Allosteric Activation Mechanism of the $\alpha1\beta2\gamma2$ γ-Aminobutyric Acid Type A Receptor Revealed by Mutation of the Conserved M2 Leucine

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ABSTRACT A conserved leucine residue in the midpoint of the second transmembrane domain (M2) of the ligand-activated ion channel family has been proposed to play an important role in receptor activation. In this study, we assessed the importance of this leucine in the activation of rat $\alpha1\beta2\gamma2$ GABA receptors expressed in *Xenopus laevis* oocytes by site-directed mutagenesis and two-electrode voltage clamp. The hydrophobic conserved M2 leucines in $\alpha1$(L263), $\beta2$(L259), and $\gamma2$(L274) subunits were mutated to the hydrophilic amino acid residue serine and coexpressed in all possible combinations with their wild-type and/or mutant counterparts. The mutation in any one subunit decreased the EC_{50} and created spontaneous openings that were blocked by picrotoxin and, surprisingly, by the competitive antagonist bicuculline. The magnitudes of the shifts in GABA EC_{50} and picrotoxin IC_{50} as well as the degree of spontaneous openings were all correlated with the number of subunits carrying the leucine mutation. Simultaneous mutation of the GABA binding site ($\beta2$Y157S; increased the EC_{50}) and the conserved M2 leucine ($\beta2$L259S; decreased the EC_{50}) produced receptors with the predicted intermediate agonist sensitivity, indicating the two mutations affect binding and gating independently. The results are discussed in light of a proposed allosteric activation mechanism.

INTRODUCTION

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system. Several different classes of GABA-gated ion channel subunits and their isoforms have been cloned: $\alpha1$–6, $\beta1$–4, $\gamma1$–3, δ, ϵ, $\rho1$–3, π, and χ (Barnard et al., 1987; Cutting et al., 1991; Garret et al., 1997; Hedblom and Kirkness, 1997; Khrestchatisky et al., 1989; Olsen and Tobin, 1990; Schofield et al., 1987; Whiting et al., 1997). These subunits all belong to a ligand-gated ion channel gene family, the acetylcholine receptor family, which includes nicotinic acetylcholine (nACh), serotonin receptor type 3 ($5\text{-}HT_3$), glycine, and GABA receptors. More recently, an invertebrate glutamate-gated chloride channel was added to this family (Cully et al., 1994). The proposed topology of a nACh receptor family subunit is a large extracellular N-terminal domain, a long intracellular loop between the third and fourth transmembrane domains, and four membrane-spanning segments (M1–M4), of which M2 is proposed to line the pore (Akabas et al., 1994; Leonard et al., 1988; Noda et al., 1982; Schofield et al., 1987; Xu and Akabas, 1996).

By analogy with other members of this family, the GABA-gated ion channel is presumed to be a pentamer (Chang et al., 1996; Cooper et al., 1991; Langosch et al., 1988; Nayeem et al., 1994). The pentameric structure can be formed by combinations of different subunit isoforms. The prototypical recombinant $\alpha1\beta2\gamma2$ GABA receptor has pharmacological and functional properties very similar to those of the typical native $GABA_A$ receptors (Pritchett et al., 1989; Sigel et al., 1990; Verdoorn et al., 1990), whereas the exogenously expressed $\rho1$ homomeric GABA receptor is similar to native $GABA_C$ receptors (Cutting et al., 1991; Johnston, 1986; Polenzani et al., 1991; Sivilotti and Nistri, 1989).

