Journal of Neurochemistry
Lippincott Williams & Wilkins, Inc., Philadelphia

Identification of an Amino Acid Defining the Distinct Properties of Murine β_1 and β_3 Subunit-Containing $GABA_A$ Receptors

*Ismar N. Cestari, †Kyeong T. Min, †John C. Kulli, and *†Jay Yang

**Neuroscience Graduate Program, University of Texas Southwestern Medical Center, Dallas, Texas; and †Departments of Anesthesiology and Pharmacology/Physiology, University of Rochester Medical Center, Rochester, New York, U.S.A.*

Abstract: Murine γ-aminobutyric acid (GABA) type A homomeric receptors made of β_1 subunits are profoundly different, when expressed in *Xenopus* oocytes, from β_3 homomeric receptors. Application of the intravenous general anesthetic pentobarbital, etomidate, or propofol to β_3 homomeric receptors allows current flow. In contrast, β_1 homomers do not respond to any of these agents. Through construction of chimeric β_1/β_3 receptors, we identified a single amino acid that determines the pharmacological difference between the two β subunits. When the serine residue present in the wild-type nonresponsive β_1 subunit is replaced by an asparagine found in the same position in the β_3 subunit, the resulting point-mutated β_1S265N forms receptors responsive to intravenous general anesthetics, like the wild-type β_3 subunits. Conversely, after mutation of the wild-type β_3 to β_3N265S, the homomeric receptor loses its ability to respond to these same general anesthetics. Wild-type-to-mutant titration experiments showed that the nonresponsive phenotype is dominant: A single nonresponsive residue within a pentameric receptor is sufficient to render the receptor nonresponsive. In $\alpha_1\beta_x$ or $\alpha_1\beta_x\gamma_2$ heteromeric receptors, the same residue manifests as a partial determinant of the degree of potentiation of the GABA-induced current by some general anesthetics. The location of this amino acid at the extracellular end of the second transmembrane segment, its influence in both homomeric and heteromeric receptor function, and its dominant behavior suggest that this residue of the β subunit is involved in an allosteric modulation of the receptor. **Key Words:** $GABA_A$ receptor—β subunit—Gating—Chimera—Site-directed mutagenesis—Intravenous general anesthetics.
J. Neurochem. **74,** 827–838 (2000).

γ-Aminobutyric acid (GABA) type A ($GABA_A$) receptor is a member of the ligand-gated ion channel superfamily of homologous receptors (Schofield et al., 1987). Binding of the ligand, GABA, to the receptor opens the integral Cl^- channel, driving the membrane potential toward the chloride equilibrium potential, thus reducing the sensitivity of the membrane to the effects of excitatory neurotransmitters. GABA and competitive GABA agonists such as muscimol, as well as antagonists such as bicuculline, bind to the extracellular N-terminal region of both the α subunit (Sigel et al., 1992) and the β subunit (Amin and Weiss, 1993). Similarly, benzodiazepines bind to the N-terminal region of the γ subunit (Mihic et al., 1994). General anesthetic drugs of diverse chemical structures, e.g., pentobarbital, propofol, etomidate, and volatile agents such as halothane, directly open the $GABA_A$ Cl^- channel in the absence of GABA (Franks and Lieb, 1994). The site on the receptor where these drugs act has not been identified, but recent evidence suggests a critical role of the second transmembrane segment (M2) in the action of volatile general anesthetics, alcohol, and etomidate on $GABA_A$ receptors (Belelli et al., 1997; Mihic et al., 1997; Moody et al., 1997).

The $GABA_A$ receptor is usually considered to be a pentamer, consisting of five homologous subunits of at least five types, α, β, γ, δ, and ε, each of which has several isoforms. The most common subunit composition of the $GABA_A$ receptor is $2\alpha{:}2\beta{:}\gamma$ (McKernan and Whiting, 1996). However, when β subunits are expressed alone, functional homomeric channels are formed. The properties of murine β_1 homomeric channels are different from those of β_3 channels (Cestari et al., 1996; Krishek et al., 1996; Davies et al., 1997; Wooltorton et al., 1997). In β_3, but not β_1, homomers pentobarbital generates a robust current that retains several pharmacological characteristics of both native and recombinant heteromeric receptors (Cestari et al., 1996; Davies et al., 1997). Similarly, etomidate (Evans and Hill, 1978; Yang and Uchida, 1996) and propofol (Hara et al., 1993), two other intravenous general anesthetics known to open chloride channels, also induced currents in β_3 but not β_1 homomers. Because of the previously

Received August 6, 1999; revised manuscript received September 30, 1999; accepted October 1, 1999.

Address correspondence and reprint requests to Dr. J. Yang at Department of Anesthesiology and Pharmacology/Physiology, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, NY 14642, U.S.A. E-mail: jyang@anes.rochester.edu

Abbreviations used: GABA, γ-aminobutryic acid; $GABA_A$, γ-aminobutyric acid type A; M2–M4, second, third, and fourth transmembrane segment, respectively; S/N site, serine–asparagine site corresponding to amino acid 265 on the β_3 wild-type subunit.

TABLE 1. *Primers used for construction of chimeric subunits and size of PCR fragments incorporated into the final construct*

Chimera no.	Forward primer	Reverse primer	PCR size and sites
I	TTCTGG**G**ATCCCATTAAATCTGACCC	TGCGACTC**GA**GCTGCAGATGCATCATAGT	359 bp, *Bam*HI[a]–*Bst*XI
II	AAATTGAG**C**TCCCCCAATTTTCAATTGTT	TGCGACTC**GA**GCTGCAGATGCATCATAGT	254 bp, *Sac*I[a]–*Sau*I
III	CTGCAGC**T**CGAGTCGCACTAGGAATCAC	GTAATACGACTCACTATAGGGC[b]	55 bp, *Xho*I[a]–*Sau*I
IV	AATTAACCCTCACTAAAGGG[c]	TGCGACTC**GA**GCTGCAGATGCATCATAGT	160 bp, *Bst*XI–*Xho*I[a]
V	ACGGGAA**T**ATTCTATTAGCACCGATGGAT	GTAATACGACTCACTATAGGGC[b]	321 bp, *Ssp*I[a]–*Not*I[d]
VI	CGCAGG**C**GC**G**CCTCACAGCTCAAAATCAAA	GTAATACGACTCACTATAGGGC[b]	129 bp, *Bss*HII[a]–*Not*I[d]
VII	AAAAAG**CTT**GCGAGCAAACAAGACCAG	GTAATACGACTCACTATAGGGC[b]	160 bp/404 bp, *Bst*XI–*Xho*I[a] and *Hin*dIII[a]–*Not*I[d]

The chimera numbers correspond to the schematic shown in Fig. 2. Actual nucleotides mutated are shown in bold, and the restriction enzyme sites thus created are underlined. The restriction enzyme sites used to subclone the PCR-amplified fragments and the final size of the PCR-derived fragments are also given.

[a] Mutated restriction enzyme site.

[b] T7 primer in the 3′ end of the vector.

[c] T3 primer in the 5′ end of the vector.

[d] *Not*I restriction enzyme site in the multiple cloning site 3′ to the insert.

demonstrated importance of M2 in the action of general anesthetics and other $GABA_A$ allosteric modulators (Wafford et al., 1994; Stevenson et al., 1995; Thompson et al., 1999), we investigated whether the difference in general anesthetic pharmacology between β_1 and β_3 subunit homomers can be explained by divergent amino acids in this region of the subunits.

MATERIALS AND METHODS

cDNA clones

The murine $GABA_A$ receptor α_1 (Wang et al., 1992) and β_1 and β_3 subunit (Kamatchi et al., 1995) cDNA clones were a gift of Dr. David R. Burt (University of Maryland Medical School). After restriction enzyme mapping to confirm the identity of the clones, the inserts were excised from the original pGEM (7z) vector using *Eco*RI and subcloned into a modified pBluescript plasmid (Stratagene), pMXT (a gift from Dr. Aguan Wei, Washington University Medical Center). The multiple cloning site of this vector is flanked by the 5′ and 3′ untranslated sequences of the *Xenopus* globin gene for increased stability of the mRNA (Gurdon et al., 1973). The numbering of amino acids is based on the mature subunit peptide resulting after cleavage of the signal peptide sequence (Kamatchi et al., 1995).

Construction of chimeric subunits

The β_1 and β_3 subunit cDNA clones were cut by restriction enzymes and then spliced to produce chimeric β_1/β_3 subunits, in which a region of one subunit was replaced by the corresponding region of the other. Because no useful restriction enzyme sites were common to both subunits, six restriction enzyme sites naturally present in one or the other subunit were selected, based on their relative locations along the sequence and convenience of use during subcloning. Correspondingly located restriction enzyme sites were then introduced as silent mutations in the other subunit clone using PCR mutagenesis (Vallette et al., 1989).

