0026-895X/99/030411-13$3.00/0

MOLECULAR PHARMACOLOGY, **55:**411–423 (1999).

A Single Hydrophobic Residue Confers Barbiturate Sensitivity to γ-Aminobutyric Acid Type C Receptor

JAHANSHAH AMIN

Department of Pharmacology and Therapeutics and The Institute for Biomolecular Science, University of South Florida, College of Medicine, Tampa, Florida

Received July 7, 1998; accepted December 18, 1998

This paper is available online at http://www.molpharm.org

ABSTRACT

Barbiturate sensitivity was imparted to the human ρ_1 homooligomeric γ-aminobutyric acid (GABA) receptor channel by mutation of a tryptophan residue at position 328 (Trp328), which is located within the third transmembrane domain. Substitutions of Trp328 with a spectrum of amino acids revealed that nearly all hydrophobic residues produced receptor channels that were both directly activated and modulated by pentobarbital with similar sensitivities. Previous studies with ligand-gated ion channels (including GABA) have demonstrated that even conservative amino acid substitution within the agonist-dependent activation domain (N-terminal extracellular domain) can markedly impair agonist sensitivity. Thus, the lack of significant variation in pentobarbital sensitivity among the Trp328 mutants attests to an intrinsic difference between pentobarbital- and the GABA-dependent activation domain. Compared with the heterooligomeric $\alpha\beta\gamma$ receptor channel, the mode of modulation for homooligomeric Trp328 mutants by pentobarbital was more dependent on the GABA concentration, yielding potentiation only at low concentrations of GABA (fractions of their respective EC_{50} values), yet causing inhibition at higher concentrations. Agonist-related studies have also demonstrated that residue 328 plays an important role in agonist-dependent activation, suggesting a functional interconnection between the GABA and pentobarbital activation domains.

Molecular events leading to anesthesia have been attributed to the modulation of the excitatory or inhibitory ligand-gated ion channels (Nicoll, 1972; Nicoll et al., 1975; Barker and Ransom, 1978; Franks and Lieb, 1994). The primary target for a number of anesthetic compounds, including pentobarbital, are the γ-aminobutyric acid type A ($GABA_A$) receptor channels (Schulz and Macdonald, 1981; Gage and Robertson, 1985; Parker et al., 1986; MacIver et al., 1991; Tanelian et al., 1993), the key components of synaptic inhibition in the central nervous system. GABA-gated chloride channels are heterooligomeric or homooligomeric pentamers composed of numerous combinations of homologous α, β, γ, δ, or ρ classes of subunits (Macdonald and Olsen, 1994). These subunits have the potential for creating a great number of GABA receptor channels with distinct pharmacology (Macdonald and Olsen, 1994). For example, GABA-evoked currents for heterooligomeric $\alpha\beta\gamma$ ($GABA_A$; Schofield et al., 1987; Levitan et al., 1988; Macdonald and Olsen, 1994) and homooligomeric β receptor channels (Blair et al., 1988; Sanna et al., 1995) are potentiated in the presence of low concentrations of pentobarbital (modulatory action). Moreover, pentobarbital at higher concentrations can directly activate these channels (agonistic action; Mathers and Barker, 1980; Nicoll and Wojtowicz, 1980; Akaike et al., 1991; Sanna et al., 1995; Rho et al., 1996). In contrast, the homooligomeric ρ_1 receptor channel ($GABA_C$; Cutting et al., 1991) is insensitive to pentobarbital (Shimada et al., 1992).

In this study, experiments were conducted to gain insight into the mechanisms of barbiturate modulation and activation of GABA-gated chloride channels. Results with ρ-β chimeras and site-directed mutagenesis of ρ_1 indicate that hydrophobic amino acid substitution for Trp328 within the third transmembrane domain (TM3) imparts modulatory and agonistic properties of pentobarbital to ρ_1 homooligomeric receptor channel. In addition, residue 328 plays an important role in agonist-dependent activation. Collectively, these results provide important clues concerning the mechanism of barbiturate action on GABA-gated ion channels.

Materials and Methods

All chimeras were constructed using either conserved restriction sites between the ρ_1 and β_2 subunits (e.g., *Hin*cII for ρ346/β305), or synthetic oligonucleotides containing designed restriction enzyme sites and polymerase chain reaction. Special care was taken not to alter the relative position of the conserved amino acids on both sides of the junction (with the exception of ρ405/β399). The DNA sequence of all chimeras was verified by DNA sequencing.

The cDNAs corresponding to ρ_1 and β_2 were cloned into the pSE-

This work was supported by grants from National Eye Institute (EY11531–01A1) and Council for Tobacco Research (SA052).

ABBREVIATIONS: GABA, γ-aminobutyric acid; TM, transmembrane domain.

LECT vector (Promega, Madison, WI) and oligonucleotide-mediated site-directed mutagenesis was achieved according to the manufacturer's protocol (Altered Sites; Promega). Successful mutagenesis was verified by DNA sequencing. The cDNAs were linearized with *Nhe*I leaving a several-hundred base pair tail (3′). These additional sequences at the 3′ end may increase cRNA stability in the oocyte. The cRNA was transcribed from the linearized cDNAs by standard in vitro transcription procedures (Megascript; Ambion, Austin, TX).

Xenopus laevis (Xenopus I; Ann Arbor MI) were anesthetized by hypothermia and oocytes were surgically removed from the frog and placed in Oocyte Ringer (OR_2) that consisted of: 82.5 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 50 U/ml penicillin, and 50 μg/ml streptomycin, pH 7.5. Oocytes were dispersed and then incubated in OR_2 minus Ca^{2+} plus 0.3% collagenase A (Boehringer Mannheim, Indianapolis, IN) for approximately 2 h. After isolation, the oocytes were thoroughly rinsed with OR_2. Stage VI oocytes were separated and maintained overnight at 18°C.

Micropipettes for injecting cRNA were fabricated on a Narishige PP-83 puller (Narishige USA, Greenvale, NY) and the tips were cut off with microscissors. The cRNA in diethylpyrocarbonate-treated water was drawn up into the micropipette with negative pressure and then injected into the oocytes by applying positive pressure using a Picospritzer II (General Valve Corporation, Fairfield, NJ). The oocytes were incubated in OR_2 at 18°C for 2 to 3 days before the experiment. To ensure that equal concentrations of cRNA for each construct were injected (especially important for comparison of maximum GABA-activated currents), set dilutions of cRNA from mutants were electrophoresed on a 1% formaldehyde-containing agarose gel. The amount of cRNA was judged and matched by interpolation of lanes containing different dilutions of the corresponding cRNA. In addition, for nearly all mutants, two independent isolates were characterized and tested.

Two to 3 days after injection, oocytes were placed on a nylon mesh suspended in a small volume chamber (~75 μl). The chamber has an inlet in the top and an outlet in the bottom that allows continuous and rapid perfusion. Twenty separate reservoirs (100-ml glass containers) were connected to four six-way valves and the outlet of each of these six-way valves (the sixth position was connected to the reservoir containing the control solution) was connected to one four-way valve. The outlet of the four-way valve lead to the chamber. In this way, up to 20 different solutions could be introduced to an individual oocyte. Switching between the different solutions was controlled manually. The oocyte was continuously perfused with recording OR_2 (OR_2 without antibiotics and the 1 mM Na_2HPO_4 replaced with 1 mM NaCl) and briefly switched to the test solution containing drug.

Recording microelectrodes were fabricated with a Narishige PP-83 puller and filled with 3 M KCl. Electrodes with resistances of 0.6–1 MΩ were used. Standard two-electrode voltage-clamp techniques (Turbo TEC-05 npi; Adams and List, Westbury, NY) were used to record currents in response to application of drugs. In all cases, membrane potential was clamped to −70 mV. Data were played out on a Gould EasyGraf chart recorder (Gould Inc., Glen Burnie, MD) during the experiment and recorded on a VCR (Instrutech PA10b; Instrutech Labs., Plymouth Meeting, PA) for off-line analysis.

The EC_{50}, and Hill numbers were estimated by fitting the concentration-response relationships to the following equation: $[I = I_{max}/(1 [EC_{50}/(A)]^n)]$ using computer software provided by Dr. David S. Weiss, where I is the peak current at a given concentration of agonist A, I_{max} is the maximum current, EC_{50} is the concentration of agonist yielding a current half the maximum, and n is the Hill coefficient.

Results

TM3 of β_2 Subunit Is Sufficient To Impart Pentobarbital Sensitivity to ρ_1. To determine the crucial domain(s) for the dual agonistic and modulatory action of pentobarbital, chimeric human ρ_1 and rat β_2 subunits were constructed. The cRNA from the different ρ-β chimeras were expressed in *Xenopus* oocytes and the responses of these receptor channels were electrophysiologically recorded in the presence of GABA, pentobarbital, and a combination of both. The summary of these experiments is depicted in Fig. 1A (see also Tables 1 and 2). The most striking result was the role of the TM3 from the β_2 subunit in conferring pentobarbital sensitivity (compare the ρ324/β283 and ρ346/β305). The ρ_1 receptor channel containing both the TM3 and the TM4 from the β_2 subunit displayed marked sensitivity to pentobarbital. In contrast, deletion of the sequences corresponding to the TM3 of the β_2 within the ρ324/β283 chimera and replacement with the TM3 of the ρ_1 subunit abolished pentobarbital sensitivity in the resulting receptor channels (Fig. 1A, ρ346/β305 and ρ405/β399).

To determine whether both the TM3 and the TM4 of the β_2 subunit are needed to confer pentobarbital sensitivity, or whether the TM3 of the β_2 subunit alone is sufficient to impart pentobarbital sensitivity to the ρ_1 receptor channels, the TM3 of the ρ_1 subunit was replaced with the equivalent domain of the β_2 subunit (Fig. 1B, ρ324/β283–304/ρ347). The expression of the cRNA for this chimera yielded a receptor channel highly sensitive to pentobarbital (Table 2; EC_{50} = 77.2 ± 0.5 μM). For comparison, TM1 from the ρ_1 subunit was replaced with the equivalent domain from the β_2 subunit. The expression of this chimera, however, produced a receptor channel that was insensitive to pentobarbital (Fig. 1B and Table 2, ρ268/β227–242/ρ285). These results indicate the importance of the amino acid sequence within the TM3 of the β_2 subunit in imparting pentobarbital sensitivity to the ρ_1 receptor channel.