Activation of GABA-gated ion channels includes agonist binding and gating of the integral chloride-selective pore. The structural determinants of GABA binding have been found to be in the N-terminal domain of the $\alpha1$ subunit (F64; Sigel et al., 1992) and $\beta2$ subunit (Y157, T160, T202, and Y205; Amin and Weiss, 1993) for $\alpha1\beta2\gamma2$ GABA receptors. In contrast to binding, the structural determinants of gating are still poorly understood. A leucine residue in the midpoint of the M2 region is conserved through all subunit isoforms in this receptor-operated ion channel family and has been postulated to correspond to the kink point of the pore-lining rod observed with electron microscopy (Unwin, 1995). Unwin proposed that the M2 helices, by bending toward the central axis, would allow the leucine side chains to project inward and associate in a tight ring via hydrophobic interactions and maintain the pore in the closed state. When agonist binds to the receptor, the hydrophobic interactions are weakened, the M2 regions twist, and the pore opens (Unwin, 1995). Studies employing cysteine-scanning mutagenesis, however, suggest that the gate is more cytoplasmic than this conserved leucine (Akabas et al., 1994; Wilson and Karlin, 1998; Xu and Akabas, 1996). Whatever the precise role this leucine plays in receptor activation, its absolute conservation across all members of this receptor family, as well as its position within the presumed pore, seems to warrant the attention it has received (Auerbach et al., 1996; Chang et al., 1996; Chang

Received for publication 7 May 1999 and in final form 2 August 1999.

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0006-3495/99/11/2542/10 $2.00

and Weiss, 1998; Filatov and White, 1995; Labarca et al., 1995; Revah et al., 1991; Tierney et al., 1996; Unwin, 1995; White and Cohen, 1992; Yakel et al., 1993).

In this study, we mutated the conserved M2 leucine to serine in rat $\alpha1$, $\beta2$, and $\gamma2$ subunits and observed that the mutation in any one subunit shifted the GABA dose-response curve of the $\alpha1\beta2\gamma2$ GABA receptors to the left. We previously took advantage of this shift in the EC_{50} to determine the stoichiometry of the $\alpha1\beta2\gamma2$ GABA receptor (Chang et al., 1996), but here we report a more detailed investigation of the activation and inhibition properties of these mutant receptors. In addition to the shift in EC_{50}, the leucine mutations created spontaneously opening channels, evident as an increase in the holding current at −70 mV. The spontaneously opening channels could be blocked by the GABA receptor antagonist picrotoxin and, surprisingly, by the competitive antagonist bicuculline. Based on our results, a Monod-Wyman-Changeux allosteric model (Chanegeux and Edelstein, 1998; Colquhoun, 1973; Edelstein and Changeux, 1996; Karlin, 1967; Monod et al., 1965) was adopted to account for the activation features of the $\alpha1\beta2\gamma2$ wild-type and mutant $GABA_A$ receptors.

MATERIALS AND METHODS

Site-directed mutagenesis and in vitro transcription

Rat $\alpha1$, $\beta2$, and $\gamma2L$ subunits were obtained by polymerase chain reaction from a rat brain cDNA library (Amin et al., 1994). The three subunits were cloned into pALTER-1 (Promega, Madison WI) between *Hin*dIII and *Xba*I for $\alpha1$ and $\gamma2$ or *Sal*I and *Bam*HI for $\beta2$. The mutagenic oligonucleotides used for making point mutations were previously described (Chang et al., 1996). The mutagenesis was conducted by following the Altered Sites protocol (Promega). All mutations were confirmed by dideoxyribonucleotide DNA sequencing (Sanger et al., 1977). A double mutation, $\beta2$(Y157S + L259S), was produced by subcloning a cDNA fragment containing the βY157S mutation into the βL259S cDNA.

The wild-type and mutant cDNAs of the $\alpha1$, $\beta2$, and $\gamma2$ subunits were linearized by *Ssp*I, which left a several hundred base pair tail for RNA stability. For cRNA synthesis, RNase-free DNA templates were prepared by treating linearized DNA with proteinase K. The capped cRNAs were then transcribed by SP6 RNA polymerase, using standard protocols. After degradation of the DNA template by RNase-free DNase I, the cRNAs were purified and resuspended in diethylpyrocarbonate-treated water. cRNA yield and integrity were examined on a 1% agarose gel.