PCR primers containing the necessary mutations were obtained from GibcoBRL (Life Technologies) and are listed in Table 1. These primers were used to amplify the corresponding regions of linearized pMXT containing the β_1 or β_3 cDNA clones. PCR was performed using the Expand High Fidelity PCR kit (Boehringer Mannheim). High amounts of DNA template and low cycle number in the reaction protocol were also used to reduce PCR-introduced errors. PCR-amplified DNA was subcloned using the PCR-Script SK(+) Cloning Kit (Stratagene) following the manufacturer's recommended protocol. The subcloned PCR-mutated DNA fragment was restriction enzyme-digested and ligated into the corresponding region of the original pMXT containing the β subunit cDNA clone. In most cases, an additional subcloning step was performed to minimize the size of PCR-amplified DNA fragment incorporated into the final chimera. After the final subcloning step, each chimera was sequenced in an automated sequencer (Applied Biosystems) to verify that the fragments were correctly spliced and to check for the absence of unintended errors.

For chimera I, a PCR mixture [5 μl of 10× buffer with $MgCl_2$, 4 μl of deoxynucleotide triphosphates (250 μM each), and 0.5 μl of *Taq* polymerase (3.5 U/μl) in a total volume of 50 μl] was assembled using the linearized β_1-pMXT cDNA clone as template (200 ng) with primers B1BAMHI5′ and B1XHOI3′ (500 μM each). After mixing and spinning, two drops of mineral oil were added to the tube, and the reaction was performed for 15 cycles in a thermal cycler (94°C for 30 s, 62°C for 45 s, 72°C for 45 s). The PCR-amplified DNA (520 bp) was electrophoresed in 1% agarose gel, recovered with the QIAEX DNA isolation system (Qiagen), and subcloned into the PCR-Script SK(+) using the manufacturer's suggested protocol. After confirming the identity and orientation of the insert by restriction enzyme analysis, purified plasmid DNA was digested with *Bam*HI, and the fragment obtained, extending from the mutated *Bam*HI site to the vector *Bam*HI site, was subcloned into the β_3-pMXT plasmid (after *Bam*HI digestion and dephosphorylation). The resulting intermediate plasmid was then cut by *Xho*I (present in the vector upstream to the coding sequence) and *Bst*XI (site present in the wild-type β_1 sequence), and the insert was subcloned into β_1-pMXT (also cut by *Xho*I and *Bst*XI) to form chimera I, with a 359-bp DNA segment of PCR origin. Other chimeras were created in a similar manner from PCR products resulting from the primer pairs listed in Table 1.

Single amino acid mutants

Point mutations were introduced by two-round PCR mutagenesis (Vallette et al., 1989). This technique allows the introduction

TABLE 2. *Primers used for construction of point mutants and size of PCR fragments incorporated into the final construct*

Mutant	Forward primer	Reverse primer	PCR size and sites
β_1S265A	AAATTGAG**C**TCCCCCAATTTTCAATTGTT	GTCTCCCTGAGGTGAGTGG**CG**GATGGTGGTCATGGTCAG	215 bp, *Bst*XI–*Sau*I
β_1S265G	AAATTGAG**C**TCCCCCAATTTTCAATTGTT	GTCTCCCTGAGGTGAGTG**GC**GATGGTGGTCATGGTCAG	215 bp, *Bst*XI–*Sau*I
β_1S265T	AAATTGAG**C**TCCCCCAATTTTCAATTGTT	GTCTCCCTGAGGTGAGTGG**G**TGATGGTGGTCATGGTCAG	215 bp, *Bst*XI–*Sau*I
β_1S265Q	AAATTGAG**C**TCCCCCAATTTTCAATTGTT	GTCTCCCTGAGGTGAGTG**TTG**GATGGTGGTCATGGTCAG	215 bp, *Bst*XI–*Sau*I

Mutant	Inside primers	Outside primers	PCR size and sites
β_1S265N	ACCATC**AA**CACTCACCTCAGGGAGACT and GTGAGTG**TT**GATGGTGGTCATGGTCAG	AAATTGAG**C**TCCCCCAATTTTCAATTGTT and GTAATACGACTCACTATAGGGC[a]	215 bp, *Bst*XI–*Sau*I
β_1I283M	TTGAC**AT**GTATCTCATGGGCTGTTTT and TGAGATA**C**ATGTCAATCGCTTTGACG	AAATTGAG**C**TCCCCCAATTTTCAATTGTT and GTAATACGACTCACTATAGGGC[a]	421 bp, *Sau*I–*Bss*HII
β_3N265S	**AG**CACTCAC**C**TT**A**GGGAGACTCTACCCAAAA[b] and GTCTCCC**T**AAGGTGAGTG**C**TGATGGTTGTCATGGTGAG[b]	ATTTGTCCACGGAGTGACAGT and TCAACGCGTTAACATAGTACAGCCAGT	200 bp, *Xho*I–*Hin*dIII

The point mutants were constructed by either a straightforward incorporation of a base change and a unique restriction enzyme site in the primer itself (top section) or by the two-step sequential PCR method (bottom section). Actual nucleotides mutated are shown in bold, and the restriction enzyme sites thus created are underlined. The restriction enzyme sites used to subclone the PCR-amplified fragments and the final size of the PCR-derived fragments are also given.

[a] T7 primer at the 3′ end of the vector.

[b] Additional silent mutation introduced to create a new restriction enzyme site (dashed underline).

of mutations at any region of a PCR fragment by performing two consecutive PCR procedures. In the first round, two PCR products are made that overlap in the region where a mutation is to be introduced. The second round uses the products of the first round as templates and generates a fragment that is the sum of the two overlapping products. This secondary PCR-amplified DNA was subcloned into PCR-Script as described for the construction of the chimeras, and the smallest possible fragment was subcloned into the wild-type subunits. For the β_1S265N mutant, two first-round PCR assays were assembled using chimera II as template with primers B1SACI5′ and B1S265N3′, and a separate reaction was set up using chimera VI as template and the primers B1S265N5′ and B3MLU3′. The PCR products of these two first-round assays were used as templates for the second-round reaction with the primers B1SACI5′ and B3MLU3′ (outside primers) following the same protocol of the PCR assay for chimera I. The DNA fragment amplified in this reaction was digested with *Bst*XI and *Bsu*36I and directly subcloned into β_1-pMXT. The final clone contained the mutant β_1S265N site and had 210 bp of PCR-amplified DNA fragment. The oligonucleotide primers used for construction of this and other point mutants are listed in Table 2.

Oocyte expression

Oocytes were obtained from *Xenopus laevis* (Nasco or Xenopus I) following a protocol approved by the institutional animal care committee. The frogs were anesthetized in 50 ml of water containing 0.15% tricaine for 20–30 min or until the animals did not move when positioned with the dorsal side down. Pieces of the ovaries were removed through small incisions in the abdomen. Oocytes were dissociated and defolliculated by gentle shaking in calcium-free modified Barth's medium (88 m*M* NaCl, 1 m*M* KCl, 2.4 m*M* $NaHCO_3$, 0.82 m*M* $MgSO_4$, 0.41 m*M* $CaCl_2$, and 7.5 m*M* Tris-HCl, pH 7.4) with added collagenase (1 mg/ml, type I; Sigma). After dissociation, healthy stage V–VI oocytes were selected and used for cRNA injection.

The cRNA was prepared using the T3 Message Machine kit (Ambion) following the manufacturer's recommended protocol. Electrophoresis on a 1% agarose gel was performed on 1 μl of the cRNA solution and on a standard RNA sample (1 μg/10 μl; GibcoBRL), and comparison of the relative intensity of the bands on UV illumination was used to estimate the cRNA concentration. Injection micropipettes were fabricated using glass capillary tubing previously immersed in dimethyldichlorosilane and baked overnight at 180°C.

LiCl-precipitated cRNA was diluted to the desired concentration (between 0.25 and 1 μg/μl) in sterile RNase-free water and aspirated into a micropipette, and 50 nl was injected into each oocyte with a microinjector (Nanoinjector; Drumond Instruments). Injected oocytes were incubated at 18°C in modified Barth's medium [as above with 0.33 m*M* $Ca(NO_3)_2$ added] containing 100 U/ml penicillin and 100 μg/ml streptomycin, solution was changed daily, and experiments were performed after 48–72 h.

Electrophysiological recordings

Electrophysiological recordings were performed in a 200-μl chamber, with an effective volume of 50 μl (controlled by the level of the suction outlet), continuously perfused at a rate of 3 ml/min with frog Ringer's solution (115 m*M* NaCl, 2 m*M* KCl, 1.3 m*M* Na_2HPO_4, and 1.8 m*M* $CaCl_2$, pH 7.4). A separate inlet was connected to a solenoid valve system, permitting the application of any of 24 different drug-containing solutions. The change from Ringer's solution to drug-containing solution was performed by simultaneously closing the control solution inlet and opening the drug-containing solution inlet, with timing synchronized with data collection and controlled by pClamp version 5.5 software (Axon Instruments). Recording microelectrodes were made of borosilicate glass (outside diameter, 1.2 mm) and had a resistance of 1–3 MΩ when filled with filtered 3 *M* KCl solution. Standard two-electrode recording was performed using a GeneClamp 500 amplifier (Axon Instruments),

with the membrane potential held at −60 mV. All drugs and chemicals were purchased from Sigma Chemical Co. except etomidate (Amidate; Abbott Laboratories), and propofol (Aldrich Chemical). Experiments were performed at room temperature (21–24°C).