Methionine Substitution for Trp328 Within TM3 of ρ_1 Confers Pentobarbital Sensitivity. Comparison of the amino acid sequences encoding the TM3 of ρ_1 and β_2 subunits revealed nonconserved differences with respect to size and hydrophobicity mainly at three positions, Trp328, Val329, and Ser330. The corresponding residues within the β_2 subunit were Met286, Gly287, and Cys288 (Fig. 1C). Using site-directed mutagenesis, the Trp328, Val329, and Ser330, within the ρ_1 subunit, singly or in combination, were mutated to Met, Gly, and Cys, respectively. All mutant receptor channels that included Trp328 to Met substitution (ρW328M, ρWV328,329 MG, and ρWVS328–330 MGC) were sensitive to pentobarbital. For these mutant receptor channels, pentobarbital at low concentrations mediated the potentiation of the GABA responses (see below, e.g., Fig. 1D) and displayed agonistic properties at higher concentrations (Table 2). In contrast, ρV329G, ρS330C, or ρVS329,330GC receptor channels were insensitive to both modulatory and agonistic action of pentobarbital (Table 2). The specificity of position 328 in conferring pentobarbital sensitivity is corroborated by the lack of response of the ρV329G, ρS330C, or ρVS329,330GC receptor channels to pentobarbital, given the proximity of Val and Ser residues to Trp328.

Hydrophobic Residues at Position 328 Impart Pentobarbital Sensitivity to ρ_1 Receptor Channel. The Trp328 within the ρ_1 subunit was replaced with an array of diverse amino acids differing in hydropathy index (HI) (Kyte and Doolittle, 1982), charge, and size. Remarkably, as with Met (HI = 1.9), substitutions of Trp328 (HI = −0.9) with other hydrophobic residues such as Leu, Ile, and Val (HI of

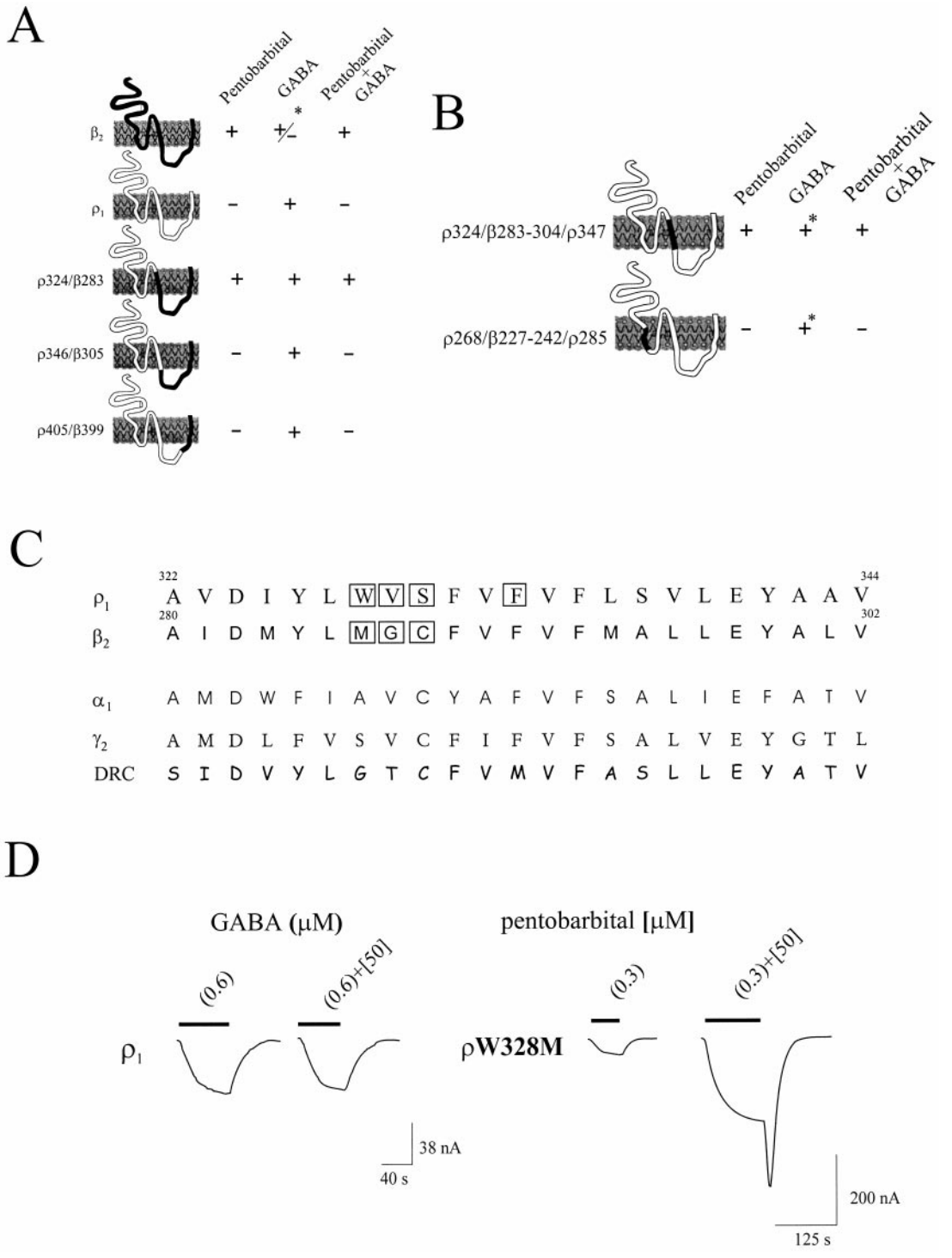

Fig. 1. Determination of crucial residue in conferring pentobarbital-sensitivity to ρ_1 receptor channel. A, chimeras between human ρ_1 and rat β_2 subunits were constructed. Special care was taken not to alter relative position of conserved amino acids on both sides of junction (with exception of ρ405/β399). cRNAs from different ρ-β chimeras were expressed in *Xenopus* oocytes and resulting receptor channels were examined using GABA (up to 20 mM), pentobarbital (up to 2.5 mM), or both; * indicates spontaneously open channels (see *Results*). In these channels (*), chloride leak (judged based on reversal potential for chloride) was directly proportional to amount of injected cRNA (data not shown). β_2 receptor channels had a severe depression in maxima when tested with GABA alone. All receptor channels that responded to GABA or pentobarbital or both are scored with +. All numbers indicate amino acid position for ρ_1 and β_2 subunit, respectively. Many constructed chimeras did not yield functional channels (data not shown). It is important to note that rat β_2 subunit is highly suited to form heterooligomeric receptor channels. This is corroborated by nearly three orders of magnitude greater maximal current when equivalent cRNA concentrations for β_2 subunit is coexpressed with α and γ subunit. B, TM3 (but not TM1) of β_2 subunit confers pentobarbital sensitivity to ρ_1 receptor channel. C, alignment of amino acid sequences corresponding to TM3 of human ρ_1, rat β_2, α_1, γ_2, and *Drosophila* (DRC) GABA subunits. Boxed residues represent amino acids that were mutated in this study. D, pentobarbital-dependent modulation of GABA responses from ρ1 and ρW328M receptor channels. Mutation of Trp328 to Met, imparted pentobarbital sensitivity to ρ_1 receptor channel. For ρW328M receptor channel, GABA (0.3 μM) responses were markedly potentiated in presence of 50 μM pentobarbital. In contrast, GABA currents (0.6 μM) for ρ_1 receptor channel were not altered in presence of equivalent concentration of pentobarbital. Thick line above each current trace represents duration of GABA application or coapplication of GABA and pentobarbital.

3.8, 4.5, and 4.2, respectively) and Ala (Fig. 1C, found at the corresponding position on the α_1 subunit, HI = 1.8), imparted both the agonistic and modulatory properties of pentobarbital to the mutated ρ_1 receptor channels. GABA responses from the ρ_1 receptor channel containing the Phe substitution (HI = 2.5), however, were only weakly enhanced by pentobarbital. The homooligomeric *Drosophila* GABA receptor channel responds to the modulatory action of pentobarbital at high concentrations (Chen et al., 1994). The TM3 of the *Drosophila* subunit contains Gly (HI = −0.4), Thr, and Cys at the equivalent position with respect to the TM3 of the ρ_1 (Fig. 1C). The corresponding residues (Trp, Val, and Ser) within ρ1 receptor channel were mutated to Gly, Thr, and Cys. Pentobarbital potentiated the GABA-evoked currents from the ρWVS328,330GTC receptor channel (data not shown) and was also found to be an agonist for this mutant receptor channel (depressed maximum, Table 2). In contrast, the Tyr (HI = −1.3) substitution (ρW328Y) yielded receptor channel with a pharmacological profile resembling that of the wild type. Pentobarbital neither directly activated nor modulated the GABA-evoked currents for the ρW328Y receptor channel (see below). Finally, substitutions of Trp328 by Glu or Ser (Fig. 1C, found at the corresponding position on the γ_2 subunit) or Pro (HI of −3.5, −0.8, and −1.6, respectively) failed to yield functional channel when tested with GABA (Table 1, up to 30 mM) or pentobarbital (Table 2, 2.5 mM). Thus, the HI of the substituted amino acid at position 328 appears to be crucial not only in imparting pentobarbital sensitivity, but also in GABA-dependent activation.