Oocyte preparation and cRNA injection

Female *Xenopus laevis* (*Xenopus* I, Ann Arbor MI) were anesthetized by 0.2% MS-222, and ovarian lobes were surgically removed and placed in a Ca^{2+}-free incubation solution consisting of (in mM) 82.5 NaCl, 2.5 KCl, 5 HEPES, 1 $MgCl_2$, 1 Na_2HPO_4, 50 U/ml penicillin, 50 μg/ml streptomycin (pH 7.5). The lobes were cut into small pieces and digested with 0.3% collagenase A (Boehringer Mannheim, Indianapolis, IN) in the above solution at room temperature with continuous stirring until the oocytes were dispersed (1–2 h). The oocytes were then thoroughly rinsed with the above solution plus 1 mM Ca^{2+}. Stage VI oocytes were selected and incubated at 18°C.

Micropipettes for cRNA injection were pulled from borosilicate glass on a P87 horizontal puller (Sutter Instrument Co., Novato, CA), and the tips were cut with scissors to ~40 μm OD. The cRNA for each subunit was diluted 50- to 60-fold and mixed at a ratio of 1:1:1 for the α:β:γ subunits. Previous studies have indicated a fixed stoichiometry over a wide ratio of injected wild-type and mutant α, β, and γ cRNAs (Chang et al., 1996). The cRNA was injected into the oocytes with a Nanoject microinjection system (Drummond Scientific, Broomall, PA). The volume of the microinjection into each oocyte was varied from 27 to 84 nl to provide a range of expression levels. Typically, a total of 0.1–1 ng of cRNA was injected into each oocyte.

Voltage clamp

One to three days after injection, oocytes were placed in a small volume chamber (<100 μl) with a 300-μm nylon mesh support. The oocyte was continuously perfused at a rate of 150–200 μl/s with the oocyte Ringer's solution (OR2), consisting of (in mM) 92.5 NaCl, 2.5 KCl, 5 HEPES, 1 $CaCl_2$, 1 $MgCl_2$ (pH 7.5) and briefly switched to the solution (OR2) with drug (e.g., GABA, picrotoxin, etc.). GABA was obtained from Calbiochem Corp. (La Jolla, CA); picrotoxin and bicuculline were from Sigma Chemical (St. Louis, MO); gabazine (SR95531) was from RBI (Natick, MA). All drugs were prepared daily from powder, except bicuculline and gabazine, which were prepared from stock solution that was previously aliquoted and kept at −20°C.

Recording microelectrodes were formed by pulling a filamented borosilicate glass (OD = 1.0 mm and ID = 0.75 mm) with a P87 Sutter horizontal puller. The electrodes were filled with 3 M KCl and had resistances of 1–3 MΩ. The perfusion chamber was grounded through a KCl agar bridge. The standard two-electrode voltage-clamp technique was carried out using the GeneClamp 500 voltage-clamp amplifier (Axon Instruments, Foster City, CA). The current signal was filtered at 10 Hz and recorded on paper with a Gould EasyGraf chart recorder (Gould Instrument Systems, Valley View, OH). At the same time, on-line digitization of the signal at 20 Hz with 12-bit resolution was carried out by using the MacADIOS Data Acquisition Board (GW Instruments, Somerville, MA) and Igor software (Wavemetrics, Lake Oswego, OR) in conjunction with a set of macros to drive the GW board (Bob Wyttenbach, Cornell University, Ithaca, NY) in a Macintosh (Apple Computer, Cupertino, CA).

Data analysis

Dose-response relationships of the agonist or antagonist were fit with one of the following equations, using a nonlinear least-squares method:
Activation:

$$I = \frac{I_{max}}{1 + (EC_{50}/[A])^n} \quad (1)$$

Inhibition:

$$I = \frac{I_{max}}{1 + ([A]/IC_{50})^n} \quad (2)$$

where I is the peak current response at a given concentration of drug A (agonist or antagonist), I_{max} is the maximum current response, EC_{50} is the concentration of the agonist with a half-maximum activation, IC_{50} is the antagonist concentration yielding a half-maximum inhibition, and n is the Hill coefficient.