Data analysis

Concentration–response curves were obtained by fitting a function of the form

$$I/I_{max} = C^n/(C^n + EC_{50}{}^n),$$

where I is the peak current attained during a drug application, I_{max} is the maximal current obtained for that cell, C is the drug concentration, n is the Hill coefficient, and EC_{50} is the concentration giving half-maximal current. Averaged values are presented as mean ± SEM values. Statistical significance of an effect was determined by the two-sided t test with the criterion set at $p < 0.05$.

Peak current induced by a constant concentration of pentobarbital was frequently observed to decrease progressively on repeated exposure, even when the interval between applications was long (>15 min). Correction for this effect was made by applying a standard dose of the agonist at the beginning of the data set and after every two test applications. Corrected peak current amplitude of the test application was calculated using the formula

$$I_{corrected} = I_{observed} \times (I_o/I_j)$$

where $I_{observed}$ is recorded peak current amplitude value (test), I_o is the peak current amplitude obtained during the application of the standard dose at the beginning of the data set, and I_j is the current value obtained for the standard dose preceding the test application (Amin et al., 1994). For quantitation of current potentiation by pentobarbital or etomidate, the true potentiation over additive response was calculated as percent potentiation $= 100 \times I_{GABA+etomidate}/(I_{GABA} + I_{etomidate})$.

RESULTS

Identification of amino acids necessary for pentobarbital activation of the $GABA_A$ β subunit homomeric channels

Figure 1 shows the effect of GABA, pentobarbital, and picrotoxin on homomeric β_1 and β_3 receptors. GABA up to 500 μM had no effect on either type of receptor. In contrast, 250 μM pentobarbital induced a large inward current in the β_3 homomeric receptor. At a higher pentobarbital concentration (2.5 mM) the peak current was less, and the characteristic rebound current observed on washout back to the control solution was seen (Akaike et al., 1985). This pentobarbital-induced current is known to be modulated by lanthanides and zinc but not to be blocked by the $GABA_A$ competitive antagonist bicuculline as reported previously (Cestari et al., 1996). The same concentrations of pentobarbital evoked no robust inward current in the β_1 homomers. Instead, we observed a dose-dependent outward current, probably representing the pentobarbital-induced blockade of spontaneously opening channels. For both subunit isoforms, picrotoxin evoked an outward-going current, again consistent with the blockade of spontaneously opening channels. In some murine β_1-injected oocytes, pentobarbital induced small inward currents (<50 nA), consistent with previous reports on human β_1 homomers (Sanna et al., 1995). We considered these small currents to represent a fundamentally different phenomenon and did not pursue it further in the present study.

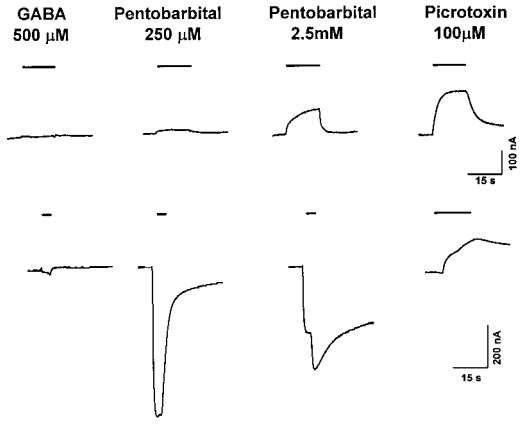

FIG. 1. β_1 and β_3 homomeric receptors have distinct pharmacological properties. Murine $GABA_A$ receptor (**top**) β_1-subunit or (**bottom**) β_3-subunit cRNA was injected into *Xenopus* oocytes. At 48–72 h later, GABA, pentobarbital, or picrotoxin was applied to oocytes (voltage-clamped at −50 mV). Currents elicited are shown (time of application is denoted by the solid bar). Opening of the chloride ionophore in β_3 homomers induces an inward-going (downward) current, and closing of spontaneously opening channels in β_1 homomers induces an outward-going (upward) current. Note the difference in the recovery from picrotoxin for the two subunit isoforms (rightmost traces).

To locate the site determining the different functional properties of the β_1 and β_3 homomers, a series of chimeric subunits were constructed in which a segment of β_1 cDNA was replaced by a corresponding segment of β_3 cDNA. Figure 2 shows a schematic representation of the parent and chimeric subunits. Each chimeric subunit was expressed as a homomeric receptor in oocytes voltage-clamped at −60 mV, and the response to the application of 500 μM pentobarbital was measured. A positive response was defined as a current >50 nA (noted in Fig. 2 by an asterisk next to the positive chimeras). To exclude the possibility of a nonfunctional chimera, all six chimeras were coexpressed with the α_1 subunit. All α_1:β_1/β_3 chimeric receptors were functional, responding to both pentobarbital and GABA (n = 4 for each chimera). Because both β_1 and β_3 parent subunits form functional receptors when coexpressed with the α_1 subunit (Ymer et al., 1989), this result was not surprising.

Of the first six chimeras made, only chimera IV displayed the positive response to pentobarbital (Fig. 3). In this chimera, the β_1 sequence replaced the β_3 sequence from the beginning of M2 all the way to the C-terminal end; this is nearly the entire second half of the subunit. Chimera V, in which the β_1 sequence replaced the β_3 sequence from the middle of the second intracellular loop between the third (M3) and fourth (M4) transmembrane segments to the C-terminal end, did not show a positive response. Thus, the region of the responsive

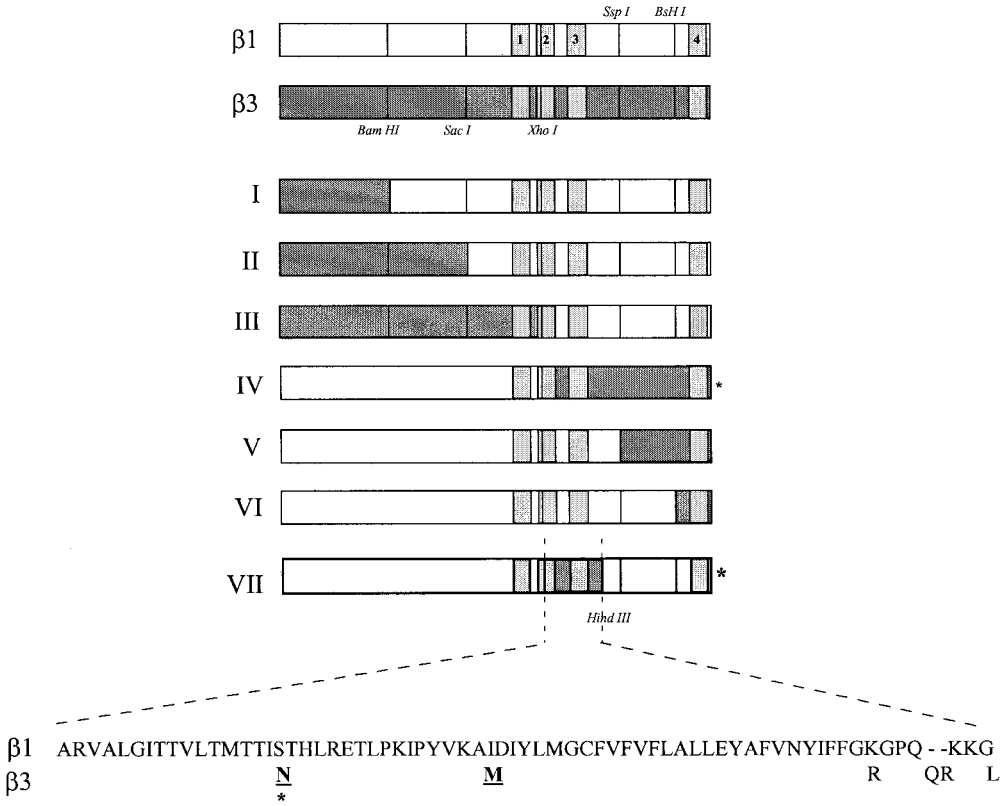

FIG. 2. A scheme of β_1/β_3 chimeras. Open bars represent the parent β_1 subunit sequence, and solid bars represent the β_3 subunits. The four transmembrane segments (M1–M4) are shown as light gray. The restriction enzyme sites used in constructing the chimeras are labeled. The asterisks (to the right of the bars) identify chimeras that, when expressed as homomeric receptors, are responsive to pentobarbital. At the bottom is the amino acid sequence from β_1 (**top**) and β_3 (**bottom**) subunits in the critical region responsible for the difference in response to pentobarbital defined by the chimeras. The serine-265 and isoleucine-283 on the β_1 subunit selected for site-directed mutagenesis are underlined.

chimera IV not common to the nonresponsive chimera V is required for the transfer of function. This region contains 93 amino acids, of which 19 differ between the β_1 and β_3 sequences. To narrow further the region conferring the positive response to pentobarbital, chimera VII was made by replacing the β_1 sequence between the *Xho*I and *Hin*dIII sites by the corresponding β_3 sequence, as shown in Fig. 2. The transferred region had four amino acid substitutions and two additional amino acids present only in the β_3 sequence. This chimera, when expressed as a homomer, retained the positive response, as shown in Fig. 3, strongly suggesting that one or more of the six divergent amino acids were responsible for the observed phenotypic differences.