Mutation of Trp328 Transforms GABA Sensitivity. Figure 2 shows the current traces elicited by different concentrations of GABA, as well as the GABA concentration-response relationships from oocytes expressing ρ_1, ρW328L, ρW328I, ρW328V, ρW328M, ρW328A, ρW328F, and ρW328Y receptor channels. GABA activated these mutants with similar efficacy (with the exception of ρW328Y, with the maximum current of approximately 10% of the wild type), when matched cRNA concentrations (see *Materials and Methods*) for these individual mutants were injected into oocytes and their maximal GABA-evoked currents were compared. These mutations, however, induced marked transformation in the GABA potency (Table 1). The ρW328L and ρW328I receptor channels displayed approximately a 3-fold increase in the sensitivity. The GABA EC_{50}s for ρW328L and ρW328I were 0.35 and 0.39 μM, respectively (in comparison with 1.03 μM for the wild type). In contrast, the Phe and Ala substitution produced a drastic 30-fold reduction in GABA sensitivity, when compared with ρ_1 receptor channel. The EC_{50} values estimated form GABA concentration-response relationships for ρW328A and ρW328F receptor channels were 32 and 30 μM, respectively. The GABA concentration-response relationship for ρWVS328–330GTC receptor channel was also altered (Table 1). In comparison with wild type, the GABA EC_{50} for the ρWVS328–330GTC receptor channel was increased by approximately 8-fold. Finally, the Met, Val, and Tyr substitutions at position 328 produced receptor channels with similar GABA sensitivity. The EC_{50} values for these receptor channels deviated 20 to 30% from the EC_{50} value for

TABLE 1

Parameters determined from fitting Hill equation to GABA concentration-response relationships. Numbers in parentheses indicate number of oocytes tested. Concentration of GABA required for half-maximal activation (EC_{50}) and Hill coefficient are mean ± S.D.

Subunit	EC_{50} μM	Hill Coefficient
ρ_1 (12)	1.03 ± 0.26	2.26 ± 0.48
β_2 (10)	Reduced maximum[a, b]	
$\alpha_1\beta_2\gamma_2$ (4)	46.5 ± 4.7	1.52 ± 0.12
Chimeras		
ρ324/β283 (3)	3.46 ± 4.7	1.52 ± 0.15
ρ346/β305 (3)	4.58 ± 1.72	0.99 ± 0.17
ρ405/β399 (4)	0.54 ± 0.11	2.04 ± 0.32
ρ268/β227–242/ρ285 (3)	GABA activated[a, b]	
ρ324/β283–304/ρ347 (6)	GABA activated[a, b]	
Point mutations		
ρWV328,329MG (6)	0.83 ± 0.17	2.22 ± 0.59
ρWVS328–330MGC (7)	1.06 ± 0.22	2.55 ± 0.52
ρW328M (6)	1.31 ± 0.30	2.39 ± 0.15
ρV329G (4)	0.42 ± 0.05	2.86 ± 0.65
ρS330C (4)	1.12 ± 0.15	2.35 ± 0.09
ρVS329,330GC (3)	0.23 ± 0.05	1.49 ± 0.06
ρW328L (4)	0.35 ± 0.22	2.62 ± 1.26
ρW328I (4)	0.39 ± 0.04	2.38 ± 0.19
ρW328V (4)	0.85 ± 0.01	3.13 ± 0.49
ρW328A (6)	31.74 ± 5.53	1.32 ± 0.08
ρW328F (7)	29.92 ± 4.44	1.51 ± 0.08
ρW328Y (5)	0.83 ± 0.06	3.05 ± 0.69
ρW328E (12)	No response up to 30 mM	
ρW328S (10)	No response up to 30 mM	
ρW328P (11)	No response up to 30 mM	
ρY198S (4)	3077.1 ± 724.2	1.56 ± 0.07
ρY198S/W328M (3)	3331.8 ± 346.7	1.61 ± 0.16
ρY198S/WVS328–330MGC (6)	2538.7 ± 616.0	1.52 ± 0.21
βM286W (6)	Reduced maximum[a, b]	
βMGC286–288WVS (5)	Reduced maximum[a, b]	
ρWVS328–330GTC (3)	7.69 ± 0.91	1.93 ± 0.10
ρF333M (7)	No response up to 30 mM	

[a] Concentration-response relationship could not be fitted to these data points.
[b] Spontaneously open.

wild type. These results suggest that position 328 is not only crucial in conferring pentobarbital sensitivity, but also plays a key role in GABA-dependent activation.

Pentobarbital-Dependent Potentiation Versus Inhibition of GABA Responses Occurs Over a Narrow Range of GABA Concentration for ρ_1 328 Mutants. Enhancement of the GABA-evoked currents by pentobarbital from the homooligomeric Trp328 mutants was dependent on GABA concentration. Figure 3A shows the pentobarbital-mediated (50 μM) modulation of GABA-evoked currents at different agonist concentrations for ρW328L, ρW328I, ρW328V, ρW328M, ρW328A, and ρW328F receptor channels. It is noteworthy that pentobarbital alone at a concentration (50 μM) applied in this experiment does not activate these Trp328 mutants.

Pentobarbital elicited potentiation of GABA responses only at low concentrations of GABA (fractions of their respective EC_{50} values; e.g., EC_5). However, at higher concentrations of GABA, pentobarbital appeared to act as a antagonist. For ρW328M receptor channels, the relationship between the fold potentiation by pentobarbital (50 μM) versus three different GABA concentrations is plotted in Fig. 3B. At 0.2 μM GABA (~0.15 of the EC_{50} for ρW328M), pentobarbital (50 μM) increased the peak GABA responses by approximately 18-fold, whereas the potentiation by pentobarbital was reduced to 5-fold in the presence of 0.3 μM GABA. In comparison, pentobarbital failed to potentiate the currents evoked by 0.5 μM GABA (~0.38 of the EC_{50} for ρW328M) and at higher concentrations of GABA, displayed antagonistic properties (Fig. 3A). For ρW328L and ρW328I receptor channels with higher sensitivity to GABA, pentobarbital (50 μM) was inhibitory at GABA concentrations as low as 0.2 μM (~50% of EC_{50}) but not at 0.1 μM (data not shown).

For ρW328F receptor channel, pentobarbital appeared to be less potent than other pentobarbital-sensitive ρ_1 mutants. The GABA responses from ρW328F were only weakly enhanced by pentobarbital. Finally, the substitution of Trp328 to a Tyr residue (ρW328Y) yielded a receptor channel with a pharmacological profile resembling that of wild type. Pentobarbital neither directly activated nor modulated the GABA-evoked currents for ρW328Y receptor channel (Fig. 3A and Table 2).

Another characteristic of the pentobarbital modulation of these Trp328 mutants was the increase in the residual current following the removal of pentobarbital and GABA. This phenomenon was most prominent in mutant receptor channels with the highest sensitivity to GABA. For instance, the residual current for ρW328L and ρW328I receptor channels nearly quadrupled in amplitude, following the removal of GABA and pentobarbital (Fig. 3A). In comparison, for mutants with greater GABA EC_{50}s (e.g., ρW328A), the rate and extent of current rise following the wash were less pronounced.

Pentobarbital also increased the rate of deactivation for these Trp328 mutants. For example, the time for the current amplitude to fall to 50% ($T_{1/2}$) of maximum following removal of GABA (0.5 μM) was 7.8 ± 0.6 s (n = 5) for ρW328M receptor channels, whereas the deactivation $T_{1/2}$ for the same

TABLE 2

Parameters determined from fitting Hill equation to pentobarbital concentration-response relationships. Numbers in parentheses indicate number of oocytes tested. For all pentobarbital-sensitive mutants, 2.5 mM pentobarbital was used (with exception of ρY198S/W328M and ρY198S/WVS328–330MGC receptor channels, where 1 mM pentobarbital was used) to obtain pentobarbital I_{max} (Pb I_{max}). Concentration of pentobarbital required for half-maximal activation (EC_{50}), Hill coefficient and Pb I_{max}/GABA I_{max} are mean ± S.D.

Subunit	EC_{50} μM	Hill Coefficient	Pb I_{max}/GABA I_{max}
ρ_1 (7)	No response up to 2.5 mM		
$\alpha_1\beta_2\gamma_2$ (4)	659.3 ± 53.7	3.44 ± 0.66	0.16 ± 0.03 (3)
β_2 (3)	49.7 ± 5.8[a]	0.96 ± 0.06	
Chimeras			
ρ324/β283 (6)	pentobarbital activated[b]		
ρ346/β305 (3)	No response up to 2.5 mM		
ρ405/β399 (4)	No response up to 2.5 mM		
ρ268/227–242/ρ285 (4)	No response up to 2.5 mM[a]		
ρ324/β283–304/ρ347 (2)	77.2 ± 0.5	1.74 ± 0.06	
Point mutations			
ρWV328,329MG (5)	819.1 ± 156.1	1.42 ± 0.19	Not determined
ρWVS328–330MGC (6)	956.6 ± 140.3	2.19 ± 0.48	Not determined
ρW328M (4)	801.9 ± 144.7	1.32 ± 0.05	0.21 ± 0.12 (2)
ρV329G (4)	No response up to 2.5 mM		
ρS330C (4)	No response up to 2.5 mM		
ρVS329,330GC (3)	No response up to 2.5 mM		
ρW328L (3)	862.63 ± 139.9	1.45 ± 0.08	0.15 ± 0.06 (4)
ρW328I (2)	2449.78 ± 163.38	1.06 ± 0.02	0.11 ± 0.05 (4)
ρW328V (3)	1627.72 ± 224.54	1.81 ± 0.07	0.1 ± 0.05 (2)
ρW328A (3)	1563.53 ± 181.75	1.78 ± 0.053	0.31 ± 0.06 (10)
ρW328F (7)	No response up to 2.5 mM		
ρW328Y (7)	No response up to 2.5 mM		
ρW328E (12)	No response up to 2.5 mM		
ρW328P (8)	No response up to 2.5 mM		
ρY198S/W328M (3)	202.7 ± 23.5	1.14 ± 0.09	0.23 ± 0.01 (2)
ρY198S/WVS328–330MGC (3)	176.5 ± 29.8	1.90 ± 0.21	0.35 ± 0.02 (2)
ρW328S (9)	No response up to 2.5 mM		
ρWVS328–330GTC (5)	Depression in the maximum[b]		
βM286W (5)	No response up to 2.5 mM		
βMGC286–288WVS (5)	No response up to 2.5 mM		
ρF333M (6)	No response up to 2.5 mM		

[a] Spontaneously open.
[b] Concentration-response relationship could not be fitted to these data points.

mutant increased to 35.2 ± 2.4 s (n = 5) following removal of GABA (0.5 μM) and pentobarbital (50 μM).

The ρW328L and ρW328I receptor channels also exhibited higher sensitivity to isoguvacine (a less potent GABA agonist for ρ_1 receptor channel) when compared with ρ_1 receptor channel. The isoguvacine EC_{50} values for ρW328L and ρW328I were 23.69± 3.38 μM (n = 3) and 37.31± 0.96 μM (n = 3) in comparison to 104 ± 3.62 μM (n = 4) for the wild-type receptor channel. In the presence of 8 μM isoguvacine ($<EC_{10}$), pentobarbital (50 μM) enhanced the isoguvacine-induced currents for ρW328L and ρW328I by 257 ± 48% (n = 4) and 292 ± 16% (n = 4), respectively (Fig. 4A).

