a tryptophan scan through the M4 segment of the β_2 subunit. We hypothesized that mutating one or more amino acids in this segment would alter the action of intravenous general anesthetics on $GABA_A$-R function, thus better defining a general anesthetic binding site on a ligand gated ion channel.

Materials and Methods

Point mutations in the $GABA_A$-R β_2 subunit complementary DNA (cDNA) were created using the QuikChange®

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site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutations were confirmed by double-stranded DNA sequencing. Wild-type or mutant $GABA_A$-R cDNAs (gift from Neil L. Harrison, Ph.D., Professor, Department of Anesthesiology, Weill Cornell Medical College, New York, NY) were expressed *via* the vector pCIS2 in human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA) as previously described.[14,15] HEK 293 cells were maintained in culture on poly-D-lysine–treated glass coverslips in a solution containing Eagle minimum essential medium supplemented with 5% fetal bovine serum (Hyclone, Logan, UT), L-glutamine (0.292 μg/ml), penicillin G sodium (100 U/ml), and streptomycin sulfate (100 mg/ml). Cells were passaged weekly by trypsin treatment up to 20 times before being discarded and replaced with early passage cells. For the transient expression of $GABA_A$-Rs, cells were transfected as described previously using the $CaPO_4$ precipitation technique.[14–16]

The coverslips were transferred 48–72 h after cDNA removal to a recording chamber and perfused continuously with extracellular solution (145 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 6 mM D-glucose, and 10 mM HEPES–NaOH adjusted to pH 7.4). Whole cell patch clamp recordings from fluorescing HEK 293 cells (voltage clamped at −60 mV) were made using the Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) as described previously.[14] The resistance of the patch pipette was 4–6 MΩ when filled with intracellular solution (145 mM *N*-methyl-D-glucamine hydrochloride, 5 mM dipotassium ATP, 1.1 mM EGTA, 2 mM $MgCl_2$, 5 mM HEPES–KOH, and 0.1 mM $CaCl_2$ adjusted to pH 7.2). In addition to the continuous bath perfusion with extracellular medium, solutions including GABA and/or modulators were applied rapidly to the cell by local perfusion using a motor-driven solution exchange device (Rapid Solution Changer RSC-160; Molecular Kinetics, Indianapolis, IN). Solutions were exchanged within approximately 50 ms. Laminar flow out of the rapid solution changer head was achieved by driving all solutions at identical flow rates (1.0 ml/min) *via* a multichannel infusion pump (KD Scientific, Holliston, MA). The solution changer was driven by protocols in the acquisition program of pCLAMP version 9.2 (Molecular Devices).

For GABA concentration–response studies, cells were typically superfused with extracellular saline, before switching into one of eight GABA concentrations for 2 s followed by a return to saline for at least 8 s before any subsequent GABA application. Below 100 μM GABA, the responses did not desensitize; at and above 100 μM, the amplitude of the responses declined by 10–15% in the continued presence of the agonist. Responses were low-pass-filtered (100 Hz, −3 dB, four-pole Bessel) and digitized with a 1322A interface (Molecular Devices) using pCLAMP 9.2 and stored for off-line analysis. Because intracellular and extracellular solutions contained equal chloride concentrations (145 mM), the chloride equilibrium potential was 0 mV. All experiments were performed at room temperature (21°–24°C).

The propofol concentration–effect study used a similar protocol as above, except eight concentrations of propofol were applied along with an EC_{20} of GABA. Each coapplication was preceded by 3 s of propofol application to ensure equilibrium between propofol and receptors had been reached.

Stock solutions of GABA and modulators were diluted in extracellular solutions shortly before use. Clinically relevant concentrations of the anesthetics etomidate and propofol were used throughout the study (with the exception, by necessity, of the propofol concentration–effect study). A comparable concentration of pregnenolone was determined by performing a pregnenolone concentration–response study and using the calculated EC_{50}. The anesthetic EC_{50} for propofol was taken to be 2 μM,[17] and the anesthetic EC_{50} for etomidate was taken to be 3 μM.[18] Propofol (2,6 di-isopropylphenol) and pregnenolone were obtained from Sigma (St. Louis, MO), and etomidate was obtained from Abbott Laboratories (Chicago, IL).

