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Propofol and Other Intravenous Anesthetics Have Sites of Action on the γ-Aminobutyric Acid Type A Receptor Distinct from That for Isoflurane

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ABSTRACT

Both volatile and intravenous general anesthetics allosterically enhance γ-aminobutyric acid (GABA)-evoked chloride currents at the GABA type A ($GABA_A$) receptor. Recent work has revealed that two specific amino acid residues within transmembrane domain (TM)2 and TM3 are necessary for positive modulation of $GABA_A$ and glycine receptors by the volatile anesthetic enflurane. We now report that mutation of these residues within either $GABA_A$ α2 (S270 or A291) or β1 (S265 or M286) subunits resulted in receptors that retain normal or near-normal gating by GABA but are insensitive to clinically relevant concentrations of another inhaled anesthetic, isoflurane. To determine whether receptor modulation by intravenous general anesthetics also was affected by these point mutations, we examined the effects of propofol, etomidate, the barbiturate methohexital, and the steroid alphaxalone on wild-type and mutant $GABA_A$ receptors expressed in human embryonic kidney 293 cells. In most cases, these mutations had little or no effect on the actions of these intravenous anesthetics. However, a point mutation in the β1 subunit (M286W) abolished potentiation of GABA by propofol but did not alter direct activation of the receptor by high concentrations of propofol. These data indicate that the receptor structural requirements for positive modulation by volatile and intravenous general anesthetics may be quite distinct.

The $GABA_A$ receptor is modulated positively by a wide variety of structurally diverse general anesthetics (Harris *et al.*, 1995; Whiting *et al.*, 1995). In particular, halogenated ethers such as enflurane and isoflurane (Nakahiro *et al.*, 1989; Wakamori *et al.*, 1991; Jones *et al.*, 1992) along with intravenous anesthetic agents such as the barbiturates (Barker and Ransom, 1978), propofol (Hales and Lambert, 1991), etomidate (Uchida *et al.*, 1995), and steroid anesthetics (Peters *et al.*, 1988) all enhance the function of the $GABA_A$ receptor at clinically relevant concentrations. In addition, intravenous and volatile anesthetics can activate the $GABA_A$ receptor directly in the absence of GABA (Barker and Ransom, 1978; Robertson, 1989; Hales and Lambert, 1991; Yang *et al.*, 1992).

The $GABA_A$ receptor is a heteromeric complex formed by different glycoprotein subunits (α1–6, β1–4, γ1–4, δ, and ϵ) that coassemble to form a chloride channel (Whiting *et al.*, 1995). Most $GABA_A$ receptors *in vivo* consist of pentameric complexes of α, β, and γ subunits with a stoichiometry of ααβγ (Chang *et al.*, 1996), although receptors lacking the γ subunit can be expressed *in vitro* and are fully sensitive to general anesthetics (Pritchett *et al.*, 1989; Jones *et al.*, 1995).

$GABA_A$ receptors are members of a ligand-gated ion channel superfamily that also includes the glycine, $serotonin_3$, GABA ρ ($GABA_C$), and nicotinic acetylcholine receptors (Ortells and Lunt, 1995). $GABA_A$ receptors share significant amino acid sequence homology with these receptors (Ortells and Lunt, 1995). Glycine receptor function is modulated positively by clinical concentrations of volatile anesthetics (Harrison *et al.*, 1993; Downie *et al.*, 1996) but is affected only weakly by barbiturates (Koltchine *et al.*, 1996; Mascia *et al.*, 1996) and etomidate (Mascia *et al.*, 1996). In addition, some general anesthetics have potent actions on neuronal (but not muscle) nicotinic acetylcholine receptors (Flood *et al.*, 1997; Violet *et al.*, 1997) or $serotonin_3$ receptors (Jenkins *et al.*, 1996). In contrast to other members of the ligand-gated channel superfamily, GABA ρ receptors are insensitive to nearly

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ABBREVIATIONS: $GABA_A$, γ-aminobutyric acid type A; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEK, human embryonic kidney; TM, transmembrane domain; MAC, minimum alveolar concentration.

all anesthetic compounds, including propofol (Mihic and Harris, 1996), barbiturates (Shimada *et al*., 1992), volatile anesthetics (Harrison *et al*., 1993; Mihic and Harris, 1996), and steroid anesthetics (Mihic and Harris, 1996).

The dissimilar pharmacology of ρ receptors helped identify residues crucial for positive modulation by general anesthetics. Recently, Mihic *et al*. (1997) constructed glycine α1/GABA ρ1 receptor chimeras and characterized a 45-amino acid residue domain within the glycine α1 receptor that was necessary for positive modulation by enflurane. Mutagenesis within this domain led to the identification of specific amino acid residues in TM2 and TM3 that seem to be necessary for modulation or direct activation of $GABA_A$ and glycine receptors by enflurane.

The current study demonstrates that mutation of these specific residues in TM2 and TM3 also abolishes the enhancement of submaximal GABA-activated currents by isoflurane. Additional experiments were performed to test whether these specific mutations in TM2, TM3, or both also affected modulation and direct activation by intravenous general anesthetics.

Materials and Methods

Site-directed mutagenesis. The S270I (i.e., serine at position 270 mutated to isoleucine), S270H, and A291W mutations of the human $GABA_A$ α2 subunit (Hadingham *et al*., 1993) and the M286W mutation of the $GABA_A$ β1 subunit (Hadingham *et al*., 1993) were introduced by the unique site elimination method (Deng and Nickoloff, 1992) with use of the USE kit (Pharmacia Biotech, Piscataway, NJ). The method uses a two-primer system in which one oligonucleotide primer encodes the desired mutation and the other alters a unique *Ssp*I restriction site on the pCIS2 plasmid to an *MluI* site. The mutagenic reaction mixture was digested with *Ssp*I restriction endonuclease to eliminate the parental template. Positive clones of transformed *Escherichia coli* DH5α (Pharmacia Biotech) then were screened for the appearance of the *MluI* site, and mutations were confirmed further by double-stranded sequencing (Sequenase 2.0; United States Biochemical, Cleveland, OH). Both *MluI* and *Ssp*I restriction enzymes were from New England Biolabs (Beverly, MA). The sequences and locations of the primers (Operon Technologies, Alameda, CA) used are α2(S270I): 5′-GACAACTCTAATCATCAGTGCTCGGAATTC-3′, corresponding to bases 879–908 of the α2 cDNA sequence; α2(S270H), 5′-GACAACTCTACACATCAGTGCTCGGAATTC-3′, corresponding to bases 879–908 of the α2 cDNA sequence; α2(A291W), 5′-CATGGACTGGTTTATTTGGGTTTGTTATGCATTTG-3′, corresponding to bases 936–970 of the α2 cDNA sequence; β1(M286); and 5′-GATTGATATTTATCTGTGGGGTTGCTTTGTG-3′, corresponding to bases 915–945 of the β1 cDNA sequence.