Pentobarbital Potentiation Occurs Over a Wide Range of GABA Concentrations for $\alpha_1\beta_2\gamma_2$ Receptor Channel. Figure 4B shows the pentobarbital modulation of the heterooligomeric $\alpha_1\beta_2\gamma_2$ receptor channel in the presence of different concentrations of GABA. The GABA concentrations used were equivalent (with respect to their EC_{50}s) to those used for the ρW328M receptor channel (see Fig. 3A). For the $\alpha_1\beta_2\gamma_2$ receptor channel (Table 1, GABA EC_{50} = 46.5 ± 4.7 μM), pentobarbital at a concentration of 50 μM markedly potentiated the GABA responses evoked by 7, 10, 17, and 34 μM GABA (Fig. 4B). Moreover, at concentrations of GABA (135 μM) approximately three times the EC_{50}, moderate enhancement of the GABA response was still present. This experiment demonstrates that for the heterooligomeric $\alpha_1\beta_2\gamma_2$ receptor channel, the potentiation by pentobarbital occurs over a much wider range of GABA concentrations (relative to EC_{50}) than pentobarbital-sensitive Trp328 mutants. The contrast in pentobarbital mode of modulation for the Trp328 ρ_1 mutants and $\alpha_1\beta_2\gamma_2$ receptor channels is intriguing, given that these two classes of receptor channels may be activated differently by GABA (Amin and Weiss, 1996; see *Discussion*).

Impairment of GABA Sensitivity Does Not Alter Unique Pentobarbital Modulation of ρ_1 328 Mutants. The ρ_1 receptor channel is approximately 40 times more sensitive to GABA than $\alpha_1\beta_2\gamma_2$ receptor channel. To examine whether this difference in GABA sensitivity could account for the contrast in pentobarbital modulation between the two classes of receptor channels, a Tyr at position 198 (presumably located with in the extracellular receptor domain) was

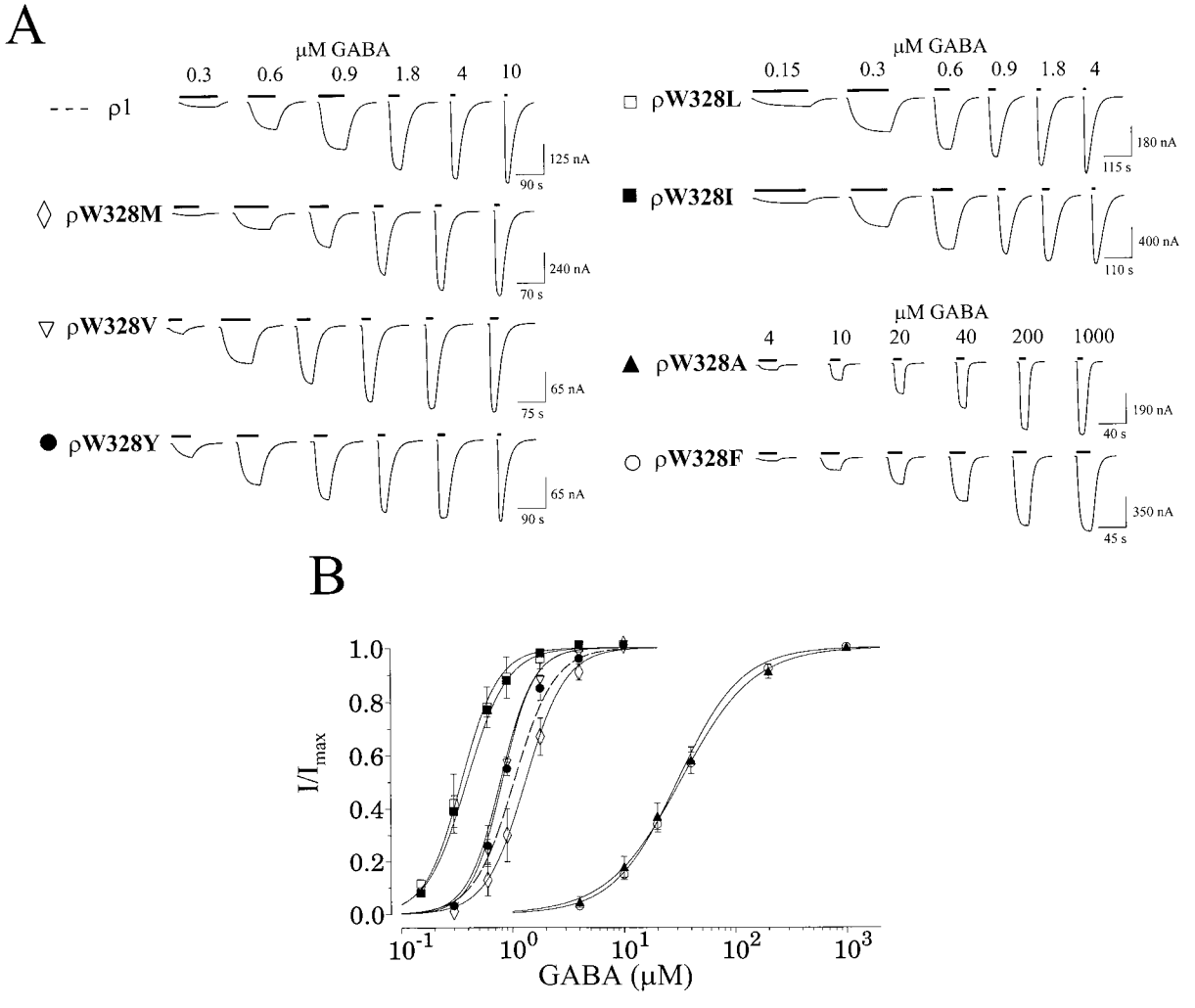

Fig. 2. GABA-dependent activation of ρ_1 Trp328 mutant receptor channels. A, current traces evoked by different concentrations of GABA for ρ_1 and ρ_1 328 mutants. Thick line above each current trace represents duration of GABA application. Symbol representing each mutant in B (concentration-response relationships) is shown on left of current traces corresponding to that mutant. B, GABA concentration-response relationship for ρ_1 Trp328 mutants. Each plot represents average of normalized peak currents versus GABA concentrations from three oocytes expressing ρ_1 or ρ_1 Trp328 mutants. Lines are best fit of Hill equation to data points, and error bars represent S.D. (n = 3). Note that there are approximately two orders of magnitude variation in concentration of GABA required to elicit half-maximal currents (EC_{50}) among these mutants.

mutated to Ser within both ρW328M and ρWVS328–330 MGC mutant subunits. Previous studies have demonstrated that Tyr198 to Ser substitution within the ρ_1 receptor channel results in a 2500-fold decrease in GABA sensitivity (Table 1, ρY198S, also Amin and Weiss, 1994). Similar to ρY198S, both ρY198S/W328M and ρY198S/WVS328–330MGC receptor channels exhibited a three orders of magnitude reduction in GABA sensitivity (Table 1, EC_{50}s of ~3000 μM). Nevertheless, the mode of pentobarbital modulation for these receptor channels remained the same as other Trp328 ρ_1 mutants. Pentobarbital at a concentration of 20 μM synergistically potentiated the GABA (200 μM) responses from oocytes expressing ρY198S/W328M (Fig. 4C) or ρY198S/WVS328–330MGC by 390 ± 14% (n = 3) and 780 ± 160% (n = 3), respectively. However, at concentrations of 750 μM and 2800 μM of GABA (below the EC_{50} values for ρY198S/W328M), pentobarbital displayed antagonistic properties. Moreover, in the presence of 30 mM GABA, the pentobarbital effect was consistent with a channel block (or desensitization; see Fig. 4D for ρY198S/W328M).

Thus, marked decrease in GABA potency in ρW328M (or ρWVS328–330MGC) did not alter the paradigm in pentobarbital modulation for the homooligomeric ρ_1 mutants. Pentobarbital yielded potentiation (for these activation-impaired Trp328 mutants) only in the presence of GABA concentrations equivalent to fractions of their respective EC_{50}s, yet caused inhibition at higher concentrations.

Pentobarbital at Higher Concentrations Is an Agonist for ρ_1 Trp328 Mutants. In addition to the modulatory effect, pentobarbital at higher concentrations is also an agonist for ρW328L, ρW328I, ρW328V, ρW328M, and ρW328A receptor channels. Figure 5A shows current traces evoked by different concentrations of pentobarbital for ρW328L and ρW328M receptor channels. In pentobarbital-direct activation studies with $GABA_A$ receptor channels, the current amplitude increases before returning to the baseline following removal of pentobarbital (at high concentrations, Rho et al., 1996; J. Amin, unpublished observations). This phenomenon was absent in pentobarbital-direct activation of the Trp328 mutants. Note that for ρW328L and ρW328M receptor channels, even at the highest concentration of pentobarbital (2.5 mM), the evoked currents did not increase in amplitude following pentobarbital wash.