For each GABA exposure, the peak current amplitudes were measured. The GABA concentration–response data for each cell were extracted from the raw data using our own software package. The analysis software was written to calculate nonlinear dose–response curve parameters using visual basic macros within Microsoft Excel (Microsoft Corp., Redmond, WA) to facilitate efficient data organization. Dose–response parameters were optimized using GRG2, a version of the Generalized Reduction Gradient algorithm included in Microsoft Excel.[19] Using iterative processing and extensively automated file handling, we were able to process multiple data streams simultaneously. The current peaks were fitted to a Hill equation of the form: $I = I_{max} * [GABA]^{nH}/([GABA]^{nH} + EC_{50}{}^{nH})$, where I is the peak of each current, I_{max} is the maximum whole cell current amplitude, [GABA] is the GABA concentration, EC_{50} is the GABA concentration eliciting a current equal to half of I_{max}, and n_H is the Hill coefficient.

Modulator-induced potentiation was calculated as the percentage increase in the peak current response to the application of an EC_{20} GABA response in the presence of the anesthetics, relative to the control (EC_{20}) response. Modulators were always preapplied for 3 s before coapplication with agonist to ensure that the modulator had reached equilibrium with the receptors. Statistical significance was assessed using a one-way analysis of variance with Dunnett posttest (Prism 3.0; GraphPad Software Inc., San Diego, CA). Data are presented as mean ± SEM with n number of cells tested.

Results

We successfully mutated seven C-terminal residues of the human $GABA_A$-R β_2 subunit M4 segment to a trypto-

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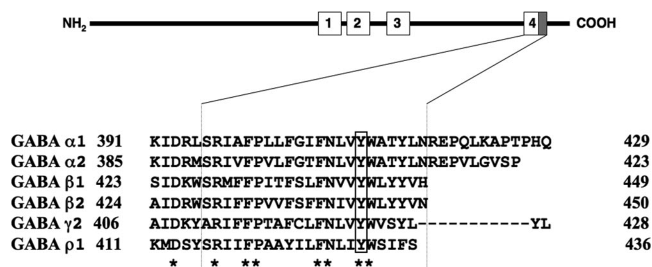

Fig. 1. Amino acid alignment of the M4 segments of six γ-aminobutyric acid (GABA) type A subunits. * Residues that are 100% conserved among subunits. The sequences were aligned using the Clustal-W method.

phan residue (fig. 1). Wild-type and mutant β_2 subunits were transfected into HEK 293 cells with wild-type human α_1 and γ_{2s} subunits, and an adenosine-associated virus green fluorescent protein. Cells that had undergone a successful transfection were identified using fluorescence microscopy. Fluorescing cells were voltage clamped under whole cell patch clamp conditions. Inward Cl^- currents were activated by the application of GABA. To investigate the effects of the mutations on receptor activity, full concentration–response relations for GABA were constructed in the wild-type $GABA_A$-R, and for each mutant receptor. Figure 2A shows representative recordings of GABA-activated currents for the wild-type $\alpha_1\beta_2\gamma_{2s}$ receptor.

Tryptophan was introduced at seven positions: I442W, V443W, Y444W, L446W, Y447W, Y448W, and V449W (the wild-type subunit contains a tryptophan at residue 445). All of the mutations expressed well, as evidenced by robust maximal currents (typically several nA). Figures 2B–D show the concentration-dependent activation (0.3–1000 μM GABA) of receptors harboring the Y444W, L446W, and Y448W mutations (also see table 1). With the exception of β_2(I442W), the GABA EC_{50} for each mutant receptor did not differ from significantly from wild type (table 1). Normalized data from multiple cells are plotted for wild type and receptors harboring the Y444W, L446W, and Y448W mutations in figure 2E.