The S265I mutation in the β1 subunit was introduced with use of the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The method uses the fact that DNA isolated from most strains of *E. coli* is *dam* methylated and therefore susceptible to *Dpn*I endonuclease digestion (target sequence, 5′-G^{m6}ATC-3′). Two oligonucleotide primers, containing the same desired mutations and complementary to each other, were extended during temperature cycling by means of *Pfu* DNA polymerase (Stratagene). The product was digested with *Dpn*I (Stratagene) to eliminate the parental template and transformed into the XL-1 Blue strain of *E. coli* (Stratagene). Positive clones were confirmed again by double-stranded sequencing.

Cell culture and transfection. Wild-type or mutant receptor cDNAs were expressed via the pCIS2 vector, which contains the strong promoter from cytomegalovirus and a polyadenylation sequence from Simian virus 40. HEK 293 cells (American Type Culture Collection, Rockville, MD) were cultured in Eagle's minimal essential medium (Sigma Chemical, St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), L-glutamine (0.292 μg/ml; GIBCO BRL, Grand Island, NY), penicillin G sodium (100 units/ml; GIBCO BRL), and streptomycin sulfate (100 μg/ml; GIBCO BRL). For electrophysiological experiments, cells were plated onto glass coverslips coated with poly-D-lysine (Sigma). Each coverslip of cells was transfected individually according to the calcium phosphate precipitation technique (Pritchett *et al*., 1989; Harrison *et al*., 1993). There are reports of endogenous $GABA_A$ receptor subunit expression in HEK 293 cells (Ueno *et al*., 1996). Our own experience with HEK 293 cells to date does not concur with this finding. In fact, similar to other reports (Davies *et al*., 1997), we do not see significant GABA-induced currents in untransfected or sham-transfected HEK 293 cells or by transfection with either $GABA_A$ α2 or β1 subunit cDNAs alone (Koltchine *et al*., 1996).

Electrophysiology. Electrophysiological recordings were performed at room temperature using the whole-cell patch-clamp technique as described previously (Harrison *et al*., 1993; Koltchine *et al*., 1996). The coverslips were transferred 24–72 hr after removal of the cDNA to a 70-ml chamber that was continuously perfused (2–3 ml/min) with extracellular medium containing 145 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM D-glucose, and 10 mM HEPES, pH 7.4, osmolarity 320–330 mOsM. The intracellular solution contained 145 mM *N*-methyl-D-glucamine hydrochloride, 5 mM K_2ATP, 5 mM HEPES/KOH, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 1.1 mM EGTA, pH 7.2, osmolarity 315 mOsM. Pipette-to-bath resistance was 4–6 MΩ. Cells were voltage-clamped at −60 mV. Because the intracellular and extracellular solutions contained equal concentrations of chloride, the chloride reversal potential was ≈0 mV.

GABA and anesthetics were rapidly (<50-msec exchange time) applied to the cell by local perfusion (Koltchine *et al*., 1996) using a motor-driven solution-exchange device (Bio Logic Rapid Solution Changer RSC-100; Molecular Kinetics, Pullman, WA). Laminar flow was maintained by applying all solutions at identical flow rates via a multichannel infusion pump (Stoelting, Wood Dale, IL). The solution changer was driven by protocols in the acquisition program pCLAMP5 (Axon Instruments, Foster City, CA). Responses were low-pass-filtered at 5 kHz, digitized (TL-1–125 interface; Axon Instruments) using pCLAMP5, and stored for off-line analysis.

Data analysis. Modulator-induced potentiation of an agonist-induced current was defined as the percentage increase of the peak control agonist response (0% indicates no difference from control response). Concentration-response data were fitted (KaleidaGraph, Reading, PA) with the logistic equation: $I/I_{max} = 100 * [drug]^n/([drug]^n + (EC_{50})^n)$, where I/I_{max} is the percentage of the maximum obtainable agonist response, EC_{50} is the concentration producing a half-maximal response, and n is the Hill coefficient. The current elicited by direct activation of the receptor by an anesthetic compound was expressed relative to the maximal current that could be elicited by GABA. Any direct activation produced by a modulator during pre-equilibration was subtracted from the total current elicited by coapplication of GABA and modulator. The subtraction of direct anesthetic activation is appreciable only at high anesthetic concentrations (e.g., ≥10 μM propofol or etomidate). In general, for the screening of potentiation, concentrations of anesthetics were chosen carefully (see Results) so little or no direct activation contributed to the observed current. Pooled data are presented throughout as mean ± standard error. Statistical significance was determined by Student's two-tailed, unpaired *t* test.

Throughout this study, potentiation by the various general anesthetic agents was always assessed with test concentrations of GABA that correspond to EC_{20} value on the concentration-response curve for the particular receptor under study. In this fashion, the percentage potentiation produced by coapplication of a given modulator can be compared across different receptors and will not be influenced by potential shifts in GABA concentration-response curves among receptors. Across all the potentiation experiments for wild-type and mutant receptors reported here, the actual percentage of maximal

GABA response for the test concentrations used were: $\alpha2\beta1$ wild-type (19.7 ± 2.5%, 30 experiments), $\alpha2$(S270I)$\beta1$ (22.3 ± 2.5%, 33 experiments), $\alpha2$(S270H)$\beta1$ (22.2 ± 2.0%, 35 experiments), $\alpha2$(A291W)$\beta1$ (20.5 ± 2.5%, 25 experiments), $\alpha2\beta1$(S265I) (18.6 ± 2.6%, 53 experiments), and $\alpha2\beta1$(M286W) (19.4 ± 2.6%, 36 experiments).

Drugs. Stock solutions of GABA (Sigma) and anesthetic compounds were diluted into extracellular solution daily before use. The other drugs used in this study were propofol (2,6-diisopropylphenol; Aldrich, Milwaukee, WI), methohexital sodium (Brevital sodium; Eli Lilly and Co., Indianapolis, IN), alphaxalone, picrotoxin (both from Research Biochemicals, Natick, MA), and isoflurane (Forane; Ohmeda Caribe, Guayama, PR). Propofol, etomidate, picrotoxin, and alphaxalone were first prepared as stock solutions in dimethylsulfoxide (Sigma) before being dissolved in the extracellular medium. The maximum final concentration of dimethylsulfoxide was 0.05% (v/v), which was determined during carrier control experiments to have no significant effect on GABA-induced currents in the receptor constructs analyzed in this study. Preparation and measurement of isoflurane solutions have been described previously (Jones *et al.*, 1992). The MAC equivalent for isoflurane at 25° used for this study was 0.5 mM (Jones *et al.*, 1992). Research-grade etomidate was a generous gift from Prof. Alfred Doenicke (Institute of Anesthesiology, Ludwig Maximilians University of München, Germany).