Figure 5B depicts pentobarbital concentration-response relationships for ρW328L, ρW328I, ρW328V, ρW328M, and ρW328A receptor channels. Each plot represents the average

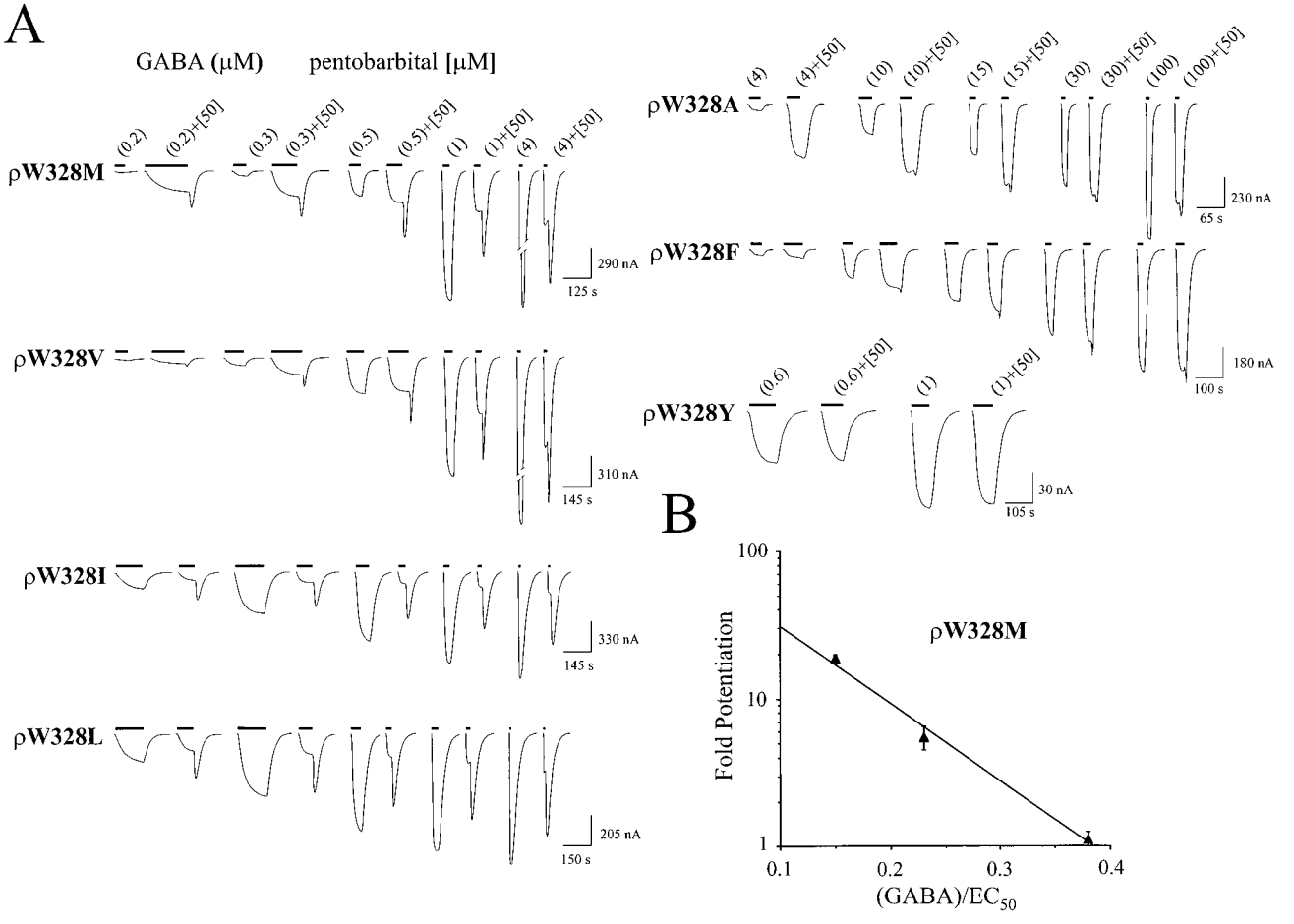

Fig. 3. Pentobarbital modulation of GABA-evoked currents for Trp328 mutant receptor channels. A, pentobarbital modulation of GABA-evoked currents (at different concentrations of GABA) for ρW328L, ρW328I, ρW328V, ρW328M, ρW328A, and ρW328Y receptor channels. Note that pentobarbital potentiation versus inhibition occurs over a narrow range of GABA concentrations. Magnitude of disrupted current traces for ρW328M and ρW328V at 4 μM GABA are 3.0 and 2.1 μA, respectively. Thick line above each current trace represents duration of GABA application or coapplication of GABA and pentobarbital. B, plot of pentobarbital potentiation for ρW328M receptor channel in presence of 0.2, 0.3, and 0.5 μM GABA versus ratio of GABA concentrations to EC_{50} for ρW328M. Note exponential relationship between relative potentiation by pentobarbital and GABA concentrations. Pentobarbital potantiation occurs at GABA concentrations below GABA EC_{50} value for ρW328M. Error bars are S.D.s (n = 3).

of normalized peak (to the extrapolated maximum) currents versus pentobarbital concentrations from oocytes ($n = 3$ except for ρW328I, $n = 2$) expressing the above Trp328 mutant receptor channels. Comparison of the pentobarbital and the GABA EC_{50} values for the same set of mutants is plotted in Fig. 5C. Unlike the effect on GABA potency, the difference in the pentobarbital potency is subtle (Table 2, pentobarbital EC_{50}s of 0.8 to 2.4 mM). For example, the difference between the ρW328A and ρW328L receptor channels in pentobarbital potency is less than 2-fold. However, for the same mutants, there is approximately a 100-fold contrast in the GABA potency.

Pentobarbital is also an agonist for the activation-impaired ρY198S/W328M and ρY198S/WVS328–330MGC receptor channels. Interestingly, pentobarbital exhibited approximately a 4-fold higher potency for these mutants (EC_{50}s of 202.7 ± 23.5 and 176.5 ± 29.8 μM, respectively) than for ρW328M and ρWVS328–330MGC receptor channels.

Similar to heterooligomeric $\alpha_1\beta_2\gamma_2$ receptor channels (Table 2) pentobarbital is a partial agonist for all pentobarbital-

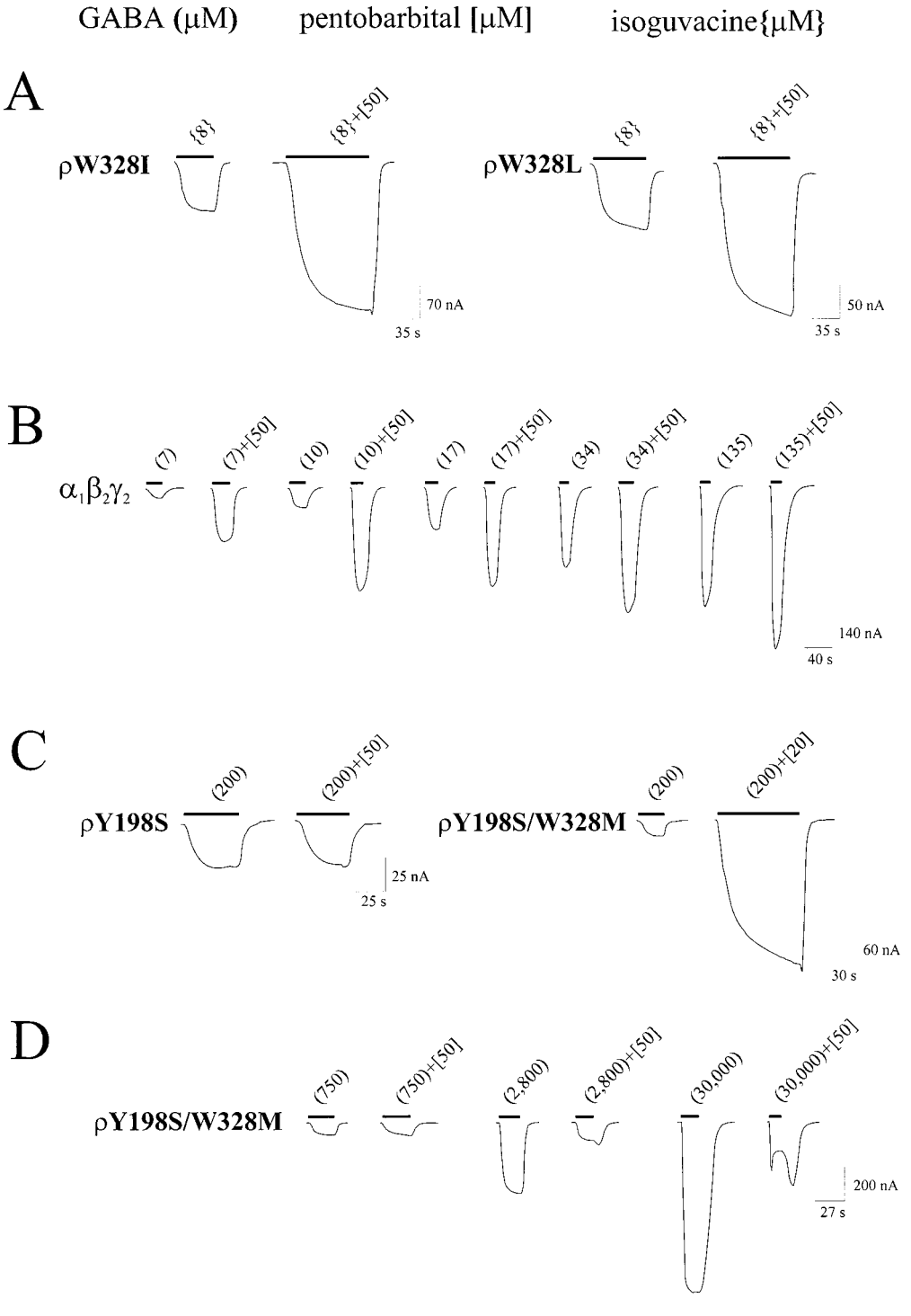

Fig. 4. A, pentobarbital potentiation of isoguvacine-evoked currents for ρW328I and ρW328L. Pentobarbital (50 μM) markedly potentiated isoguvacine (8 μM) responses for ρW328I and ρW328L receptor channels. Concentration of isoguvacine (8 μM) used in this study is equivalent to fraction of isoguvacine EC_{50}s for ρW328I and ρW328L receptor channels. B, pentobarbital modulation of heterooligomeric $\alpha_1\beta_2\gamma_2$ receptor channel. GABA concentrations used were equivalent (with respect to their EC_{50}s) to those used in Fig. 3A for ρW328M receptor channel. Thick line above each current trace represents duration of GABA application or coapplication of GABA and pentobarbital. Note contrast in pentobarbital action between $\alpha_1\beta_2\gamma_2$ and ρW328M, where potentiation for $\alpha_1\beta_2\gamma_2$ receptor channel occurs over a wide range of GABA concentration (relative to EC_{50} value). C and D, a mutation within GABA activation domain of ρW328M markedly reduces GABA potency but does not alter GABA concentration-dependent (relative to EC_{50} value) modulation by pentobarbital. C, at GABA concentration (200 μM) equivalent to a fraction of EC_{50} value (3331 ± 346.7 μM), pentobarbital (20 μM) markedly enhanced GABA-evoked currents for homooligomeric ρY198S/W328M receptor channel. D, pentobarbital depression of GABA responses occurred at concentrations of GABA below EC_{50} for ρY198S/W328M receptor channels. At GABA concentration equivalent to 0.84 of EC_{50} (2800 μM GABA), pentobarbital (50 μM) inhibited GABA responses and at higher concentration (30,000 μM GABA) caused an apparent block desensitization. Thick line above each current trace represents duration of GABA application, or coapplication of GABA and pentobarbital.

sensitive mutants. Table 2 lists the ratio of maximal current (I_{max}) evoked by 2.5 mM pentobarbital to the I_{max} for GABA. Among these pentobarbital-sensitive mutants, the apparent I_{max} for pentobarbital varied from 10 to 30% of I_{max} for GABA (Table 2).