To determine whether mutations could alter the ability of intravenous anesthetics and a neuroactive steroid to modulate $GABA_A$-R function, the effects of propofol, etomidate, and pregnenolone were investigated. The concentrations of modulator chosen (2 μM propofol, 3 μM etomidate, and 50 nM pregnenolone) were close to those that are required for surgical anesthesia in humans in the case of the intravenous anesthetics (propofol and etomidate). All three modulators potentiated EC_{20} GABA responses in the wild-type receptor (fig. 3A). All of the mutants retained their sensitivity to the modulators, except for Y444W, which was selectively insensitive to the action of 2 μM propofol, while retaining normal sensitivity to etomidate and pregnenolone (figs. 3B and 4).

To determine whether the mutation was decreasing anesthetic sensitivity, rather than removing it altogether,

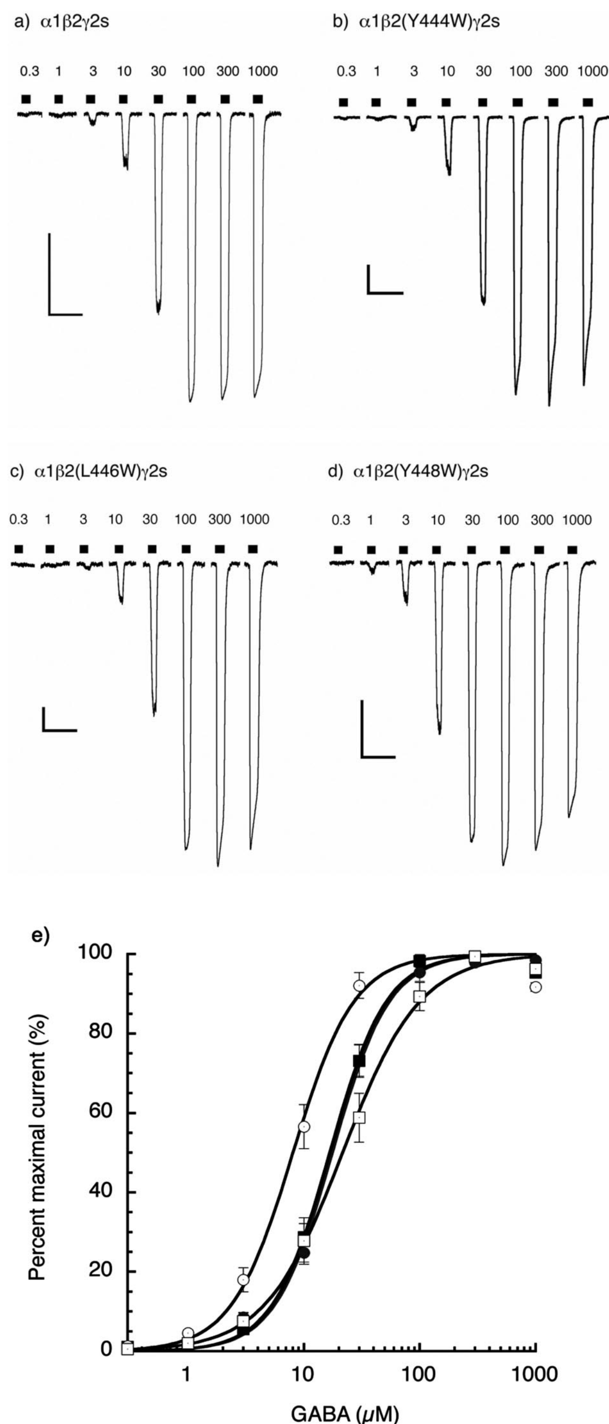

Fig. 2. Gamma-aminobutyric acid (GABA)–activated Cl^- currents recorded from human embryonic kidney 293 cells expressing (*A*) wild-type $\alpha_1\beta_2\gamma_{2s}$, (*B*) $\alpha_1\beta_2$(Y444W)γ_{2s}, (*C*) $\alpha_1\beta_2$(L446W)γ_{2s}, and (*D*) $\alpha_1\beta_2$(Y448W)γ_{2s}. Cells were whole cell voltage clamped at −60 mV. *Bars* over current traces indicate the duration of GABA application and are labeled with the concentration applied (μM). *Calibration bars* denote 10 s and 500 pA. (*E*) Concentration–response relations for wild-type type (*filled circles*), $\alpha_1\beta_2$(Y444W)γ_{2s} (*filled squares*), $\alpha_1\beta_2$(L446W)γ_{2s} (*open squares*), and $\alpha_1\beta_2$(Y448W)γ_{2s} (*open circles*). GABA currents were expressed as a fraction of the maximal GABA response, and these normalized data were fitted by a Hill equation. *Data points* are shown as the means, and the *error bars* indicate SEM of recordings from at least 10 cells. Where not shown, *error bars* are smaller than the *symbol*.