Results

Expression and characteristics of wild-type and mutant receptors. Six wild-type and mutant $GABA_A$ receptors were expressed by transfection of HEK 293 cells: $\alpha2\beta1$ wild-type, $\alpha2$(S270I)$\beta1$, $\alpha2$(S270H)$\beta1$, $\alpha2$(A291W)$\beta1$, $\alpha2\beta1$(S265I), or $\alpha2\beta1$(M286W). After transient expression in HEK 293 cells, all of the wild-type and mutant receptors tested in this study produced inward currents in response to application of GABA via the rapid solution changer. The EC_{50} and Hill slope values estimated from GABA concentration-response curves for receptors that contain either mutant $\alpha2$ or $\beta1$ subunits demonstrate that these receptors retain GABA concentration-response relationships that are similar to those of wild-type $\alpha2\beta1$ receptors: $\alpha2\beta1$ wild-type (EC_{50} = 8.7 ± 0.4 μM, n_H = 1.9 ± 0.2, nine experiments), $\alpha2$(S270I)$\beta1$ (EC_{50} = 14.6 ± 0.1 μM, n_H = 2.4 ± 0.1, six experiments), $\alpha2$(S270H)$\beta1$ (EC_{50} = 3.5 ± 0.2 μM, n_H = 1.5 ± 0.1, five experiments), $\alpha2$(A291W)$\beta1$ (EC_{50} = 2.4 ± 0.1 μM, n_H = 1.7 ± 0.2, five experiments), $\alpha2\beta1$(S265I) (EC_{50} = 37.5 ± 8.5 μM, n_H = 1.2 ± 0.2, seven experiments), and $\alpha2\beta1$(M286W) (EC_{50} = 8.7 ± 0.4 μM, n_H = 0.8 ± 0.2, seven experiments).

The EC_{50} values for the mutant receptors do not differ by >4.3-fold from wild-type. The Hill slopes for the GABA concentration-response relationships for the $\alpha2\beta1$(S265I) and $\alpha2\beta1$(M286W) mutant receptors are significantly lower compared with wild-type $\alpha2\beta1$ receptors ($p < 0.001$ and < 0.05, respectively). In addition, the maximal current amplitude of all mutant receptors in response to GABA did not differ by more than 1.6-fold from wild-type: $\alpha2\beta1$ wild-type (699 ± 75 pA, 37 experiments), $\alpha2$(S270I)$\beta1$ (437 ± 33 pA, 46 experiments), $\alpha2$(S270H)$\beta1$ (606 ± 88 pA, 45 experiments), $\alpha2$(A291W)$\beta1$ (1032 ± 157 pA, 34 experiments), $\alpha2\beta1$(S265I) (694 ± 97 pA, 58 experiments), and $\alpha2\beta1$(M286W) (557 ± 90 pA, 59 experiments).

An interesting property of mutations at the A291 position in TM3 ($GABA_A$ $\alpha2$ numbering) is the propensity to form tonically open receptors. In particular, $GABA_A$ $\alpha2$(A291W)$\beta1$(M286W) and glycine $\alpha1$(A288W) mutant receptors seem to be tonically open in the absence of agonist. The application of picrotoxin results in "outward" currents, consistent with closure of open channels (Mihic *et al.*, 1997). For the $GABA_A$ $\alpha2$(A291W)$\beta1$(M286W) mutant receptor, picrotoxin (1–100 μM) produces outward currents whose maximal amplitude was 38.5 ± 6.5% (six experiments) of the amplitude of the maximal inward current produced by GABA. Neither the $\alpha2$(A291W)$\beta1$ nor the $\alpha2\beta1$(M286W) mutant receptors seem to be tonically active because application of picrotoxin at concentrations up to 100 μM resulted in no deflection of the base-line current (four experiments for each). Consistent with the approach taken in earlier work (Mihic *et al.*, 1997), we did not attempt anesthetic modulation or direct activation experiments on tonically open channels such as the $GABA_A$ $\alpha2$(A291W)$\beta1$(M286W) mutant receptor.

Mutations in TM2 and TM3 of the $GABA_A$ $\alpha2$ and $\beta1$ subunits abolish positive modulation by isoflurane. At the clinically relevant concentrations of 0.25 and 0.5 mM (0.5 and 1.0 MAC equivalents, respectively), isoflurane strongly enhanced responses to an EC_{20} concentration of GABA in wild-type $\alpha2\beta1$ receptors (Fig. 1A). In contrast, coapplication of isoflurane up to 1.0 mM (2 MAC) produced no enhancement of submaximal GABA currents at mutant $\alpha2$(S270H)$\beta1$ or $\alpha2$(A291W)$\beta1$ receptors (Fig. 1, B and C). In fact, all five mutant receptors studied in this report were insensitive to isoflurane concentrations up to 1.0 mM (Table 1). To further examine the mechanism by which these mutations altered potentiation by isoflurane, we examined whether the addition of a $\gamma2$s subunit to one of the mutant receptors would lead to any potentiation by isoflurane. Submaximal GABA currents at the $\alpha2$(A291W)$\beta1\gamma2$s mutant receptor, however,

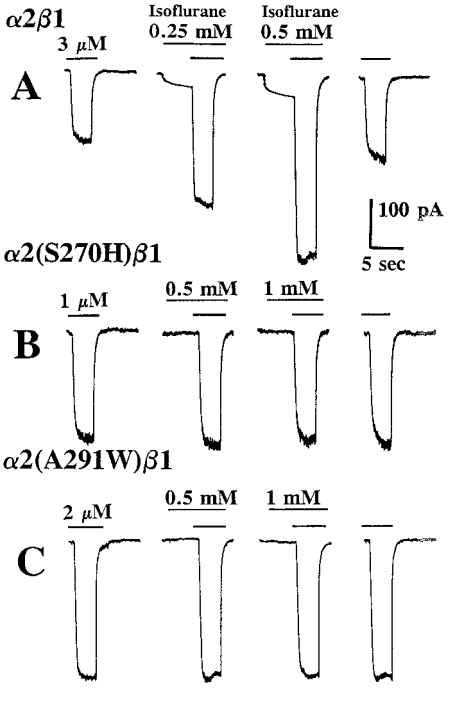

Fig. 1. Mutations within TM2 and TM3 abolish positive modulation by isoflurane. A, Submaximal GABA currents in wild-type $GABA_A$ $\alpha2\beta1$ receptors are strongly enhanced by coapplication of clinically relevant concentrations of isoflurane (0.25 and 0.5 mM). B and C, In contrast, submaximal GABA currents in $\alpha2$(S270H)$\beta1$ or $\alpha2$(A291W)$\beta1$ mutant receptors are not enhanced by coapplication of isoflurane concentrations up to 1 mM. Individual recordings are from HEK 293 cells transfected with cDNAs encoding the indicated subunit combination.