Thiopental and Phenobarbital Modulation of Trp328 Mutants. Thiopental and phenobarbital were also effective at potentiating GABA responses for pentobarbital-sensitive mutants, albeit with different potencies. As shown in Fig. 6, in the presence of 4 μM GABA, thiopental (50 μM) was nearly as potent as pentobarbital (50 μM) for ρW328A homooligomeric receptor channel. Thiopental and pentobarbital increased the GABA responses for ρW328A receptor channel by 659 ± 72.8% (n = 4) and 836.8 ± 90.4% (n = 4), respectively.

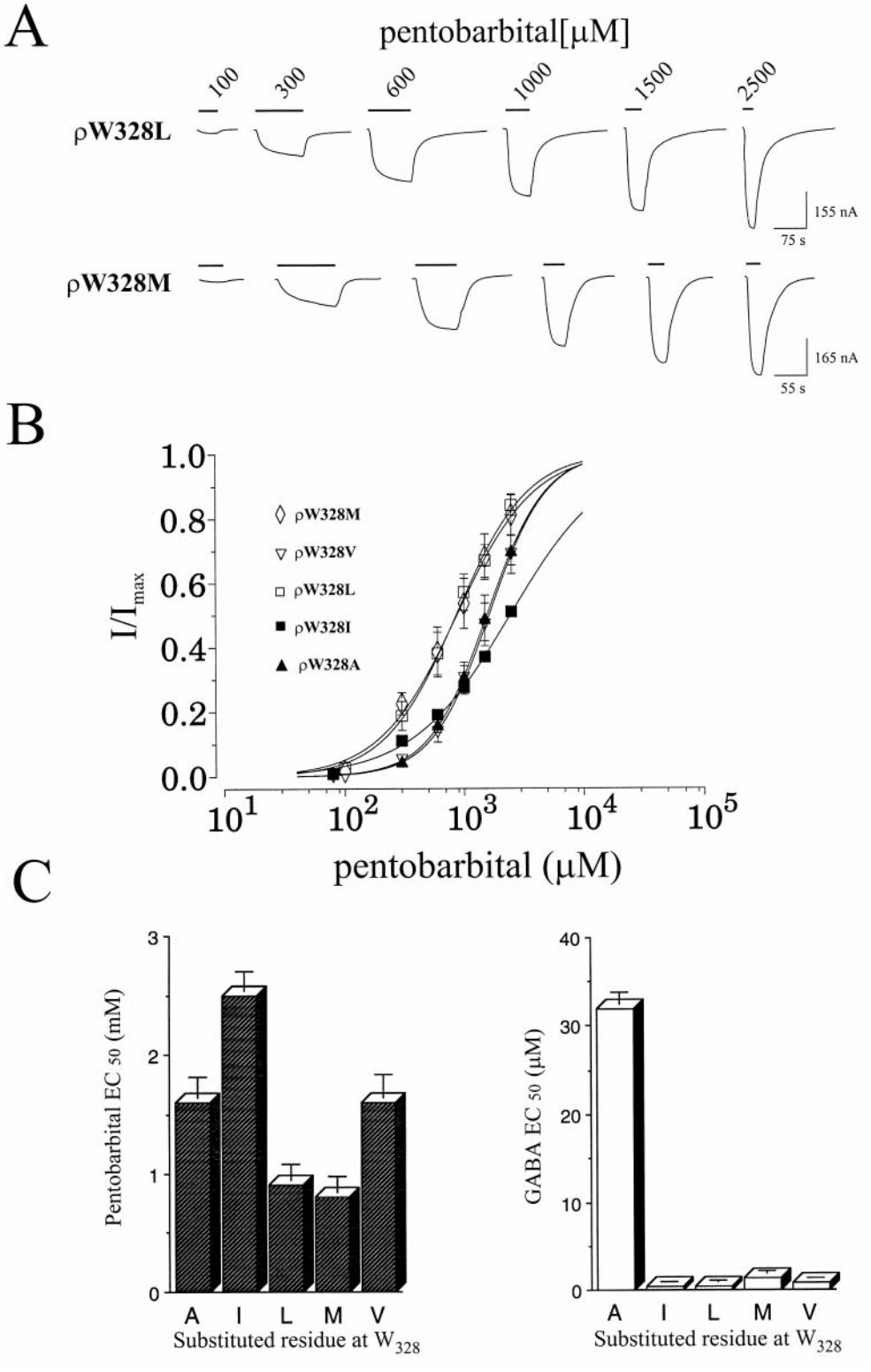

Fig. 5. A, current traces evoked by different concentrations of pentobarbital for ρW328M and ρW328L receptor channels. Thick line above each current trace represents duration of GABA application. B, pentobarbital concentration-response relationship for ρ_1Trp328 mutants. Each plot represents average of normalized peak (to extrapolated maximum) currents versus pentobarbital concentrations from three oocytes (except for ρW328I, two oocytes) expressing ρW328L, ρW328I, ρW328V, ρW328M and ρW328A receptor channels. Lines are best fit of Hill equation to data points, and error bars represent S.D. C, comparison of ρW328L, ρW328I, ρW328V, ρW328M, and ρW328A receptor channels' EC_{50} values for pentobarbital and GABA. Note that for these mutants, there are marked alteration in EC_{50} values for GABA, while there are only moderate difference in EC_{50} values for pentobarbital.

On the other hand, phenobarbital at twice the concentration (100 μM), was less effective at potentiating ρW328A's responses to GABA (452.5 $\pm$ 16.1%, $n = 4$). Preliminary results indicate that ρW328L, ρW328I, ρW328V, and ρW328M receptor channels were also modulated by thiopental and phenobarbital with similar relative potencies (data not shown). The comparative potencies of these barbiturates for Trp328 mutants are consistent, in general, with their relative clinical potencies (Franks and Lieb, 1994).

Tryptophan Substitution for Met286 within TM3 of β_2 Subunit Abolishes Pentobarbital Sensitivity. Within the β_2 subunit, the Met at position 286 alone or in combination with Gly287 and Cys288 were mutated to their corresponding amino acid counterparts found in the ρ_1 subunit. Figure 7 illustrates the currents elicited by bath application of GABA (5 μM) or both GABA (5 μM) and pentobarbital (30 μM) to oocytes expressing β_2 or βM286W receptor channel. Similar to β_2, the expression of the cRNA for βM286W (or βMGC286–288WVS) yielded spontaneously open channels. The magnitude of the chloride ion leak (judged by the reversal potential for chloride) in these ion channels was proportional to the amount of injected cRNA (data not shown). In addition, β_2 wild-type and mutant receptor channels displayed severe depression in the I_{max} when tested with GABA (see legend to Fig. 1). Nonetheless, in contrast to β_2, coapplication of pentobarbital and GABA to oocytes expressing βM286W (or βMGC286–288WVS, data not shown) receptor channels failed to increase the GABA-evoked currents (Fig. 7).

The coexpression of cRNA for the rat α_1 subunit with either βM286W or βMGC286–288WVS yielded receptor channels highly responsive to GABA (similar potency and efficacy as wild-type $\alpha_1\beta_2$ receptor channel, data not shown). However, in contrast to βM286W (or βMGC286–288WVS), the $\alpha_1\beta$M286W or the $\alpha_1\beta$MGC286–288WVS receptor channel was pentobarbital sensitive (data not shown). Comparison of the amino acid sequence encoding the TM3 domain of β_2 and α_1 subunits revealed that the α subunit contains an Ala residue at the corresponding position (Fig. 1C). In the $\alpha_1\beta$M286W or the $\alpha_1\beta$MGC286-288WVS receptor channel, the lost pentobarbital function of the mutated β_2 subunit may be reverted by the presence of the Ala residue within the TM3 of the α subunit.

Discussion

The data presented here indicate that replacing residue 328 with a spectrum of amino acid residues can confer barbiturate modulation as well as alter GABA-dependent activation of the mutated ρ_1 receptor channel. The apparent major determinant for pentobarbital sensitivity of the mutated ρ_1 receptor channel was, however, the hydrophobicity of the substituted amino acid at position 328. There were also key differences in the pentobarbital modulation between the homooligomeric ρ_1 328 mutants and the heterooligomeric $\alpha\beta\gamma$ receptor channels.