Serine at position 265 is the functional determinant of pentobarbital action on GABA$_A$ β subunit homomers

Of the six discordant amino acids, those at positions 265 and 283 were selected for mutagenesis, based on their locations at the extracellular end of M2 and M3 and the role of the amino acid at position 265 in other properties of the GABA$_A$ receptor. Two mutants of the β_1 subunit were constructed. In the first, denoted β_1I283M, the isoleucine at position 283 was replaced by the β_3's methionine; β_1I283M did not acquire the response to pentobarbital (data not shown). In the second, β_1S265N, the serine at position 265 was replaced by β_3's asparagine.

Like wild-type β_3 homomeric receptors, β_1S265N homomers responded to pentobarbital in a concentration-dependent manner, with a decreased peak current at high concentrations and an "off-response" on beginning of washout, as shown in Fig. 4A. Figure 4B shows the best fit of the normalized responses to the Hill equation, with values for n_H of 1.7 ± 0.2 and EC_{50} of 38.2 ± 3.7 μM (solid line) comparable to the wild-type β_3 response (dotted line). Also like the β_3 wild-type, β_1S265N responded to etomidate and propofol (Fig. 5). To ensure that the converse mutation of the β_3 homomer would convert its phenotype to the β_1 type, we generated the mutant β_3N265S. Figure 6 shows that β_3N265S homomers indeed possess a β_1 phenotype: Pentobarbital caused no inward current. Sensitivity to pentobarbital, etomidate, and propofol can be conferred on β_1 homomers by a single amino acid substitution, which is then necessary and sufficient for the expression of this phenotype. This position will be referred in the subsequent text as the S/N site (for serine–asparagine site

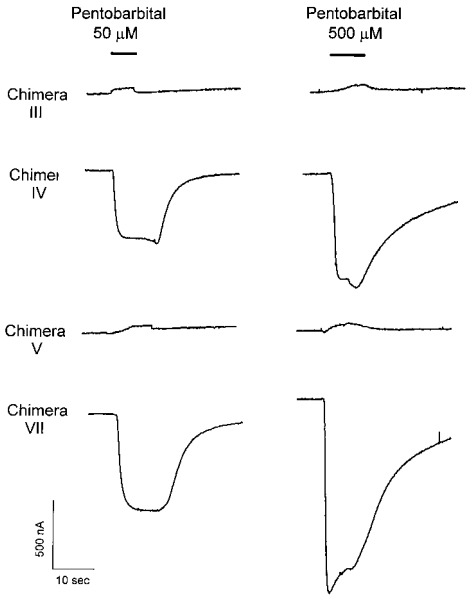

FIG. 3. Responses to pentobarbital applications in β_1/β_3 chimeras. Current response to application of 50 (**left**) or 500 μM (**right**) pentobarbital in oocytes with homomeric chimeric GABA receptors is shown. Nonresponsive chimeras (III and V) showed an outward-going (upward) current, suggesting blockade of spontaneously opening receptors similar to those in the β_1 wild-type homomers. Chimera numbers are those shown in Fig. 2.

corresponding to amino acid 265 on the β_3 wild-type subunit).

The effect of four other amino acids—alanine, glycine, threonine, and glutamine—at the S/N site was then investigated in the β_3 subunit. These four substitutions were chosen because of their side chains. Alanine has a methyl group, and in general its substitution in an α-helix is well tolerated (Overington, 1992). Glycine has the minimal possible side chain, offering a single hydrogen, and is known to be poorly tolerated in α-helices (Overington, 1992). Threonine, like serine, has a hydroxyl-containing side chain. Glutamine is a conservative substitution for the asparagine present in the wild-type β_3 subunit. None of the above mutants, when expressed as homomers, responded to pentobarbital (n = 5 cells for each mutation from two separate injections). When coexpressed with the α_1 subunit, however, all responded to both pentobarbital and GABA. Thus, only β subunit homomeric receptors with an asparagine residue at the S/N site respond to the intravenous general anesthetics used in these experiments.

Dominant role of serine at the S/N site in receptor function

Functional recombinant homomeric receptors, like native heteromeric receptors, are thought to be pentamers (Amin and Weiss, 1996). If we assume all five positions to be equivalent, there are six possible combinations of wild-type and mutant subunits (Table 3). To determine the minimal proportion of wild-type β_3 subunits necessary to preserve the response to pentobarbital, different mixtures of cRNA for β_3 wild-type and for β_3N265S mutant were injected, ranging from all wild-type to all mutant. For this analysis we assume that (a) subunit expression is proportional to the amount of injected cRNA for both wild-type and mutants, (b) the amount of cRNA injected into each oocyte is known accurately enough to allow comparison of averaged values, and (c) wild-type and mutant subunits associate freely with equal probabilities. Finally, we assume that the total current reflects the averaged contribution of all the individual channels present in the cell. Using the binomial equation, we can then calculate the relative proportions of the six different pentamers expected for each of the mixtures used (see Table 3).

The probability of each pentamer for a given subunit mixture ratio is shown in Fig. 7A. Curve 5 represents the probability of finding a pentaoligomer with all five wild-type subunits, curve 4 that with four wild-type and one mutant, and so on. The curve for the all-mutant pentamer is not shown; it is the mirror image of curve 5. The probability of finding a pentaoligomer of a particular subunit combination is shown in Fig. 7B. For example, the probability of finding a pentamer with either four or

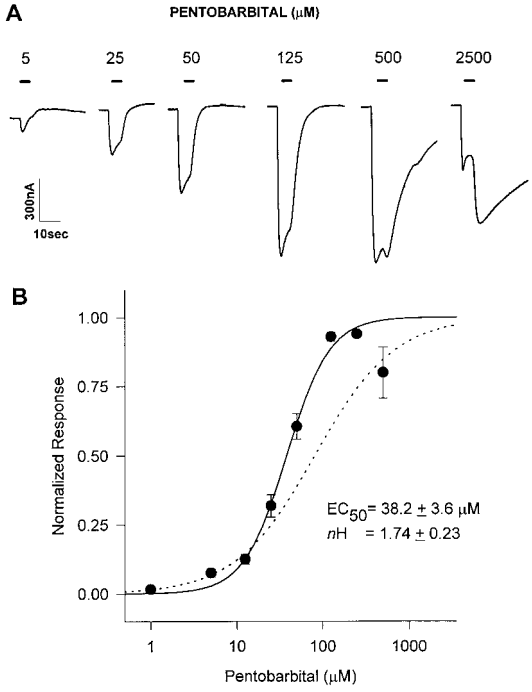

FIG. 4. Pentobarbital induces current in the β_1S265N point mutant. **A:** Current elicited by increasing concentrations of pentobarbital applied to an oocyte injected with the β_1S265N point mutant cRNA is shown. Note the characteristic "off-response" seen as an increase in the current on washout of the high concentration of pentobarbital. **B:** The solid line shows the best fit of the responses of four to six oocytes to the Hill equation with the two parameter values noted. Data from 2.5 mM pentobarbital were not included in the fit because of the decrease in the peak current at this concentration. The dotted line is the best-fitting dose–response curve for the β_3 wild-type (EC_{50} = 81 ± 9 μM, n_H = 0.94 ± 0.8) taken from Cestari et al. (1996).

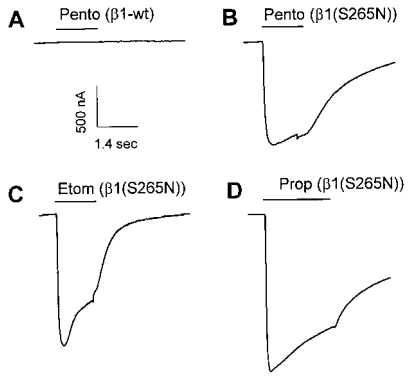

FIG. 5. Etomidate (Etom) and propofol (Prop) cause current flow in the β_1S265N point mutant homomeric receptor. Pentobarbital (Pento; 125 μM) failed to induce a current in the β_1 wild-type (wt)-injected oocyte (**A**) but induced an inward-going current in a β_1S265N point mutant-injected oocyte (**B**). Etom (100 μM; **C**) and Prop (300 μM; **D**) also elicited current flow in the β_1S265N homomeric receptor. The responses for the point mutant are from different oocytes and were verified in at least four oocytes from two separate injections. Calibration bar = 0.5 μA for A and B, 0.24 μA for C, and 1.0 μA for D.

more wild-type subunits is represented by curve b, which is a simple sum of curves 5 and 4 from Fig. 7A. When pentobarbital is applied to cells with different ratios of subunits, the relationship between current and subunit ratio should match one of the curves in Fig. 7B, depending on the number of wild-type subunits required for pentobarbital sensitivity.

Pentobarbital at 250 μM, sufficient to maximize channel activation and minimize blockade, was applied to oocytes (n = 7 cells for each group) injected with six different wild-type-to-mutant ratios of cRNAs, ranging from 1.0 to 0, and the peak current was measured. Figure 7C shows the normalized peak currents observed for the different ratios of wild-type β_3 to β_3N265S cRNA. (Actual current magnitudes obtained for each cRNA injection ratios were normalized by the average response to 100% wild-type β_3-injected oocytes.) The results are clearly consistent with a dominant behavior of the mutated subunit: Only receptors with all five wild-type subunits contribute to the response (compare Fig. 7B, curve a with Fig. 7C). The inclusion of only one mutant subunit lacking the critical asparagine residue is sufficient to render the receptor nonresponsive.