TABLE 1

Potentiation of wild-type and mutant receptors by anesthetics

Values indicate mean ± standard error for percent potentiation of an EC_{20} test concentration of GABA by the anesthetic. Numbers in parentheses give number of experiments contributing to the mean value.

Combination	Isoflurane (0.5 mM and 1.0 mM)	Propofol (1 μM)	Etomidate (10 μM)	Methohexital (5 μM)	Alphaxalone (1 μM)
α2β1	161 ± 46 (6) 226 ± 43 (8)	124 ± 46 (7)	213 ± 39 (7)	234 ± 37 (6)	287 ± 158 (5)
α2(S270I)β1	5 ± 12 (6)[a] 2 ± 6 (6)[a]	88 ± 29 (7)	236 ± 49 (5)	147 ± 41 (5)	211 ± 36 (5)
α2(S270H)β1	−9 ± 2 (4)[a] −6 ± 2 (4)[a]	123 ± 25 (6)	249 ± 56 (6)	152 ± 37 (8)	344 ± 100 (6)
α2(A291W)β1	−5 ± 5 (5)[a] −5 ± 3 (7)[a]	106 ± 9 (6)	65 ± 27 (7)[c]	53 ± 14 (6)[b]	272 ± 178 (5)
α2β1(S265I)	2 ± 6 (6)[a] 3 ± 2 (6)[a]	68 ± 30 (8)	266 ± 55 (11)	238 ± 63 (10)	487 ± 115 (5)
α2β1(M286W)	9 ± 4 (6)[a] 3 ± 11 (6)[a]	−13 ± 9 (9)[b]	122 ± 57 (11)	70 ± 21 (9)[b]	383 ± 56 (6)

[a] $p < 0.001$ compared with the control group.
[b] $p < 0.005$ compared with the control group.
[c] $p < 0.01$ compared with the control group.

also were insensitive to potentiation by isoflurane: potentiation of EC_{20} GABA currents by 0.5 and 1.0 mM isoflurane was −3.8 ± 2.7% and −8.3 ± 4.7%, respectively (five experiments for both concentrations; negative values indicate slight inhibition).

Propofol potentiation of GABA. A propofol concentration of 1 μM was chosen to assay GABA potentiation because it is within the estimated clinically relevant concentration range (Franks and Lieb, 1994) and produces no direct activation in most of the receptors tested (see below). Mutations

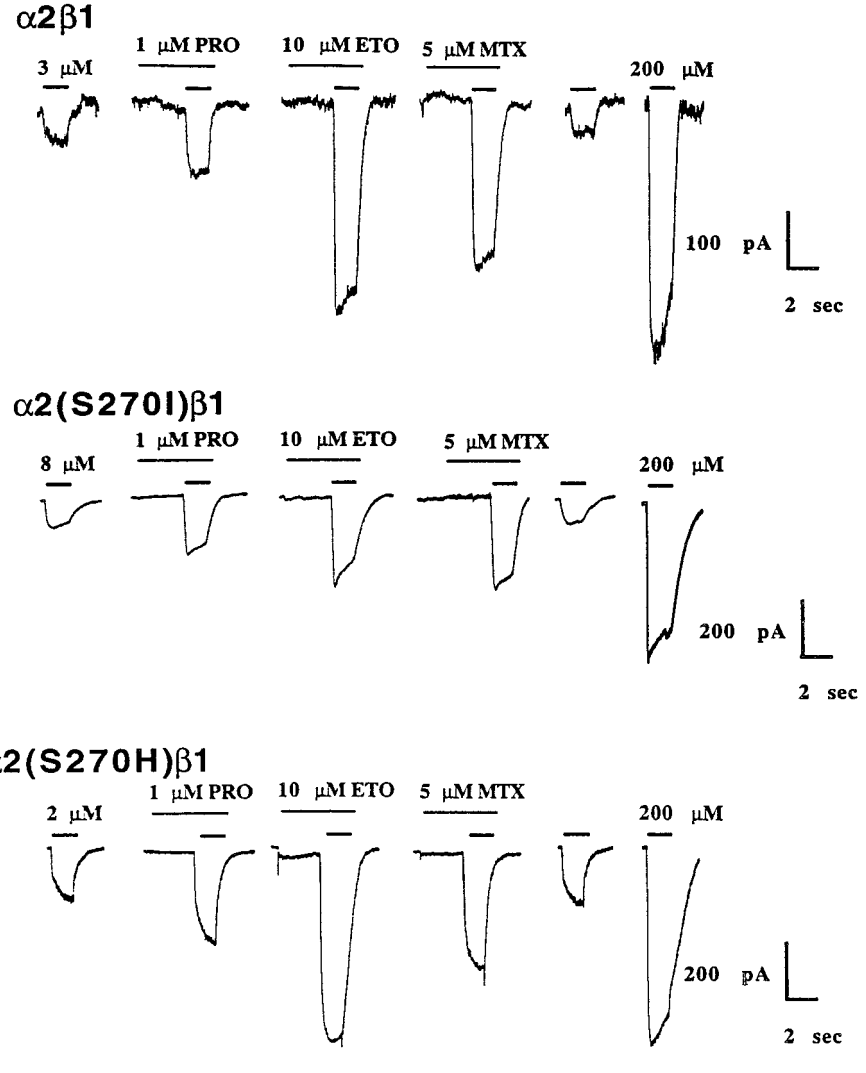

Fig. 2. Mutations in the GABA$_A$ α2 subunit that abolish potentiation by isoflurane do not alter potentiation by propofol (*PRO*), etomidate (*ETO*), and methohexital (*MTX*). Submaximal GABA currents in wild-type GABA$_A$ α2β1 receptors are strongly enhanced by anesthetic concentrations of propofol (1 μM), etomidate (10 μM), and methohexital (5 μM). Similarly, submaximal GABA currents at α2(S270I)β1 or α2(S270H)β1 mutant receptors are enhanced by propofol, etomidate, and methohexital. Individual recordings are from HEK 293 cells transfected with cDNAs encoding the indicated subunit combinations.

in the $\alpha 2$ subunit had little or no effect on GABA potentiation by 1 μM propofol (Fig. 2, Table 1). A point mutation in TM3 of the $\beta 1$ subunit (M286W), however, eliminated GABA potentiation by 1 μM propofol (Table 1). In fact, submaximal GABA currents at the $\alpha 2\beta 1$(M286W) mutant receptor were not enhanced by propofol at concentrations up to 10 μM (Fig. 3). The addition of a $\gamma 2$s subunit did not alter the effect of this $\beta 1$ mutation because submaximal currents at the $\alpha 2\beta 1$(M286W)$\gamma 2$s mutant receptor also were insensitive to potentiation by propofol at concentrations up to 10 μM (Fig. 3B).