Pentobarbital Versus GABA. The lack of stringency for amino acid side chains (except for hydrophobicity) at position 328 to confer pentobarbital sensitivity is unique. For instance, the Met side chain is different from that of Ala in both size and the constituent elements, whereas the EC_{50} for pentobarbital between ρW328M and ρW328A receptor channels varied by less than 2-fold. Mutational analysis of different ligand-gated ion channels (including GABA) has shown that even conservative amino acid substitutions (such as Tyr to Phe) within the agonist-dependent activation domain can markedly impair the agonist sensitivity (Vandenberg et al., 1992; Amin and Weiss, 1993). The differences in amino acid side chain requirement between the agonist and the pentobarbital activation domains is perhaps best manifested by the nature of the bond they form. The interaction between pentobarbital and its site of action may be mediated through the butyl side chain of the amphipathic pentobarbital mole-

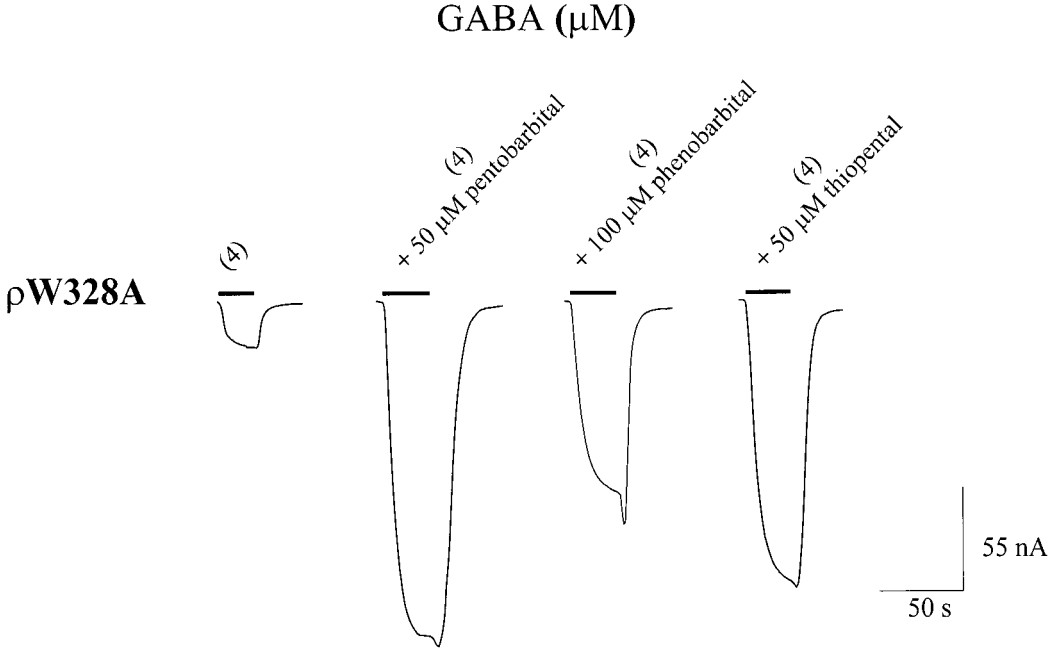

Fig. 6. Comparison of pentobarbital, phenobarbital, and thiopental in modulating GABA responses from ρW328A receptor channel. Among tested barbiturates, pentobarbital and thiopental appear to be most potent positive modulators of GABA responses for ρW328A receptor channels. Thick line above each current trace represents duration of GABA application or coapplication of GABA and barbiturates.

cule via the relatively weak hydrophobic interaction, whereas the GABA agonist may interact with its activation domain by more specific and stronger hydrogen bonding. In the pentobarbital-mediated potentiation of the GABA responses, the apparent sequential release of pentobarbital followed by GABA may attest to this notion, because there appeared to be a direct correlation between the magnitude of current rise following the wash, and the EC_{50} of the Trp328 mutants (Fig. 3A, smallest rise in the current for ρW328A and largest for ρW328L and ρW328I).

Target-Specific or General Perturbation? The view that general anesthetics indirectly affect membrane-embedded ion channels by altering the fluidity of lipid membranes is being gradually replaced by a target-specific model (Franks and Lieb, 1994). Two chief arguments, namely the identification of protein targets such as luciferase, as well as the discovery of the stereoselectivity of anesthetic agents, support the target-specific model for anesthetic action (Huang and Barker, 1980; MacIver and Roth, 1987; Franks and Lieb, 1991). Recently, several groups (Belelli et al., 1997; Mihic et al., 1997) have shown that the residues within the TM2 and the TM3 appear to be crucial for the action of the general anesthetics etomidate and enflurane. Interestingly, Mihic et al. (1997) have conversely mutated the corresponding 328 residue within the α subunit of the glycine receptor, or α and β subunit of the $GABA_A$ receptor channel to a Trp, to abolish the action of enflurane. Pentobarbital and enflurane are two structurally diverse anesthetics that appear to exert their action through the same site. This notion, together with the lack of amino acid side chain specificity for pentobarbital-dependent modulation, rekindles the debate over the mechanism of anesthetic action. It is tempting to speculate that substitution of the Trp328 to a hydrophobic residue may cause the TM2 (gate) to be readily accessible to the pentobarbital's induced local lateral pressure within the membrane bilayer (Gaines, 1966; Gruner and Shyamsunder, 1991; Cantor, 1997). In this scenario, anesthetics may only need the exposure of the channel's gating component to the membrane bilayer to shift equilibrium between the open and closed states.

Pentobarbital Modulation of ρ_1 Versus $\alpha_1\beta_2\gamma_2$ Receptor Channels. The contrast in the pentobarbital modulation between homooligomeric ρ_1 and heterooligomeric $\alpha_1\beta_2\gamma_2$ receptor channels is intriguing given that the ρ_1 receptor channel shows approximately 40-fold greater sensitivity to GABA than $\alpha_1\beta_2\gamma_2$, and ρ_1 displays unique activation and deactivation kinetics.

The aforementioned difference in GABA sensitivity, however, does not appear to play a key role in pentobarbital's unique modulation of ρ_1 Trp328 mutants. In experiments in which the GABA sensitivity of ρW328M was decreased by (mutation of Tyr to Ser at position 198) nearly three orders of magnitude, the dual modulatory action of pentobarbital for the resulting receptor channel persisted. Therefore, pentobarbital's unique modulation of (pentobarbital-dependent potentiation versus inhibition) ρ_1 Trp328 mutants is independent of GABA potency.

Could the difference in activation mechanism between these two classes of receptor channels account for the contrast in pentobarbital modulation? Experiments with coexpression of different ratios of wild-type ρ_1 and activation-impaired ρ_1 subunits (Y198S) have demonstrated previously that the agonist-dependent activation of homooligomeric ρ_1 receptor channel appears to be preceded by three binding steps (Amin and Weiss, 1996) rather than two binding steps observed for heterooligomeric receptor channels (Blount and Merlie, 1989). The three-step activation scheme for ρ_1 receptor channel was derived based on the assumption of one binding site per subunit in a pentameric configuration (five potential binding sites). A speculative view is that in the presence of pentobarbital the forward rates for GABA are increased for pentobarbital-sensitive $\rho_1$receptor channel and at relatively higher concentrations of GABA, two additional binding sites could become occupied, leading the channel into a closed/desensitized state. Consistent with this, binding studies for the $GABA_A$ receptor channel (e.g., $\alpha_1\beta_2\gamma_2$) have shown that GABA binding is enhanced in the presence of pentobarbital (Olsen et al., 1991; Wakamori et al., 1991; Lin et al., 1993). Alternatively, pentobarbital antagonistic action could arise from pentobarbital binding to an inhibitory site

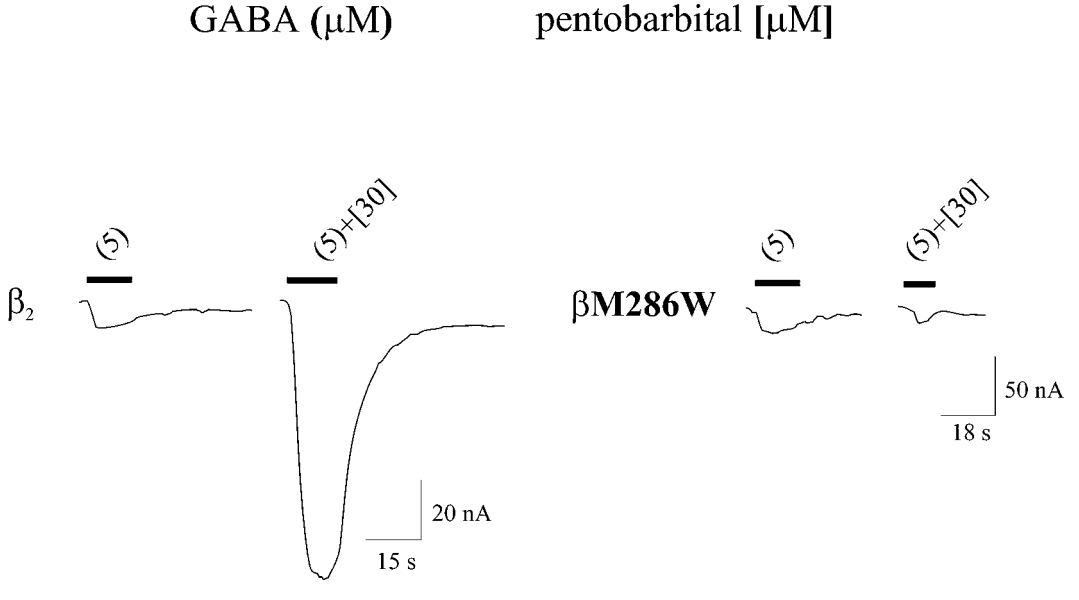

Fig. 7. Pentobarbital-dependent modulation of GABA responses from β2 or βM286W receptor channel. Mutation of Met 286 to Trp (βM286W), abolished pentobarbital sensitivity of β_2 receptor channel. On other hand, GABA currents for wild-type β_2 receptor channel markedly increased by coapplication of pentobarbital. Thick lines above each current trace represent duration of GABA application or coapplication of GABA and pentobarbital.

within the ρ_1Trp328 mutants. Rho et al. (1996), based on barbiturate studies on $GABA_A$ receptor channels, has proposed the presence of a low-affinity inhibitory site for pentobarbital. The depression in the pentobarbital-direct activation I_{max} (with respect to GABA I_{max}) could also be due to occupation of this postulated inhibitory site by pentobarbital.

Tryptophan Residue. What architectural features within the ρ_1 receptor channel might be created by Trp328 substitutions? The Trp residue is unique not only with respect to size, but also because of the indole moiety on its side chain. This residue can potentially anchor the TM3 to the extracellular side of the membrane. In this scenario, mutation of Trp328 to hydrophobic amino acids such as Met, Leu, Ile, Ala, or Val may dislodge the N-terminal amino acids of the TM3 from the interface of the extracellular side of the membrane and subsequently allow the residue 328 to rest deep within the membrane. This structural perturbation in the TM3 may then expose the gate of the channels to the membrane components (see above). Alternatively, the TM3 in the new configuration along with other TMs may constitute a binding cavity for pentobarbital. This phenomenon in which membrane-spanning domains interact to create a binding site, is not unique among membrane-embedded proteins. For example, the interactions of different TMs in the rhodopsin molecule constitute a binding cavity for the retinal molecule (Unger et al., 1997). Finally, Trp328 may impede the interaction of the pentobarbital with its binding/sensor domain solely based on its size. Consistent with this hypothesis, ρ_1 receptor channels containing the Tyr at position 328 did not respond to pentobarbital. Furthermore, in comparison with other hydrophobic amino acid substitutions, ρ_1 receptor channel containing the Phe (contains an aromatic ring on its side chain) substitution (ρW328F) exhibited lower pentobarbital sensitivity.