Significance of β subunit S/N site in heteromeric receptors

With the functional significance of the β subunit S/N site in β subunit homomers defined, we investigated whether this particular residue affects pentobarbital gating of current in heteromeric $GABA_A$ receptors. Wild-type β_1 and β_3, and their respective S/N site mutants, were coexpressed with a wild-type α_1 subunit. Pentobarbital directly induced currents in all four subunit combinations: $\alpha_1\beta_{1wt}$, $\alpha_1\beta_{1(S/N)}$, $\alpha_1\beta_{3wt}$, and $\alpha_1\beta_{3(N/S)}$. The EC_{50} and Hill slopes for the four subunit combinations did not statistically differ from one another (Fig. 8). Therefore, although pentobarbital gating of homomeric β receptors is profoundly influenced by the S/N site residue, in heteromeric receptors the amino acid at this site has no effect.

Belelli et al. (1997) reported that the S/N site residue influences the degree of GABA current potentiation by the intravenous general anesthetic etomidate. We therefore examined the effect of both etomidate and pentobarbital on the potentiation of GABA-induced current in the same four heteromeric receptors. Figure 9A shows that coapplication of etomidate or pentobarbital with GABA potentiated the GABA-induced current in all subunit combinations. Receptors with β_{3wt} or $\beta_{1(S/N)}$ subunits showed significantly greater potentiation by etomidate than receptors containing β_{1wt} or $\beta_{3(N/S)}$ subunits (Fig. 9B), confirming the results of Belelli et al. (1997). It is interesting that pentobarbital also showed subunit-dependent potentiation of GABA-induced current, but the potentiation was greater with either β_1 or β_3 wild-type subunits than with either of the mutants (Fig. 9C).

Lastly, we explored whether the particular amino acid at the S/N site had a dominant effect on etomidate's potentiation of the GABA-induced current. This issue is important because naturally occurring heteromeric receptors most likely contain a mixture of β subunit isoforms (Benke et al., 1994; Li and De Blas, 1997). The logic of the procedure was identical to that used to demonstrate the dominant effect of the S/N site amino acid in pentobarbital gating of homomeric receptors (Fig. 7). We varied the ratio of β_{3wt} to $\beta_{3(N/S)}$ mutant cRNA combined with fixed amounts of α_1 and γ_2 subunit cRNAs. Figure 10A shows etomidate potentiation of GABA-induced currents in oocytes with wild-type $\alpha_1\beta_3\gamma_2$ (bottom) and mutant $\alpha_1\beta_{3(N/S)}\gamma_2$ (top) penta-

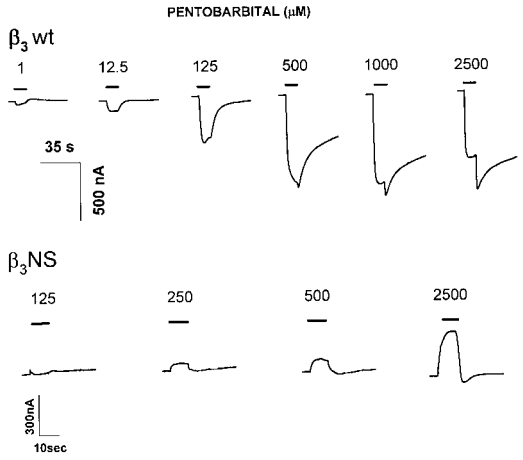

FIG. 6. The β_3N265S (β_3NS) point mutant does not respond to pentobarbital. Pentobarbital elicits inward-going current when applied to the β_3 wild-type (wt) homomeric receptor (**top**) but not the β_3NS point mutant (**bottom**). The point mutant shows an outward-going (upward) current during pentobarbital application, just like the β_1 wt homomeric receptor (see Fig. 1). Comparable observations were made in six oocytes injected with this point mutant.

TABLE 3. *Theoretical distribution of the wild-type and mutant subunits in a pentameric receptor oligomer based on the binomial distribution for different ratios of subunit availability*

Wild-type: mutant ratio	Fraction of each combination					
						
1:4	0.0003	0.006	0.051	0.205	0.410	0.327
2:3	0.010	0.077	0.230	0.346	0.259	0.078
3:2	0.078	0.259	0.346	0.230	0.077	0.010
4:1	0.327	0.410	0.205	0.051	0.006	0.0003

The combinations represent a β_x pentameric assembly. The open and solid circles represent the wild-type and the mutant subunits, respectively. The calculation is based on a random incorporation of each subunit, where the probability of incorporation is determined by the availability of the subunits, i.e., a binomial distribution $b(x) = \Sigma[n!/x!(n - x)!]p^x (1 - p)^{(n - x)}$, where $n = 5$, p is the probability of finding a wild-type for a given injection ratio, and n is the number of wild-type subunits in a pentamer. This holds true because each subunit necessarily has to be a wild-type or a mutant. It is assumed that all receptors are pentamers and that all subunit positions within the oligomer are equivalent.

meric receptors. In heteromeric receptors of $\alpha_1\beta_x\gamma_2$ composition, etomidate potentiation was greater for β_{3wt} than for $\beta_{3(N/S)}$, similar to the finding for the $\alpha_1\beta_x$ receptors above (Fig. 9). As the proportion of mutant $\beta_{3(N/S)}$ subunits relative to β_{3wt} subunits increased, the degree of etomidate potentiation decreased (Fig. 10B). Assuming that heterooligomeric receptors composed of α, β, and γ subunits assemble in a 2:2:1 ratio (Chang et al., 1996), the relative number of pentamers containing two β_{3wt}, one β_{3wt} and one $\beta_{3(N/S)}$, or two $\beta_{3(N/S)}$ subunits was calculated from the binomial distribution (see Fig. 10 and Table 3). The etomidate potentiation data are best approximated by the $\beta_{3(N/S)}$ dominant phenotype model (Fig. 10B, dotted line).

DISCUSSION

Homomeric β subunit receptors

Functional receptors in the ligand-gated ion channel superfamily are usually heteromeric, composed of two or more types of subunits. The homomeric receptor formed of murine $GABA_A$ β_3 subunits is unique, in that it does not respond to the natural ligand GABA but is gated by other ligands instead. Because of the structural homology among members of this superfamily, there may be other homomeric receptors opened by an unexpected ligand. Previously we reported that murine β_3 homomeric $GABA_A$ receptors possess functional chloride ionophores gated by several intravenous general anesthetics but not by GABA (Cestari et al., 1996); this observation was subsequently confirmed by Wooltorton et al. (1997). In contrast, murine β_1 homomeric $GABA_A$ receptors (having 82% amino acid identity with β_3 subunits) are not gated by these anesthetics (Cestari et al., 1996).

We exploited the dramatic difference in responsiveness to pentobarbital of the β_1 and β_3 homomers to identify a single amino acid near the outer mouth of M2, the S/N site, as the critical determinant of this pharmacological difference. The pentobarbital-induced current was abolished when the asparagine-265 normally present on the β_3 subunit was replaced by serine normally present at the same position on the β_1 subunit. Conversely, mutation of β_1 serine-265 to asparagine resulted in a receptor responsive to pentobarbital and also to propofol and etomidate, two other intravenous general anesthetics of different chemical classes. Thus, the identity of a single amino acid alters the responsiveness of the homomeric receptor to three different intravenous general anesthetics.

The same $GABA_A$ receptor β subunit S/N site has previously been reported to alter the loreclezole (Wingrove et al., 1994), β-carboline (Stevenson et al., 1995), volatile general anesthetic (Mihic et al., 1997), and etomidate (Belelli et al., 1997) potentiation of GABA-induced currents and alcohol modulation of glycine receptors (Wick et al., 1998; Ye et al., 1998). Wingrove et al. (1994) found less loreclezole modulation of GABA-induced current in β_1-containing receptors than in β_2- or β_3-containing receptors and that this difference depended on the S/N site residue. Asparagine resulted in a greater degree of potentiation than serine. These authors concluded that the S/N site is part of the loreclezole binding site, a site also shared by the β-carbolines. Mihic et al. (1997), starting from the observation that enflurane, a volatile general anesthetic agent, enhances glycine α_1 but inhibits GABA ρ_1 homomeric receptors, converged on two residues near the outer M2–M3 linker region as the critical determinant of the allosteric action. Our S/N site corresponds to their M2 domain residue.