Etomidate potentiation of GABA. Etomidate (1–20 μM) potentiated submaximal GABA currents at wild-type $\alpha 2\beta 1$ receptors with an estimated EC_{50} value of 3.4 μM, Hill slope of 1.6, and predicted maximal potentiation (E_{max}) of 216% (six or seven experiments for all concentrations). Etomidate (10 μM) significantly potentiated submaximal GABA currents in all wild-type and mutant receptors tested (Table 1). The magnitude of potentiation by etomidate was markedly reduced in the $\alpha 2$(A291W)$\beta 1$ mutant receptor, whereas all other mutant receptors retained normal potentiation by 10 μM etomidate (Table 1).

Methohexital potentiation of GABA. Previous work from our laboratory has shown that the barbiturate methohexital induces GABA potentiation, direct activation, and blocking effects on wild-type $GABA_A$ $\alpha 2\beta 1$ receptors (Koltchine *et al.*, 1996). A concentration of 5 μM methohexital was used for the potentiation experiments because it produces substantial GABA potentiation without any direct activation of $GABA_A$ $\alpha 2\beta 1$ receptors (Koltchine *et al.*, 1996). Methohexital potentiated the action of GABA at all receptors, although two receptors harboring mutations in TM3, $\alpha 2$(A291W)$\beta 1$ and $\alpha 2\beta 1$(M286W), showed significantly lesser degrees of potentiation by 5 μM methohexital (Table 1).

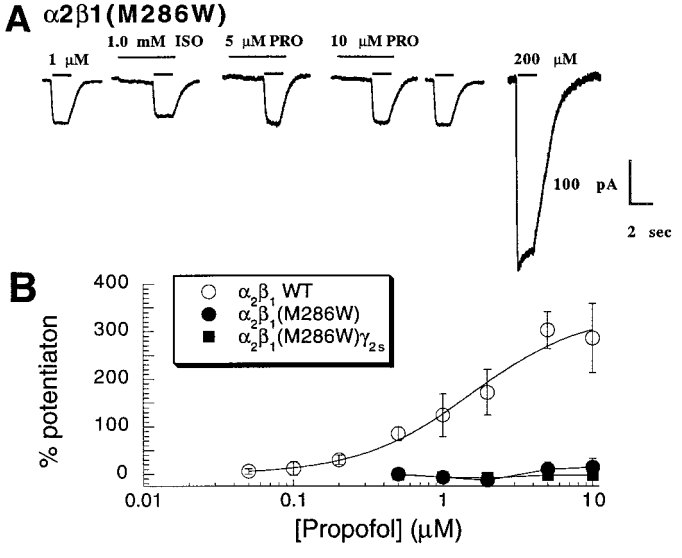

Fig. 3. Potentiation by both propofol and isoflurane is abolished in the $\alpha 2\beta 1$(M286W) mutant receptor. A, Submaximal GABA currents in the $\alpha 2\beta 1$(M286W) receptor are not enhanced by coapplication of 1.0 mM isoflurane (*ISO*) or supra-anesthetic propofol (*PRO*) concentrations (5 and 10 μM). Recording is from a single HEK 293 cell transfected with cDNAs encoding the $\alpha 2\beta 1$(M286W) receptor combination. B, Concentration-response relationships for potentiation of GABA by propofol. For wild-type (*WT*) $GABA_A$ $\alpha 2\beta 1$ receptors, significant potentiation of an EC_{20} test concentration of GABA occurs at all concentrations of ≥ 0.2 μM ($p < 0.05$ for each concentration ≥ 0.2 μM; 5–12 experiments for each data point). From the curve fit, the EC_{50} value for potentiation of wild-type $\alpha 2\beta 1$ receptors by propofol is 1.6 μM with a Hill slope of 1.1. In contrast, propofol (≤ 10 μM) does not potentiate submaximal GABA currents at $\alpha 2\beta 1$(M286W) (●, 6–9 experiments) or $\alpha 2\beta 1$(M286W)$\gamma 2$s (■, 7 experiments) mutant receptors.

Alphaxalone potentiation of GABA. None of the mutant receptors studied showed altered potentiation of submaximal GABA currents by 1 μM alphaxalone relative to wild-type $\alpha 2\beta 1$ receptors (Table 1).

Effects of mutations in TM2 and TM3 on direct receptor activation by intravenous and volatile anesthetics. Direct activation by isoflurane was not studied in detail because isoflurane produces only minimal direct activation (5–10% of maximal GABA currents) in wild-type $GABA_A$ $\alpha 2\beta 1$ receptors. The direct activation of wild-type $\alpha 2\beta 1$ receptors by isoflurane (Fig. 1A) was not evident in mutant receptors (Fig. 1, B and C). In fact, concentrations of isoflurane up to 5 mM (10 MAC) failed to elicit any direct activation of the $\alpha 2$(A291W)$\beta 1$ mutant receptor (data not shown).

Direct $GABA_A$ receptor activation by propofol. Direct activation of wild-type $GABA_A$ $\alpha 2\beta 1$ receptors by propofol also was described previously (Jones *et al.*, 1995). Concentrations of propofol of >50 μM can produce a profound block of the effect of GABA responses (Hales and Lambert, 1991), which is often followed by a "rebound" or "surge" current during washout. In our experiments, blocking and rebound effects were most evident at propofol concentrations of ≥ 100 μM, although small rebound currents immediately after washout of applied propofol were occasionally evident at 50 μM (Fig. 4). A propofol concentration of 50 μM thus was suitable to compare near-maximal direct activation by propofol in different receptors, with minimal interference from block.