The amino acid residues in the center of the TM2 (leucine, the presumed gate) and the TM3 (Phe and Val) are conserved among all GABA subunits. Hypothetically, pentobarbital binding may induce interaction of these conserved residues leading to an increase in agonist affinity for its receptor, given that the binding of the agonist to its receptor and the gating of the channel are closely coupled. Interestingly, mutation of conserved Phe residue (ρF333 M) within the center of the TM3 resulted in receptor channels that responded to neither GABA nor pentobarbital (Tables 1 and 2). Alternatively, in a situation in which the agonist binding cleft resides proximal to the extracellular side of the membrane, the polar moiety of the pentobarbital can alter the agonist binding cleft and thereby change the affinity of the agonist for its receptor. In either proposed mechanism, marked variation in GABA sensitivity among the Trp328 mutants as well as the increase in pentobarbital potency concomitant with impairment of GABA activation domain (ρY198S/W328M), may attest to the close coupling of the agonist and pentobarbital binding/sensor site.

Within the TM3, Trp328 is positioned 5 amino acids from the presumed extracellular interface and 14 amino acids from the intracellular compartment. This positioning of residue 328 within the membrane is intriguing, because anesthetics in general exhibit membrane asymmetry in exerting their effect. It is also interesting that the length of the hydrophobic side chain of pentobarbital (also thiopental), which is nearly 5 angstroms in length, closely matches the depth in which position 328 may penetrate within the lipid bilayer in a presumed α-helical structure.

Acknowledgments

I am indebted to Dr. N. P. Franks, Dr. W. R. Lieb, and Dr. E. Bennett for constructive suggestions; Dr. L. Carlacci for helpful discussion; Dr. D. Fitzpatrick and Dr. P. Gottschall for reading, and Dr. V. Pollock and K. Morris for help in the preparation of this manuscript.

References

Akaike N, Shirasaki T and Yakushiji T (1991) Quinolones and fenbufen interact with $GABA_A$ receptor in dissociated hippocampal cells of rat. *J Neurophysiol* **66**:497–504.
Amin J and Weiss DS (1993) $GABA_A$ receptor needs two homologous domains of the β-subunit for activation by GABA but not by pentobarbital. *Nature* **366:**565–569.
Amin J and Weiss DS (1994) Homomeric ρ_1 GABA Channels: Activation properties and domains. *Receptors Channels* **2:**227–236.
Amin J and Weiss DS (1996) Insights into the activation mechanism of ρ_1 GABA receptors obtained by coexpression of wild type and activation-impaired subunits. *Proc R Soc Lond B Biol Sci* **263:**273–282.
Barker JL and Ransom BR (1978) Pentobarbitone pharmacology of mammalian central neurons grown in tissue culture. *J Physiol (Lond)* **280:**355–372.
Belelli D, Lambert JJ, Peters JA, Wafford K and Whiting PJ (1997) The interaction of the general anesthetic etomidate with the γ-aminobutyric acid type A receptor is influenced by a single amino acid. *Proc Natl Acad Sci USA* **94:**11031–11036.
Blair LA, Levitan ES, Marshall J, Dionne VE and Barnard EA (1988) Single subunits of the $GABA_A$ receptor form ion channels with properties of the native receptor. *Science* **245:**577–579.
Blount P and Merlie JP (1989) Molecular basis of the two nonequivalent ligand binding sites of the muscle nicotinic acetylcholine receptor. *Neuron* **3:**349–357.
Cantor RS (1997) The lateral pressure profile in membranes: A physical mechanism of general anesthesia. *Biochemistry* **36:**2339–2344.
Chen R, Delia B, Lambert JJ, Peters JA, Antonio R and Lan NC (1994) Cloning and functional expression of a Drosophila γ-aminobutyric acid receptor. *Proc Natl Acad Sci USA* **91:**6069–6073.
Cutting GR, Lu L, O'Hara BF, Kasch LM, Montrose-Rafizadeh C, Donovan DM, Shimada S, Antonarakis SE, Guggino WB, Uhl GR and Kazazian HH Jr (1991) Cloning of the γ-aminobutyric acid (GABA) ρ_1 cDNA: A GABA receptor subunit highly expressed in the retina. *Proc Natl Acad Sci USA* **88:**2673–2677.
Franks NP and Lieb WR (1991) Stereospecific effect of inhalational general anesthetic optical isomers on nerve ion channels. *Science* **254:**427–430.
Franks NP and Lieb WR (1994) Molecular and cellular mechanisms of general anaesthesia. *Nature* **367:**607–614.
Gage PW and Robertson B (1985) Prolongation of inhibitory postsynaptic currents by pentobarbitone, halothane and ketamine in CA1 pyramidal cells in rat hippocampus. *Br J Pharmacol* **85:**675–681.
Gaines GL (1966) *Insoluble Monolayers at Lipid-Gas Interfaces.* Interscience Publishers, New York.
Gruner SM and Shyamsunder E (1991) Is the mechanism of general anesthesia related to lipid membrane spontaneous curvature? *Ann NY Acad Sci* **625:**685–697.
Huang LY and Barker JL (1980) Pentobarbital: Stereospecific actions of the (+) and (−) isomers revealed on cultured mammalian neurons. *Science* **207:**95–197.
Kyte J and Doolittle RF (1982) A simple method for displaying the hydrophobic character of a protein. *J Mol Biol* **157:**105–132.
Levitan ES, Blair LAC, Dionne VE and Barnard EA (1988) Biophysical and pharmacological properties of cloned $GABA_A$ receptor expressed in Xenopus oocytes. *Neuron* **1:**773–781.
Lin L-H, Whiting P and Harris RA (1993) Molecular determinants of general anesthetic action: Role of $GABA_A$ receptor structure. *J Neurochem* **60:**1548–1553.
Macdonald RL and Olsen RW (1994) $GABA_A$ receptor channels. *Annu Rev Neurosci* **17:**569–602.
MacIver MB and Roth SH (1987) Barbiturate effects on hippocampal excitatory synaptic responses are selective and pathway specific. *Can J Physiol* **65:**385–394.
MacIver MB, Tanelian DL and Mody I (1991) Two mechanisms for anesthetic-induced enhancement of $GABA_A$-mediated neuronal inhibition, molecular and cellular mechanisms of alcohol and anesthetics. *Ann N Y Acad Sci* **625:**91–96.
Mathers DA and Barker JL (1980) (−)Pentobarbital opens ion channels of long duration in cultured mouse spinal neurons. *Science* **209:**507–509.
Mihic JS, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, Mascia MP, Valenzuela FC, Hansen KK, Greenblatt EP, Harris RA and Harrison NL (1997) Sites of alcohol and volatile anesthetic action on $GABA_A$ and glycine receptors. *Nature* **389:**385–389.
Nicoll RA (1972) The effects of anesthetics on synaptic excitation and inhibition in the olfactory bulb. *J Physiol (Lond)* **223:**803–814.
Nicoll RA, Eccles JC, Oshima T and Rubia F (1975) Prolongation of hippocampal inhibitory postsynaptic potentials by barbiturates *Nature* **258:**625–627.
Nicoll RA and Wojtowicz JM (1980) The effects of pentobarbital and related compounds on frog motoneurons. *Brain Res* **191:**225–237.
Olsen RW, Sapp DM, Bureau MH, Turner DM and Kokka N (1991) Allosteric actions of central nervous system depressants including anesthetics on subtypes of the inhibitory γ-aminobutyric acid A receptor-chloride channel complex *Ann NY Acad Sci* **625:**145–154.

Parker I, Gundersen CB and Miledi R (1986) Actions of pentobarbital on rat brain receptors expressed in *Xenopus* oocytes. *J Neurosci* **6:**2290–2297.
Rho JM, Donevan SD and Rogawski MA (1996) Direct activation of receptors by barbiturates in cultured rat hippocampal neurons. *J Physiol (Lond)* **497:**509–522.
Schofield PR, Darlison MG, Fujita N, Burt DR, Stephenson FA, Rodriguez H, Rhee LM, Ramachandran J, Reale V, Glencorse TA, Seeburg PH and Barnard EA (1987) Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor superfamily. *Nature* **328:**221–227.
Sanna E, Garau F and Harris RA (1995) Novel properties of homomeric beta 1 gamma-aminobutyric acid type A receptor: Action of the anesthetics propofol and pentobarbital. *Mol Phamacol* **47:**213–217.
Schulz DW and Macdonald RL (1981) Barbiturate enhancement of GABA-mediated inhibition and activation of chloride ion conductance: Correlation with anticonvulsant and anesthetic actions. *Brain Res* **209:**177–188.
Shimada S, Cutting G and Uhl GR (1992) γ-Aminobutyric acid A or C receptor? γ-Aminobutyric acid ρ_1 receptor RNA induces bicuculline-, barbiturate-, and benzodiazepine- insensitive γ-aminobutyric acid responses in *Xenopus* oocytes. *Mol Pharmacol* **41:**683–687.
Tanelian DL, Kosek P, Mody I and MacIver MB (1993) The role of the $GABA_A$ receptor/chloride channel complex in anesthesia. *Anesthesiology* **78:**757–776.
Unger VM, Hargrave PA, Baldwin JM and Schertler GFX (1997) Arrangement of rhodopsin transmembrane α-helices. *Nature* **389:**203–206.
Vandenberg RJ, Handford CA and Schofield PR (1992) Distinct agonist- and antagonist-binding sites on the glycine receptor. *Neuron* **9:**491–496.
Wakamori M, Ikemoto Y and Akaike NJ (1991) Effects of two volatile anesthetics and a volatile convulsant on the excitatory and inhibitory amino acid responses in dissociated CNS neurons of the rat. *J Neurophysiol* **66:**2014–2021.

Send reprint requests to: Dr. Jahanshah Amin, University of South Florida, College of Medicine, 12901 Bruce B. Downs Blvd. MDC Box 9, Tampa, FL 33612-4799. Email: Jamin@pharm.med.usf.edu