Because the S/N site is involved in allosteric modulation by several chemically diverse drugs, it is difficult to see how the amino acid at the S/N site could participate in forming the actual binding site. Furthermore, application of the substituted-cysteine-accessibility technique to the $GABA_A$ receptor α subunit has identified the amino acids in the M2 domain with side chains exposed to the

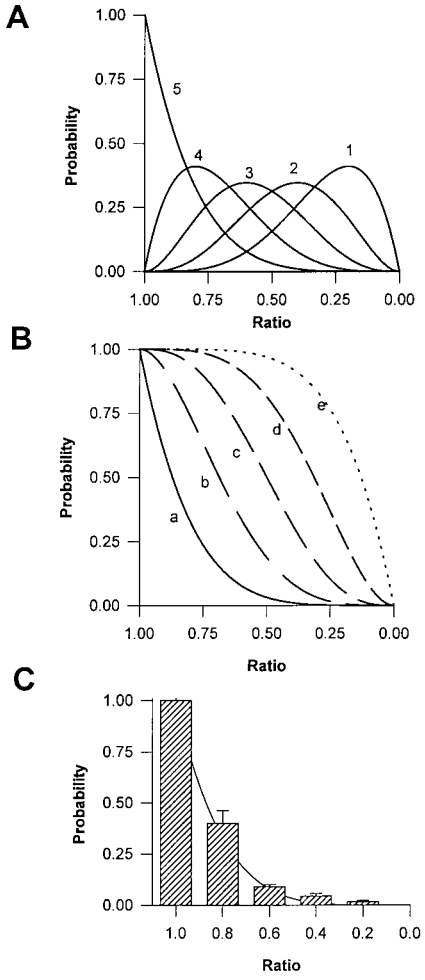

FIG. 7. The serine residue at the S/N site exerts a negative-dominant effect on pentobarbital-gating of current. **A:** Probability of finding pentamers with different composition [all wild-type (curve 5), four wild-type and one mutant (curve 4), etc.] for different ratios of wild-type to mutant cRNA assuming binomial distribution of individual subunits. **B:** Joint probability of finding (curve a) a pentamer with all wild-type subunits, (curve b) a pentamer with at least four wild-type subunits (and therefore no more than one mutant), (curve c) a pentamer with at least three wild-type subunits (and no more than two mutants), etc., as a function of different ratios of wild-type to mutant cRNA. Note that curve b is the sum of curves 5 and 4 shown in A. **C:** Average current (n = 7 for all ratios) induced by 125 μM pentobarbital from oocytes injected with different ratios of β_3 wild-type and β_3N265S cRNA. The current values were normalized to the average response at a ratio of 1.0 (all wild-type), which was 563 ± 73 nA. The solid line represents the current predicted if only receptors with all wild-type subunits were functional (see Table 3), assuming a binomial distribution of wild-type and mutant subunits in pentameric oligomers.

aqueous pore (Xu and Akabas, 1996). When the residues within M2 are plotted on an α-helical wheel representation, residues susceptible to sulfhydryl reagents lie on the lumen (Xu and Akabas, 1996). In this arrangement, the position corresponding to the S/N site is located on the opposite side, away from the aqueous lumen. Because general anesthetic molecules are very lipophilic, however, this observation alone cannot exclude the S/N site as part of the binding site.

A more parsimonious interpretation is that mutations in the region of the S/N site may interfere with the conformational changes occurring during normal gating and allosteric modulation. This interpretation is supported by a recent report that etomidate potentiation of [^{3}H]flunitrazepam binding in an $\alpha_1\beta_3\gamma_2$ receptor is abolished by the same asparagine to serine mutation at the S/N site in the β_3 subunit (Moody et al., 1997). Other evidence supporting the effect of this region of M2 on allosteric modulation is the report of Birnir et al. (1997). They found that mutation of a conserved threonine (M2 12′ locus) residue located only three amino acids toward the cytoplasmic face from the S/N site to glutamine abolished pentobarbital potentiation of GABA-induced current in an $\alpha_1\beta_1$ heteromeric receptor. Overall, these findings are consistent with the conclusion of Ueno et al. (1997) that the process of transduction of agonist-binding site occupancy into channel opening requires specific residues in the β subunit.

The M2 role in receptor function

The importance of the M2 domain to channel function derives both from its role in forming the wall of the ion

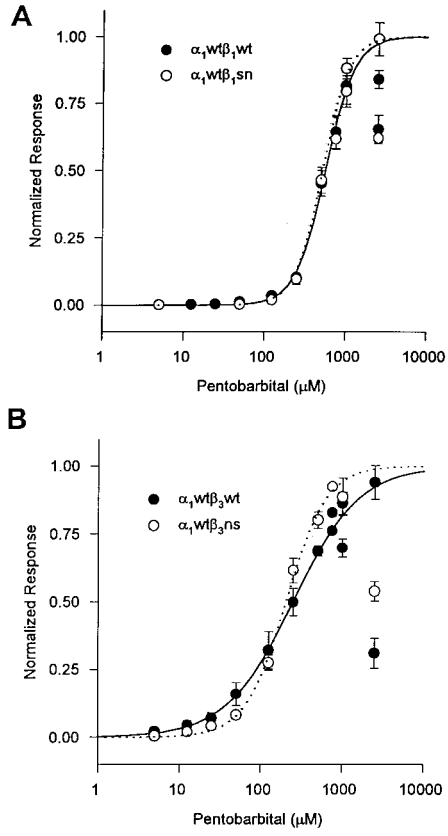

FIG. 8. Pentobarbital concentration–response curve of β subunit wild-type (wt) and β subunit point mutant $\alpha_1\beta$ heteromeric receptors. **A:** Concentration response curves of heteromeric receptors containing β_1 wt (●) and β_1 mutant (sn) heteromers (–○–). **B:** Concentration–response curves of β_3 wt (●) and β_3 mutant (ns) heteromers (–○–). Lines represent the best fit to the Hill equation.

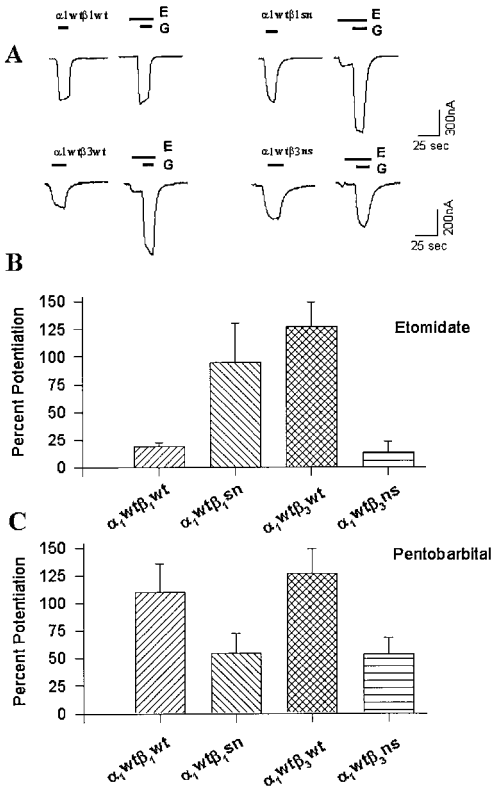

FIG. 9. The β subunit S/N site amino acid determines etomidate but not pentobarbital potentiation of $\alpha_1\beta$ heteromeric receptors. Etomidate but not pentobarbital potentiation of GABA-induced current is dependent on the β subunit M2 domain amino acid. **A:** Four groups of *Xenopus* oocytes were injected with β_{1wt}, $\beta_{1(S/N)}$, β_{3wt}, or $\beta_{3(N/S)}$ cRNA with an equal amount of α_{1wt} cRNA. GABA (G; respective EC_{20} dose) was applied alone or with the intravenous general anesthetic etomidate (E; 10 μM) or pentobarbital (25 μM). These anesthetics potentiated the GABA-induced current in all subunit combinations examined. However, the magnitude of potentiation was subunit-dependent. **B** and **C:** Summary of anesthetic potentiation. Data are mean ± SEM (bars) values from six oocytes for each combination. Etomidate, but not pentobarbital, potentiation of GABA-induced current is controlled by the critical S/N amino acid. Differences are significant at $p < 0.05$. wt, wild-type; sn and ns, mutant.

pores, crucial in defining permeation properties, and from its role in the conformational changes associated with different states of the channel. Direct evidence for these conformational changes was provided by observation of alteration in the relative position of the M2 α-helical region of the nicotinic acetylcholine receptor in resting receptors compared with activated receptors (Unwin, 1993, 1995). The homology among the members of the ligand-gated ion channel superfamily makes it likely that the M2 domain of the $GABA_A$ receptor also undergoes conformational changes during receptor activation (Smith and Olsen, 1995). Receptor processes that depend on a specific conformational state would be affected, thus explaining how different aspects of receptor function are modified without requiring a common binding site.

Similarly, mutation of the glycine receptor α_1 subunit at a position four residues away from the S/N site alters the affinity of several ligands, both agonists and antagonists. Some amino acid substitutions cause changes in distribution of the conductance levels of the receptor (Rajendra et al., 1994; Laube et al., 1995; Lynch et al., 1995). Because these point mutations have multiple effects on receptor function, Rajendra et al. (1994) concluded that this position is crucial for both the integration of information from agonist and antagonist binding sites and its transmission to the activation gate of the receptor.