Mutations in the $\alpha 2$ subunit had little effect on direct activation by 50 μM propofol (Fig. 4; Table 2). In fact, during the course of the potentiation experiments, it was noted that the receptor mutant $\alpha 2$(A291W)$\beta 1$ exhibited noticeable direct activation by 1 μM propofol, a situation not seen in the wild-type $\alpha 2\beta 1$ receptors. Further examination of the direct activation by propofol revealed that the $\alpha 2$(A291W)$\beta 1$ mutant receptor has >10-fold higher apparent affinity for propofol than the wild-type $\alpha 2\beta 1$ receptor, as shown by the leftward shift in the concentration-response relationship for direct activation by propofol (Fig. 5A). The GABA concentration-response curve for the $\alpha 2$(A291W)$\beta 1$ receptor is shifted to the left in a similar manner.

Interestingly, although submaximal GABA currents at the $\alpha 2\beta 1$(M286W) mutant receptor are not potentiated by propofol (see above), the direct activation by propofol of this mutant receptor was not different from its effect on wild-type receptors (Fig. 5A; Table 2). Propofol (50 μM) still activated the $\alpha 2\beta 1$(S265I) mutant receptor directly but elicited significantly smaller maximal currents compared with wild-type $\alpha 2\beta 1$ receptors ($p < 0.001$ compared with wild-type; Fig. 4, Table 2).

Direct $GABA_A$ receptor activation by etomidate. It has been reported in experiments with *Xenopus laevis* oocytes that the direct action of etomidate is nearly absent or that etomidate has substantially reduced efficacy and/or apparent affinity in $\beta 1$ compared with $\beta 2$ or $\beta 3$ subunit-containing receptors (Hill-Venning *et al.*, 1997; Sanna *et al.*, 1997). In our experiments using HEK 293 cells, etomidate

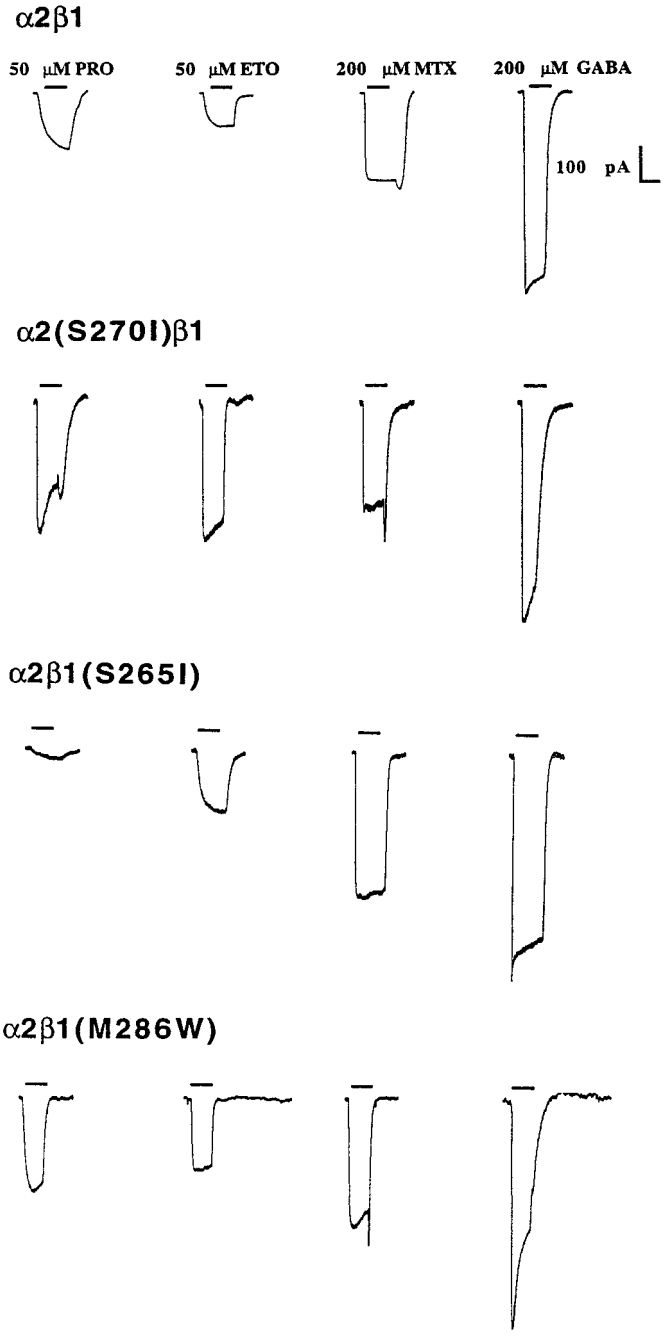

Fig. 4. Direct receptor activation by supra-anesthetic concentrations of propofol (*PRO*), etomidate (*ETO*), and methohexital (*MTX*) is similar to that for wild-type GABA$_A$ α2β1 receptors in most mutant receptors, except for α2β1(S265I), in which direct activation by propofol is reduced markedly. Wild-type GABA$_A$ α2β1 receptors are activated directly by propofol (50 μM), etomidate (50 μM), and methohexital (200 μM). Propofol, etomidate, and methohexital also directly activate α2(S270I)β1, α2β1(S265I), and α2β1(M286W) mutant receptors. Direct activation by propofol is reduced markedly in α2β1(S265I) but not α2(S270I)β1 receptors. *Horizontal scale bar*, 10 sec for direct activation by propofol, etomidate, and methohexital and 2 sec for the maximal GABA applications. The "rebound" currents at the tail end of drug applications are evident in some of the propofol and methohexital traces. Individual recordings are from HEK 293 cells transfected with cDNAs encoding the indicated subunit combinations.

produced significant direct effects in wild-type α2β1 receptors in the absence of GABA (Figs. 4 and 5B, Table 2). The direct activation by etomidate at wild-type α2β1 receptors had an EC_{50} value of 10.7 μM, a much higher apparent affinity than seen in experiments with β1-containing subunits in *X. laevis* oocytes (Hill-Venning *et al*., 1997; Sanna *et al*., 1997).

TABLE 2
Intravenous anesthetic direct activation data for wild-type and mutant receptors

Combination	Direct activation by intravenous anesthetics		
	Propofol (50 μM)	Etomidate (50 μM)	Methohexital (200 μM)
	% of maximal GABA current		
α2β1	53 ± 3 (8)	44 ± 6 (7)	57 ± 10 (6)
α2(S270I)β1	36 ± 8 (11)	46 ± 7 (11)	75 ± 9 (5)
α2(S270H)β1	40 ± 8 (11)	39 ± 4 (11)	43 ± 7 (8)
α2(A291W)β1	40 ± 8 (10)	30 ± 6 (8)	40 ± 11 (6)
α2β1(S265I)	15 ± 5 (11)[a]	18 ± 6 (11)[b]	38 ± 8 (10)
α2β1(M286W)	42 ± 4 (14)	26 ± 4 (19)[b]	45 ± 3 (9)

[a] $p < 0.001$ compared with the control group.
[b] $p < 0.05$ compared with the control group.