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Table 1. Effect of β_2 M4 Tryptophan Substitutions on GABA$_A$-R Activation

GABA$_A$-R	n	EC$_{50}$, μM	n$_H$
$\alpha_1\beta_2\gamma_{2s}$	25	19.8 ± 1.6	2.2 ± 0.1
$\alpha_1\beta_2$(I442W)γ_{2s}	17	319.7 ± 38.6†	1.4 ± 0.1*
$\alpha_1\beta_2$(V443W)γ_{2s}	17	13.9 ± 1.7	1.6 ± 0.1
$\alpha_1\beta_2$(Y444W)γ_{2s}	27	19.1 ± 1.6	2.1 ± 0.2
$\alpha_1\beta_2$(L446W)γ_{2s}	22	31.8 ± 5.8	1.9 ± 0.1
$\alpha_1\beta_2$(Y447W)γ_{2s}	20	26.4 ± 3.6	1.8 ± 0.1
$\alpha_1\beta_2$(Y448W)γ_{2s}	24	10.3 ± 1.3	2.1 ± 0.1
$\alpha_1\beta_2$(V449W)γ_{2s}	16	19.1 ± 3.3	2.2 ± 0.1

Values of the effective γ-aminobutyric acid concentration for 50% of maximal activation (EC$_{50}$) and Hill coefficient for the γ-aminobutyric acid concentration–effect relation (n$_H$) are tabulated as mean ± SEM and were determined from n cells. Statistical significance was assessed using a one-way analysis of variance with Dunnett post test for multiple comparisons (comparing all mutants with wild type).

* $P < 0.01$. † $P < 0.001$.

GABA$_A$-R = γ-aminobutyric acid type A receptor.

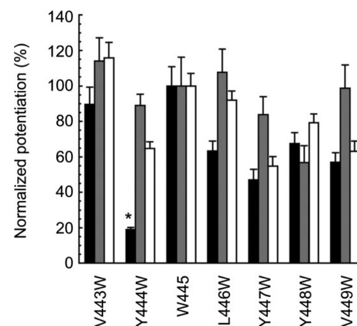

Fig. 4. The effect of tryptophan mutations in the M4 segment of the γ-aminobutyric acid type A receptor β_2 subunit on EC$_{20}$ γ-aminobutyric acid potentiation by 2 μM propofol (*black bars*), 3 μM etomidate (*gray bars*), and 50 nM pregnenolone (*white bars*). The potentiation of each compound was normalized to its effect on the wild-type receptor. Significance was assessed using a one-way analysis of variance with a Dunnett post test for multiple comparisons (comparing all mutants with wild type). * $P < 0.001$.