Dominant role of S/N site: topology and functional implications

Substitution of glycine, threonine, glutamine, or serine at the S/N site in the β_3 subunit did not reproduce the wild-type phenotype. Because the β_3 subunit is known to be glycosylated (Buchstaller et al., 1991), the requirement for asparagine suggests a possible role of N-linked glycosylation. However, asparagine-265 is not within the N-linked glycosylation consensus sequence (Kornfeld and Kornfeld, 1986; Parekh et al., 1989) and probably

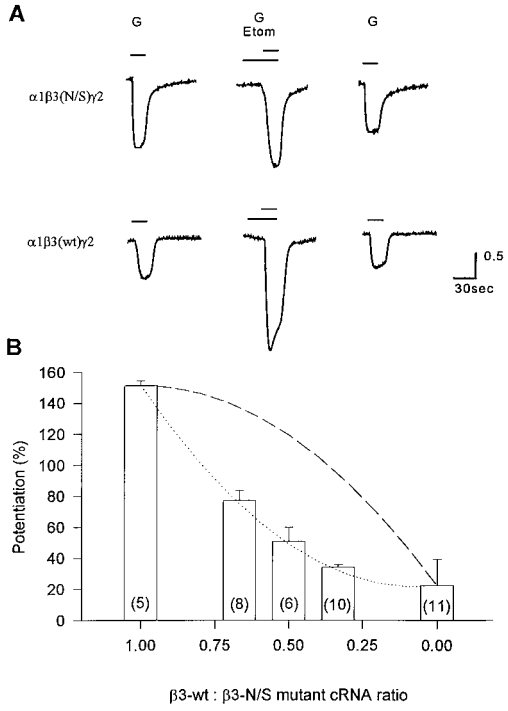

FIG. 10. The β subunit S/N site amino acid exerts a dominant effect on etomidate (Etom) potentiation of $\alpha_1\beta_x\gamma_2$ heteromeric receptors. *Xenopus* oocytes were injected with $\beta_{3(N/S)}$ or β_{3wt} cRNA with a fixed amount of α_1 and γ_2 cRNA. Etom potentiation of current evoked by GABA (G; EC_{20} dose) was examined. **A:** The current traces show control G alone (**left**), coapplication of GABA and 10 μM Etom (**middle**), and G alone (**right**). Etom potentiation of β_{3wt}-containing $\alpha\beta\gamma$ trimeric receptors was greater, as was observed for the $\alpha\beta$ dimeric combination (Fig. 7). **B:** Increasing proportions of β_{3wt} cRNA compared with $\beta_{3(N/S)}$ cRNA were injected in a dimeric combination with α_1 and γ_2 cRNA. The decrease in potentiation with increasing proportion of the point mutant was much better approximated by a $\beta_{3(N/S)}$-dominant binomial model (dotted line) than by a β_{3wt}-dominant model (dashed line). Data are mean ± SE (bars) values (no. of assays).

projects into the lipid domain away from the aqueous ion channel or the extracellular surface.

The wild-type-to-mutant cRNA ratio experiment shows that the functional role of the S/N site is negative dominant, so that the presence of a single nonfunctioning subunit (such as the β_3N265S mutant) within the pentameric assembly is sufficient to abolish the pentobarbital-induced ion channel opening. If the S/N site residue is considered to be part of the pentobarbital binding site, its dominant role is hard to understand unless the ion channel only opens when pentobarbital occupies the sites in all five subunits. Such a concordant interaction between five binding sites would be manifested as a dose–response curve with a larger Hill slope, which we did not find.

The experiments on the coexpression of wild-type and mutated β_3N265S can also be best interpreted in terms of the involvement of this site in conformational changes of the receptor. By varying the proportions of wild-type β_3 and activation-defective β_3N265S, we found that the inclusion of only one mutant subunit in a pentameric receptor was sufficient to block activation by pentobarbital. Unwin (1995) proposed that, for the nicotinic acetylcholine receptor, all five subunits need to rotate at the level of the M2 region, which allows for the open configuration to be stabilized by interactions between the helices around the pore. If a similar conformational change takes place in these homomeric receptors, the inability of even one of the subunits to assume or remain at this alternative position would preclude the open state, consistent with a negative dominant role for the serine residue at the S/N site.

Role of S/N site in heteromeric receptors

In contrast to homomeric receptors, where the β subunit S/N residue strongly affects anesthetic gating, the S/N site only partially controls the GABA-gated current in heteromeric receptors. The effect of etomidate in heteromeric $GABA_A$ is β subunit-dependent (Hill-Venning et al., 1997; Sanna et al., 1997); the effect of pentobarbital is not (Thompson et al., 1996; Sanna et al., 1997). Our results extend these observations and confirm the finding of Belelli et al. (1997) that the amino acid at the S/N site determines the different actions of etomidate at receptors with different β subunits. Other subunit combination and site-directed mutagenesis studies have shown that direct gating by intravenous general anesthetics has different receptor structural requirements than the indirect ligand-gated current-potentiating actions (Davies et al., 1997; Moody et al., 1997; Uchida et al., 1997). One parsimonious interpretation is that intravenous general anesthetics do not open a homomeric receptor the same way a ligand opens a heteromeric receptor. Because β_3 homomers are spontaneously active even in the absence of GABA (Cestari et al., 1996; Wooltorton et al., 1997), pentobarbital or other allosteric potentiators might increase the current simply through potentiation of spontaneously opening channels. In heteromeric receptors, which show negligible spontaneous opening in the absence of GABA (Angelotti and Macdonald, 1993), the same structural determinants that correlate with homomeric gating manifest as allosteric potentiation. This interpretation of homomeric receptor function cannot, however, explain the observation that pentobarbital and etomidate, both known to potentiate GABA-induced current in heteromers, fail to induce any significant current in β_1 homomers, even though they also open spontaneously (see present study, Fig. 1; Krishek et al., 1996). Further studies are necessary to define this complex relationship between homomeric and heteromeric receptor functions.

In summary, we have identified a particular asparagine residue in the M2 portion of the $GABA_A$ receptor β_3 subunit as the critical determinant for current generation by pentobarbital and two other intravenous general anesthetics. We favor the interpretation that this particular residue plays an essential role in the ion channel transduction process. We think it unlikely that it forms a binding pocket common to many compounds of diverse chemical structures. The same amino acid regulates the magnitude of allosteric potentiation by etomidate but not pentobarbital in heteromeric receptors. Further investigation of the pharmacological and physiological similarities and differences between homomeric and heteromeric receptors may lead to a deeper understanding of subunit interactions inherent in the function of these complex oligomeric $GABA_A$ receptors.

Acknowledgment: This research was supported by the Veterans Administration Merit Review and grant RO1-GM52325 from the National Institutes of Health (to J.Y.) and the CNPq of Brazil (to I.N.C.). The authors thank Li Li for technical assistance and the Department of Anesthesiology and Pain Management, University of Texas Southwestern Medical Center, where part of this work was performed. We thank Christine Seccombe for help with manuscript preparation.

REFERENCES

Akaike N., Hattori K., Inomata N., and Oomura Y. (1985) γ-Aminobutyric-acid and pentobarbitone-gated chloride currents in internally perfused frog sensory neurones. *J. Physiol. (Lond.)* **360,** 367–386.

Amin J. and Weiss D. S. (1993) $GABA_A$ receptor needs two homologous domains of the beta-subunit for activation by GABA but not by pentobarbital. *Nature* **366,** 565–569.

Amin J. and Weiss D. S. (1996) Insights into the activation mechanism of rho1 GABA receptors obtained by coexpression of wild-type and activation impaired subunits. *Proc. R. Soc. Lond. [Biol.]* **263,** 273–282.

Amin J., Dickerson I. M., and Weiss D. S. (1994) The agonist binding site of the $GABA_A$ receptor is not formed by the extracellular cysteine loop. *Mol. Pharmacol.* **45,** 317–323.

Angelotti T. P. and Macdonald R. L. (1993) Assembly of $GABA_A$ receptor subunits: $\alpha1\beta1$ and $\alpha1\beta1\gamma2s$ subunits produce unique ion channels with dissimilar single-channel properties. *J. Neurosci.* **13,** 1429–1440.

Belelli D., Lambert J. J., Peters J. A., Wafford K., and Whiting P. J. (1997) The interaction of the general anesthetic etomidate with the $GABA_A$ receptor is influenced by a single amino acid. *Proc. Natl. Acad. Sci. USA* **94,** 11031–11036.

Benke D., Fritschy J. M., Trzeciak A., Bannwarth W., and Mohler H. (1994) Distribution, prevalence, and drug binding profile of $GABA_A$ receptor subtypes differing in the beta-subunit variant. *J. Biol. Chem.* **269,** 27100–27107.