As with propofol, etomidate has an increased apparent affinity in the α2(A291W)β1 mutant receptor, as indicated by a leftward shift in the etomidate direct activation concentration-response curve (Fig. 5B). Direct activation by 50 μM etomidate of the α2β1(S265I) mutant receptor also was reduced slightly compared with the wild-type α2β1 receptor (Table 2). Although high concentrations of etomidate can produce channel block and rebound currents at GABA$_A$ receptors (Robertson, 1989), these were not seen during our experiments at etomidate concentrations up to 100 μM.

Direct GABA$_A$ receptor activation by methohexital. At high concentrations, methohexital produces substantial direct activation of GABA$_A$ α2β1 receptors (Koltchine *et al*., 1996); 200 μM methohexital was chosen for the direct activation experiments because at this concentration, methohexital elicits a large direct effect with minimal blocking effects. Direct activation of all the mutant receptors by methohexital was similar to that for the wild-type α2β1 receptor (Fig. 4, Table 2). Direct activation by alphaxalone was not studied because it is not prominent in GABA$_A$ α2β1 receptors (C. E. Rick, unpublished observations).

Discussion

Actions of general anesthetics on wild-type GABA$_A$ α2β1 receptors. Submaximal GABA currents at wild-type human GABA$_A$ α2β1 receptors were potentiated by all the volatile and intravenous anesthetics studied. Thus, in agreement with other published studies, the presence of the γ subunit is not required for potentiation by propofol (Jones *et al*., 1995), methohexital (Koltchine *et al*., 1996), etomidate (Uchida *et al*., 1995; Sanna *et al*., 1997), alphaxalone (Horne *et al*., 1993), or isoflurane (Harrison *et al*., 1993; Mihic *et al*., 1994).

A mutation in TM3 of the α2 subunit alters the concentration-response relationship for GABA and for direct receptor activation by propofol and etomidate. Although the GABA concentration-effect relationships for the mutants analyzed in this study mostly were very similar to that in the α2β1 wild-type receptor, a few changes were evident. A receptor with a mutation in TM3 of the α2 subunit, α2(A291W)β1, has a higher apparent affinity for GABA than the wild-type α2β1 receptor, as indicated by a leftward shift

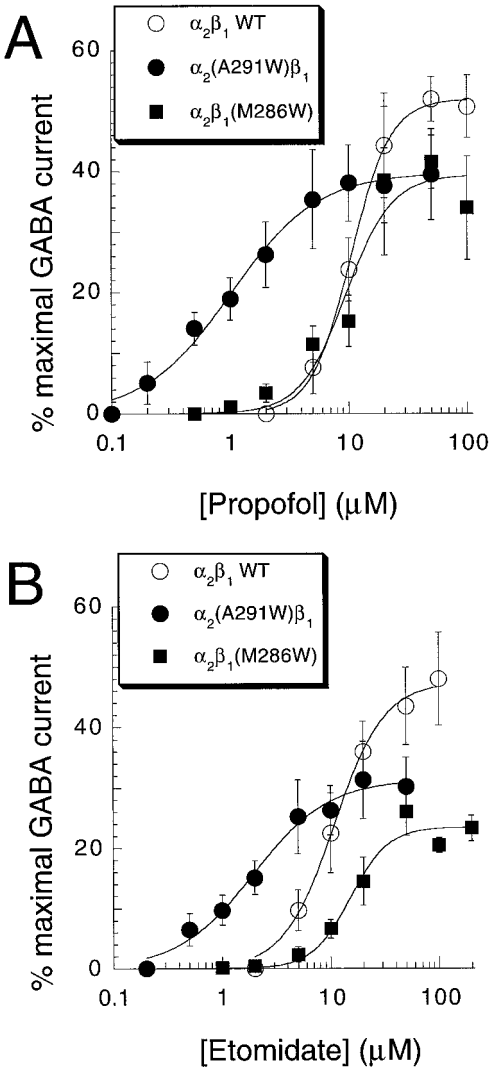

Fig. 5. Concentration-response relationships for direct activation of $GABA_A$ $\alpha2\beta1$ receptors by propofol and etomidate. A, For wild-type (*WT*) $\alpha2\beta1$ receptors (○), propofol produces significant direct activation at all concentrations of ≥5 μM ($p < 0.05$ for each concentration ≥5 μM, 4–8 experiments). The direct activation of wild-type $\alpha2\beta1$ receptors by propofol has an EC_{50} value of 10.5 μM, Hill slope of 2.6, and E_{max} value of 52.0% of the maximal GABA current. In contrast, the $\alpha2$(A291W)$\beta1$ receptor is more sensitive than the wild-type $\alpha2\beta1$ receptor to the direct actions of propofol (●). Propofol produces significant direct activation at all concentrations of ≥0.5 μM at the $\alpha2$(A291W)$\beta1$ receptor ($p < 0.01$ for each concentration ≥0.5 μM, 4–13 experiments). The direct activation of $\alpha2$(A291W)$\beta1$ receptors by propofol has an EC_{50} value of 1.0 μM, Hill slope of 1.2, and E_{max} value of 39.8%. The $\alpha2\beta1$(M286W) mutant receptor, although insensitive to potentiation by propofol, is still directly gated by propofol (■) at all concentrations of ≥2 μM ($p < 0.05$ for each concentration ≥ 2 μM, 6–15 experiments). The direct activation of $\alpha2\beta1$(M286W) receptors by propofol has an EC_{50} value of 9.5 μM, Hill slope of 2.2, and E_{max} value of 39.5%. B, For wild-type (*WT*) $\alpha2\beta1$ receptors (○), etomidate produces significant direct activation at all concentrations of ≥5 μM ($p < 0.05$ for each concentration ≥5 μM, 4–7 experiments). The direct activation of wild-type $\alpha2\beta1$ receptors by etomidate has an EC_{50} value of 10.7 μM, Hill slope of 1.9, and E_{max} value of 47.5%. Similar to propofol, the $\alpha2$(A291W)$\beta1$ receptor is more sensitive to the direct actions of etomidate (●). Etomidate produces significant direct activation at all concentrations of ≥0.5 μM at the $\alpha2$(A291W)$\beta1$ receptor ($p < 0.05$ for each concentration ≥0.5 μM, 7–10 experiments). The direct activation of $\alpha2$(A291W)$\beta1$ receptors by etomidate has an EC_{50} value of 1.9 μM, Hill slope of 1.2, and E_{max} value of 31.4%. The $\alpha2\beta1$(M286W) mutant receptor is directly gated by etomidate (■) at all concentrations of ≥10 μM ($p < 0.01$ for each concentration ≥10 μM, 7–19 experiments). The direct activation of $\alpha2\beta1$(M286W) receptors by etomidate has an EC_{50} value of 15.1 μM, Hill slope of 2.5, and E_{max} value of 23.5%. *Error bars*, standard error.