concentration–effect relations for the potentiation of EC$_{20}$ GABA responses by propofol for wild-type $\alpha_1\beta_2\gamma_{2s}$ and mutant $\alpha_1\beta_2$(Y444W)γ_{2s} receptors were constructed in a separate set of experiments summarized in figure 5. The effect of the mutation was a threefold rightward shift in the EC$_{50}$ for propofol potentiation from 2.4 ± 0.2 μM (wild type) to 7.6 ± 0.4 μM ($\alpha_1\beta_2$(Y444W)γ_{2s}). In addition to potentiation, the agonistic effect of propofol in the absence of GABA (direct activation) was measured. The effect of the mutation was to shift the concentration relation for propofol on the $\alpha_1\beta_2$(Y444W)γ_{2s} mutant rightward by a factor of 3.7 compared with the wild type: $\alpha_1\beta_2\gamma_{2s}$ EC$_{50}$ = 3.3 ± 0.3 μM, n_H = 2.2 ± 0.1, I_{max} = −932 ± 74 pA, n = 8; $\alpha_1\beta_2$(Y444W)γ_{2s}: EC$_{50}$ 12.5 ± 2.7 μM, n_H = 2.7 ± 0.1, I_{max} = −952 ± 63 pA, n = 11. To further determine if the effects on potentiation were restricted to propofol, these experiments were repeated with 0.1–3 μM etomidate. These results revealed that the β_2(Y444W) mutation had no effect on the etomidate–potentiation relation (wild type: EC$_{50}$ = 0.6 ± 0.2 μM, n_H = 1.2 ± 0.1, maximum potentiation = 320 ± 70%, n = 9; $\alpha_1\beta_2$(Y444W)γ_{2s}: EC$_{50}$ = 0.7 ± 0.2 μM, n_H = 0.9 ± 0.1, maximum potentiation = 370 ± 50%, n = 7).

Finally, to investigate whether these effects were specific to the β_2(Y444W) substitution, two additional mutants were generated: β_2(Y444F) and β_2(Y444C). The potentiation of the EC$_{20}$ GABA response of the $\alpha_1\beta_2$(Y444C)γ_{2s} by 2 μM propofol was similar to wild type (241 ± 58%, n = 5) but differed significantly (P <

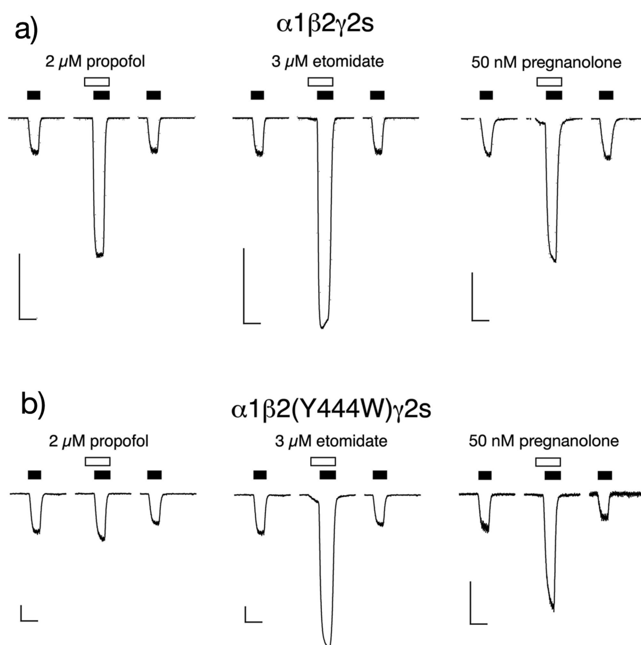

Fig. 3. Representative examples of the potentiation of EC$_{20}$ γ-aminobutyric acid responses by propofol (2 μM), etomidate (3 μM), and pregnenolone (50 nM). Whole cell current records were obtained from human embryonic kidney 293 cells expressing (*A*) wild-type $\alpha_1\beta_2\gamma_{2s}$ and (*B*) mutant $\alpha_1\beta_2$(Y444W)γ_{2s} receptors. *Bars* above the current traces indicate the duration of γ-aminobutyric acid (*filled bars*) and modulator (*open bars*) application. *Calibration bars* denote 10 s and 100 pA.

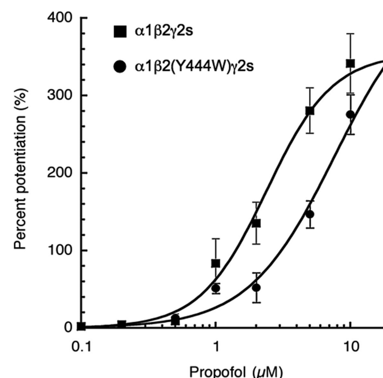

Fig. 5. Concentration–effect relations for the potentiation of EC$_{20}$ γ-aminobutyric acid responses by propofol for wild-type $\alpha_1\beta_2\gamma_{2s}$ (*filled squares*) and mutant $\alpha_1\beta_2$(Y444W)γ_{2s} receptors (*filled circles*). The effect of the mutation was a threefold rightward shift in the propofol EC$_{50}$ for potentiation from 2.4 ± 0.2 to 7.6 ± 0.4 μM.