Birnir B., Tierney M. L., Balziel J. E., Cox G. B., and Gage P. W. (1997) A structural determinant of desensitization and allosteric

regulation by pentobarbitone of the $GABA_A$ receptor. *J. Membr. Biol.* **155,** 157–166.
Buchstaller A., Adamiker D., Fuchs K., and Sieghart W. (1991) N-deglycosylation and immunological identification indicate the existence of β-subunit isoforms of the rat $GABA_A$ receptor. *FEBS Lett.* **287,** 27–30.
Cestari I. N., Uchida I., Li L., Burt D. R., and Yang J. (1996) The agonistic action of pentobarbital on GABAA beta-subunit homomeric receptors. *Neuroreport* **7,** 943–947.
Chang Y., Wang R., Barot S., and Weiss D. S. (1996) Stoichiometry of a recombinant $GABA_A$ receptor. *J. Neurosci.* **16,** 5415–5424.
Davies P. A., Kirkness E. F., and Hales T. G. (1997) Modulation by general anaesthetics of rat $GABA_A$ receptors comprised of α1β3 and β3 subunits expressed in human embryonic kidney 293 cells. *Br. J. Pharmacol.* **120,** 899–909.
Evans R. H. and Hill R. G. (1978) GABA-mimetic action of etomidate. *Experientia* **34,** 1325–1327.
Franks N. P. and Lieb W. R. (1994) Molecular and cellular mechanisms of general anaesthesia. *Nature* **367,** 607–614.
Gurdon J. B., Lingrel J. B., and Marbaix G. (1973) Message stability in injected frog oocytes: long life of mammalian alpha and beta globin messages. *J. Mol. Biol.* **80,** 539–551.
Hara M., Kai Y., and Ikemoto Y. (1993) Propofol activates $GABA_A$ receptor–chloride ionophore complex in dissociated hippocampal pyramidal neurons of the rat. *Anesthesiology* **79,** 781–788.
Hill-Venning C., Belelli D., Peters J. A., and Lambert J. J. (1997) Subunit-dependent interaction of the general anaesthetic etomidate with the $GABA_A$ receptor. *Br. J. Pharmacol.* **120,** 749–756.
Kamatchi G. L., Kofuji P., Wang J. B., Fernando J. C., Liu Z., Mathura J. R., and Burt D. R. (1995) $GABA_A$ receptor beta 1, beta 2, and beta 3 subunits: comparisons in DBA/2J and C57BL/6J mice. *Biochim. Biophys. Acta* **1261,** 134–142.
Kornfeld R. and Kornfeld S. (1986) Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **54,** 631–664.
Krishek B. J., Moss S. J., and Smart T. G. (1996) Homomeric β1 $GABA_A$ receptor–ion channels: evaluation of pharmacological and physiological properties. *Mol. Pharmacol.* **49,** 494–504.
Laube B., Langosch D., Betz H., and Schmieden V. (1995) Hyperekplexia mutations of the glycine receptor unmask the inhibitory subsite for beta-amino-acids. *Neuroreport* **6,** 897–900.
Li M. and De Blas A. L. (1997) Coexistence of two beta subunit isoforms in the same $GABA_A$ receptor. *J. Biol. Chem.* **272,** 16564–16569.
Lynch J. W., Rajendra S., Barry P. H., and Schofield P. R. (1995) Mutations affecting the glycine receptor agonist transduction mechanism convert the competitive antagonist, picrotoxin, into an allosteric potentiator. *J. Biol. Chem.* **270,** 13799–13806.
McKernan R. M. and Whiting P. J. (1996) Which $GABA_A$-receptor subtypes really occur in the brain? *Trends Neurosci.* **19,** 139–143.
Mihic S. J., Whiting P. J., Klein R. L., Wafford K. A., and Harris R. A. (1994) A single amino acid of the human gamma-aminobutyric acid type A receptor gamma 2 subunit determines benzodiazepine efficacy. *J. Biol. Chem.* **269,** 32768–32773.
Mihic S. J., Ye Q., Wick M. J., Koltchine V. V., Krasowski M. D., Finn S. E., Mascia M. P., Valenzuela C. F., Hanson K. K., Greenblatt E. P., Harris R. A., and Harrison N. L. (1997) Sites of alcohol and volatile anaesthetic action on GABAA and glycine receptors. *Nature* **389,** 385–389.
Moody E. J., Knauer C., Granja R., Strakhova M., and Skolnick P. (1997) Distinct loci mediate the direct and indirect actions of the anesthetic etomidate at $GABA_A$ receptors. *J. Neurochem.* **69,** 1310–1313.
Overington J. (1992) Structural constraints on residue substitution. *Genet. Eng. (NY)* **14,** 231–249.
Parekh R. B., Dwek R. A., Edge C. J., and Rademacher T. W. (1989) N-glycosylation and production of recombinant glycoproteins. *Trends Biotech.* **7,** 117–122.
Rajendra S., Lynch J. W., Pierce K. D., French C. R., Barry P. H., and Schofield P. R. (1994) Startle disease mutations reduce the agonist sensitivity of the human inhibitory glycine receptor. *J. Biol. Chem.* **269,** 18739–18742.
Sanna E., Garau F., and Harris R. A. (1995) Novel properties of homomeric β1 $GABA_A$ receptors: actions of the anesthetics propofol and pentobarbital. *Mol. Pharmacol.* **47,** 213–217.
Sanna E., Murgia A., Casula A., and Biggio G. (1997) Differential subunit dependence of the actions of the general anesthetics alphaxalone and etomidate at $GABA_A$ receptors expressed in *Xenopus laevis* oocytes. *Mol. Pharmacol.* **51,** 484–490.
Schofield P. R., Darlison M. G., Fujita N., Burt D. R., Stephenson F. A., Rodriguez H., Rhee L. M., Ramachandran J., Reale V., Glencorse T. A., et al. (1987) Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor superfamily. *Nature* **328,** 221–227.
Sigel E., Baur R., Kellenberger S., and Malherbe P. (1992) Point mutations affecting antagonist affinity and agonist dependent gating of $GABA_A$ receptor channels. *EMBO J.* **11,** 2017–2023.
Smith G. B. and Olsen R. W. (1995) Functional domains of $GABA_A$ receptors. *Trends Pharmacol. Sci.* **16,** 162–168.
Stevenson A., Wingrove P. B., Whiting P. J., and Wafford K. A. (1995) β-Carboline γ-aminobutyric acid A receptor inverse agonists modulate gamma-aminobutyric acid via the loreclezole binding site as well as the benzodiazepine site. *Mol. Pharmacol.* **48,** 965–969.
Thompson S. A., Whiting P. J., and Wafford K. A. (1996) Barbiturate interactions at the human $GABA_A$ receptor: dependence on receptor subunit combination. *Br. J. Pharmacol.* **117,** 521–527.
Thompson S. A., Arden S. A., Marshall G., Wingrove P. B., Whiting P. J., and Wafford K. A. (1999) Residues in transmembrane domains I and II determine gamma-aminobutyric acid type A receptor subtype-selective antagonism by furosemide. *Mol. Pharmacol.* **55,** 993–999.
Uchida I., Li L., and Yang J. (1997) The role of the $GABA_A$ receptor α1 subunit N-terminal extracellular domain in propofol potentiation of chloride current. *Neuropharmacology* **36,** 1611–1621.
Ueno S., Bracamontes J., Zorumski C., Weiss D. S., and Steinbach J. H. (1997) Bicuculline and gabazine are allosteric inhibitors of channel opening of the $GABA_A$ receptor. *J. Neurosci.* **17,** 625–634.
Unwin N. (1993) Nicotinic acetylcholine receptor at 9 Å resolution. *J. Mol. Biol.* **229,** 1101–1124.
Unwin N. (1995) Acetylcholine receptor channel imaged in the open state. *Nature* **373,** 37–43.
Vallete F., Mege E., Reiss A., and Adesnik M. (1989) Construction of mutant and chimeric genes using the polymerase chain reaction. *Nucleic Acids Res.* **17,** 723–733.
Wafford K. A., Bain C. J., Quirk K., McKernan R. M., Wingrove P. B., Whiting P. J., and Kemp J. A. (1994) A novel allosteric modulatory site on the $GABA_A$ receptor beta subunit. *Neuron* **12,** 775–782.
Wang J. B., Kofuji P., Fernando J. C. R., Moss S. J., Ruganier R. L., and Burt D. R. (1992) The alpha 1, alpha 2, and alpha 3 subunits of $GABA_A$ receptors: comparison in seizure-prone and -resistant mice and during development. *J. Mol. Neurosci.* **3,** 177–184.
Wick M. J., Mihic S. J., Ueno S., Maxcia M. P., Trudell J. R., Brozowski S. J., Ye Q., Harrison N. L., and Harris R. A. (1998) Mutations of gamma-aminobutyric acid and glycine receptors change alcohol cutoff: evidence for an alcohol receptor? *Proc. Natl. Acad. Sci. USA* **95,** 6504–6509.
Wingrove P. B., Wafford K. A., Bain C., and Whiting P. J. (1994) The modulatory action of loreclezole at the gamma-aminobutyric acid type A receptor is determined by a single amino acid in the beta 2 and beta 3 subunit. *Proc. Natl. Acad. Sci. USA* **91,** 4569–4573.
Wooltorton J. R. A., Moss J. S., and Smart T. G. (1997) Pharmacological and physiological characterization of murine homomeric β3 $GABA_A$ receptors. *Eur. J. Neurosci.* **9,** 2225–2235.
Xu M. and Akabas M. H. (1996) Identification of channel-lining residues in the M2 membrane-spanning segment of the GABA(A) receptor alpha1 subunit. *J. Gen. Physiol.* **107,** 195–205.
Yang J. and Uchida I. (1996) Mechanisms of etomidate potentiation of $GABA_A$ receptor-gated currents in cultured postnatal hippocampal neurons. *Neuroscience* **73,** 69–78.
Ye Q., Koltchine V. V., Mihic S. J., Mascia M. P., Wick M. J., Finn S. E., and Harris R. A. (1998) Enhancement of glycine receptor function by ethanol is inversely correlated with molecular volume at position alpha267. *J. Biol. Chem.* **273,** 3314–3319.
Ymer S., Schofield P. R., Draguhn A., Werner P., Kohler M., and Seeburg P. H. (1989) $GABA_A$ receptor β subunit heterogeneity: functional expression of cloned cDNAs. *EMBO J.* **8,** 1665–1670.