in the GABA concentration-response curve. In addition, the concentration-response curves for direct activation by etomidate and propofol were shifted to the left of those for the wild-type $\alpha2\beta1$ receptor by ≈6- and ≈10-fold, respectively. Thus, for the $\alpha2$(A291W)$\beta1$ mutant receptor, the apparent affinities for activation by GABA, etomidate, and propofol are all increased relative to wild-type. Also, the receptors containing mutations in the $\beta1$, but not $\alpha2$, subunit had significantly lower Hill slopes compared with the wild-type $\alpha2\beta1$ receptor for their GABA concentration-response relationships. These changes suggest altered gating mechanisms in these mutant receptors.

The higher apparent affinity of GABA, etomidate, and propofol for the $\alpha2$(A291W)$\beta1$ mutant receptor may be related to the observation that mutations at the 291 position in TM3 ($GABA_A$ $\alpha2$ numbering) have the propensity for producing tonically open $GABA_A$ or glycine receptors. This, however, is observed only in the glycine $\alpha1$(A288W) and $GABA_A$ $\alpha2$(A291W)$\beta1$(M286W) mutant receptors (Mihic *et al.*, 1997). In other words, tonically active receptors are produced only when all five subunits of the presumed pentameric receptor contain the mutation to tryptophan in TM3.

A mutation in TM3 of the $\beta1$ subunit abolishes potentiation of GABA but not direct receptor activation by propofol. For the most part, the mutations analyzed in this study had little effect on GABA potentiation and/or direct activation by the intravenous anesthetics propofol, etomidate, methohexital, or alphaxalone. An exception is the $\alpha2\beta1$(M286W) mutant receptor, at which propofol concentrations of ≤10 μM failed to enhance submaximal GABA currents. The addition of a $\gamma2$s subunit did not alter the deleterious effect of the $\beta1$(M286W) mutation because the $\alpha2\beta1$(M286W)$\gamma2$s mutant receptor also was insensitive to propofol potentiation of GABA. In contrast to the lack of potentiation, propofol still directly activates this mutant receptor, with a concentration-response relationship that overlaps that for the wild-type $\alpha2\beta1$ receptors.

Although the relationship between agonist potentiation and direct activation by intravenous anesthetics at the $GABA_A$ receptor is poorly understood, there are examples of subunit combinations in which one effect is present without the other. For example, receptors containing the $GABA_A$ $\alpha4$ subunit show agonist potentiation but not direct receptor activation by propofol and pentobarbital (Wafford *et al.*, 1996); a similar situation is seen with *Drosophila* GABA receptors (Belelli *et al.*, 1996). In the current study, a receptor with a mutation in TM2 of the $\beta1$ subunit, $\alpha2\beta1$(S265I), shows markedly reduced direct activation by propofol but retains normal GABA potentiation. These results suggest distinct requirements for potentiation and direct activation by intravenous general anesthetics. Also, in conjunction with the results of previous studies (Sanna *et al.*, 1995a, 1995b), our results further implicate the β subunit as the major determinant influencing propofol actions at the $GABA_A$ receptor.

Recent work has demonstrated that mutation of N289 within the $GABA_A$ $\beta3$ subunit (homologous with S265 in the $\beta1$ subunit) to methionine abolishes direct activation and GABA potentiation by etomidate (Belelli *et al.*, 1997). Although none of the TM2 mutants analyzed in this study significantly reduced GABA potentiation by etomidate, direct activation of the $\alpha2\beta1$(S265I) and $\alpha2\beta1$(M286W) mutant re-

ceptors was reduced significantly compared with wild-type. It will be interesting to determine whether other residues within or near TM2 of the β subunit also alter the modulatory effects or direct activation by etomidate.

Residues within TM2 and TM3 of the GABA$_A$ receptor may form part of a volatile ether anesthetic binding site. The results from this study demonstrate that specific residues within both TM2 and TM3 of GABA$_A$ α2 and β1 subunits are necessary for positive receptor modulation by the halogenated methyl ethyl ether anesthetic isoflurane. This extends the findings from a previous study that these residues also are critical for positive modulation by the related volatile anesthetic enflurane (Mihic *et al*., 1997). These same specific residues within TM2 and TM3 seem to be critical for modulation by *n*-alkanols (Mihic *et al*., 1997). In this study, mutations in either the α2 or β1 subunits in TM2 or TM3 abolished modulation by isoflurane. Similar to the situation seen with propofol potentiation of GABA at the α2β1(M286W)γ2s mutant receptor, the α2(A291W)β1γ2s mutant receptor was as insensitive to isoflurane potentiation of GABA as the α2(A291W)β1 mutant receptor. This is consistent with the hypothesis that the γ subunit is not a major determinant of volatile anesthetic modulation.

The mutations analyzed in the current study all result from the replacement of a smaller amino acid by a larger amino acid (e.g., serine to isoleucine or histidine, alanine or methionine to tryptophan). We hypothesize that the serine in TM2 (for α2 or β1 subunits) and alanine (α2 subunit) or methionine (β1 subunit) in TM3 forms part of a binding site for enflurane, isoflurane, and *n*-alkanols. If residues within TM2 and TM3 of the GABA$_A$ α and β subunits do indeed form part of a volatile anesthetic binding pocket, then substitution of larger amino acid residues at those positions (e.g., those found at the corresponding positions in the GABA ρ1 receptor) may sterically hinder volatile anesthetic binding and thereby ablate positive modulation of receptor function.

In summary, the results of this study indicate that the receptor structural requirements for volatile and intravenous general anesthetic modulation of the GABA$_A$ receptor may be quite distinct. Future work with chimeric and additional mutant receptor subunits will attempt to uncover whether TM3 in the β subunit contains a binding site for propofol or whether mutations such as β1(M286W) instead interfere with transduction step or steps necessary for agonist potentiation by propofol.

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