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0.05) from the potentiation of the $\alpha_1\beta_2$(Y444F)γ_{2s} EC_{20} response (101 ± 33%, n = 5).

Discussion

Although general anesthetics have been in use for 165 yr, the mechanism of action for this unique class of drugs has remained a relative mystery. Recent advances using chimeric proteins, site-directed mutagenesis, protein modeling, and transgenic animal model suggests that a small number of neuronal ion channels are the likely sites of action for general anesthetics.[6,20–22] In this study, we provide further evidence that $GABA_A$-Rs play a key role in the actions of intravenous general anesthetics. Specifically, our data suggest that propofol, but not etomidate, hydrogen bonds to a conserved tyrosine in the M4 segment of the $GABA_A$-R β_2 subunit.

The techniques used in this study have previously been used to identify residues critical for anesthetic action on the $GABA_A$-R. As referenced above, tryptophan scanning mutagenesis has been successfully used to identify critical residues in the M4 segment of the α_1 subunit.[8] Given the homology between the α_1 and the β_2 subunit, the tryptophan scanning mutagenesis method was also selected for this study. We found that tryptophan substitutions in the C-terminal end of the β_2 M4 segment resulted in modest changes to normal receptor function and a novel change in the sensitivity of the receptor to propofol, but not etomidate.

All of the mutant subunits examined in this study expressed well and formed functional channels. Previous studies have found that large decreases in maximal current occurred when small hydrophobic amino acids were substituted with the much larger tryptophan: leucine to tryptophan, isoleucine to tryptophan, and valine to tryptophan substitutions in the M4 segments of the $GABA_A$-R α_1 subunit and the muscle acetylcholine receptor.[8,23] This was not found to be the case for β_2(V443W) or β_2(V449W) in this study. Therefore, it is unlikely that the β_2 M4 region plays a key role in receptor assembly as hypothesized for other subunits.

The result of the β_2(I442W) mutation was to increase the GABA EC_{50} from approximately 20 to 320 μM and reduce the Hill coefficient from 2.2 to 1.4. Such a change is a hallmark of a receptor with a deficient gating pathway. This is made likely by the position of this residue deep within the membrane where the tilted transmembrane segments are thought to move over one another during receptor gating.[20] All of the other mutations investigated in this study exhibited normal GABA sensitivity (table 1). Although there were modest differences, none were statistically significant. Therefore, it would seem that no significant gating alterations have been introduced by six of the seven tryptophan switches. These results present an interesting difference in the role a conserved tyrosine plays in the α_1 and β_2 subunits during receptor activation. A tyrosine-to-tryptophan switch at position α_1(411)[8] exhibited a significantly increased GABA sensitivity as compared with the wild-type receptor, whereas the GABA sensitivity of the homologous substitution in the β_2 subunit at position 444 was not significantly different as compared with wild type.

This difference may be due in part to the relative sizes of the anesthetic binding pockets in the α and β subunits. Experiments with anesthetics with different molecular volumes and a variety of mutant subunits predict that the volume of the pocket in the β subunit is three times greater than its α subunit counterpart.[7,17] This larger cavity may be more tolerant to large tryptophan mutations and may therefore exhibit smaller changes in receptor function. Likewise, the smaller, more sterically hindered pocket in the α subunit may be more prone to nonconservative alterations. Alternatively, the difference may be due to the two subunits undergoing dissimilar isomerizations during receptor activation.

These results are in agreement with many other findings that suggest that the α and β subunits play different roles in receptor activation. For example, the M1 segment of the β subunit, but not the α subunit, is thought to play a critical role in controlling anion permeability.[24] Also, the positive surface[25–27] of the β subunit extracellular domain controls GABA binding,[28] whereas the homologous domain in the α subunit determines benzodiazepine binding.[29]

Only one mutation studied here altered the ability of propofol, etomidate, or pregnenolone to modulate channel function. The β_2(Y444W) mutant exhibited a selective attenuation of propofol's actions on the $GABA_A$-R's sensitivity to GABA, whereas the actions of etomidate and pregnenolone were preserved. This is the first example, to our knowledge, of a mutant that selectively affects propofol, but not etomidate sensitivity of the $GABA_A$-R. Previous studies have shown that mutation of β_2N265 and β_2M289 in the second and third transmembrane segments simultaneously alters the sensitivity of the receptor to both etomidate and propofol.[13,30,31] These results have been reproduced *in vivo* using knock-in mutant mouse lines harboring N265 mutations,[21,32] indicating that both agents have an overlapping site of action. However, the molecular structures of these two anesthetics is far from similar; therefore, it is perhaps not surprising that at least one amino acid in the binding pocket plays a different role in conferring the actions of one drug *versus* another. It should be noted that the selective alteration of anesthetic sensitivity of the $GABA_A$-R induced by the β_2Y444W mutation is inconsistent with the theory that all general anesthetics interact in a nonspecific manner with a common binding site. Instead, we suggest that an anesthetic molecule is able to form a specific array of low-energy bonds within its critical binding site in the nervous system. It is also

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interesting to note the similarity in the reduction in magnitude of the potentiation and direct activation of β_2(Y444W)-containing receptors. This suggests that the same site of action confers both effects. This finding is supported by a previous study with the stereoisomers of etomidate where the same stereoselectivity was observed for both direct activation and potentiation. These observations taken together suggest that these two effects do not occur *via* the anesthetic molecule binding at separate sites on the protein. Instead, it seems more likely that anesthetic binding in the transmembrane segments alters channel gating to such an extent that changes to both potentiation and direct activation occur as a result of this single interaction.

Our results from propofol concentration–response studies revealed that instead of eliminating propofol modulation, the β_2Y444W mutation selectively increased the EC_{50} for propofol potentiation from 2.4 to 7.6 μM. This threefold shift in apparent affinity is associated with a free energy change of 0.68 kCal/mol. This small energetic change could certainly be accounted for thermodynamically by the rearrangement of the hydrogen bond between water and tyrosine and the bond between propofol and tyrosine. When the phenol of tyrosine is replaced by the indole of tryptophan, a hydrogen bond donor and acceptor are both lost, both of which could interact with the 1-position hydroxyl group on the propofol molecule. If this is the case, the results described here would indicate one of the more common orientations of the propofol molecule among its array of bound conformations, because the 1 position of the propofol molecule and its flanking isopropyl groups must be facing the M4 segment in the wild-type receptor for the hydrogen bond to form. This hypothesis is supported by our observation that the modulation of non–hydrogen bonding β_2(Y444F)-containing receptors is also impaired, whereas the hydrogen bond–doning β_2(Y444C)-containing receptors retain normal sensitivity to propofol.

In conclusion, the data presented in this study represent the first investigation of the role the β_2 M4 segment plays in the activation and modulation of the $GABA_A$-R. Unlike its counterpart in the α subunit, we found that this segment does not play a key role in determining the efficacy of GABA. However, this segment is important for determining the sensitivity of the receptor to propofol. These results, taken together with those for general anesthetic action in all four transmembrane segments of both α and β subunits, paint a more complete picture of the anesthetic binding sites of the $GABA_A$-R. However, until a high-resolution structural basis for drug action on these receptors is obtained, we will rely on structural simulations of receptor activation and modulation to better understand the relative positions of these critical amino acids and the movements they may undergo. The results of these experiments and the additional mutagenesis experiments that they will undoubtedly suggest will tell us more about the physical characteristics and the dynamics of general anesthetic binding sites within neuronal ion channels. This information will greatly aid future intelligent drug design and give us a much better understanding of the molecular mechanisms of general anesthesia.

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