The measured holding current (at $V_m = -70$ mV) in oocytes expressing mutant receptors includes the current through the spontaneously opening channels (I_{spont}) in addition to the background leakage current of the oocyte. Because the mutant receptors had a dramatically impaired picrotoxin sensitivity, we were unable to determine the contribution of the leakge current by blocking I_{spont} with picrotoxin. Therefore, to approximate I_{spont}, the observed total holding current for the oocytes expressing the mutant receptors was corrected by subtracting the mean leakage current (at $V_m = -70$ mV) determined in oocytes expressing wild-type $\alpha1\beta2\gamma2$ GABA receptors (−18 ± 5 nA, mean ± SD, $n = 9$).

Dose-response relationships normalized to take into account the spontaneous openings of the mutant receptors as well as the maximum open probability of the wild-type receptor were simultaneously fit to the following allosteric model of activation (see Scheme III in the Discussion):

$$R = \frac{1}{1 + L(1 + ([\mathrm{GABA}]/K_R))^n/(1 + ([\mathrm{GABA}]/K_{R^*}))^n} \quad (3)$$

where K_R and K_R^* are the agonist binding affinities of the closed and open receptor, respectively, L is [R]/[R*] or the ratio of the equilibrium occupancies of the closed and open forms of the unbound receptor, and n is the maximum number of GABA molecules that can bind to the receptor (two in our case) (Edelstein and Changeux, 1996). K_R and K_R^* were free parameters for the fit but were assumed to be constant for all mutant and wild-type receptors. The L values were experimentally derived for the mutant receptors, but L was a free parameter for the wild-type receptor, because spontaneous activation in this case was not resolvable. The data from the $\alpha\beta\gamma_m$ combination were excluded because, as opposed to the other mutant combinations, I_{spont} was significantly higher than would be predicted from the EC_{50} shift. When $\alpha\beta\gamma_m$ was included in the fit, the sum of the squared errors increased dramatically, and simulation with these derived parameters indicated that the $\alpha\beta\gamma_m$ data were the major source of error. Furthermore, we derived K_R and K_R^* by a different method (see below) that was independent of the experimentally determined values of L. In this case, the K_R and K_R^* values were nearly identical to those derived from the fit in the absence of the $\alpha\beta\gamma_m$ data.

As an alternative method, we determined the affinity of the open state (K_R^*) from the following equation (Edelstein and Changeux, 1996):

$$EC_{50} = K_{R^*}(\sqrt[n]{2} - 1) \quad (4)$$

Simulations demonstrated that the EC_{50} approached a lower limit as L decreased (Fig. 6 *A, small open circles and dashed line*). Therefore, the lower bound EC_{50} was extrapolated from the EC_{50}s of the $\alpha\beta_m\gamma$, $\alpha\beta_m\gamma_m$, and $\alpha_m\beta_m\gamma$ combinations. In this manner we derived a value of 0.11 μM for K_R^*, which is similar to the value we determined in the simultaneous fit described above (0.12 μM). K_R (78.6 μM) and wild-type L (100744) were then determined from a nonlinear least-squares fit of Eq. 3 to the wild-type data. The fit converged to the same K_R and L regardless of the starting values, suggesting that the parameters were well defined by the data. For the simultaneous fit described above, we derived values of 78.5 μM and 88,934 for K_R and wild-type L, respectively. Equations 1–3 were fit using ChanFit, a home-written nonlinear least-squares iterative search program.

RESULTS

Hydrophilic substitution of the conserved M2 leucine in the α1, β2, or γ2 subunits increased the GABA sensitivity

The conserved leucine in the putative second transmembrane domain (M2) was mutated to serine in rat α1, β2, and γ2 subunits (α1L263S, β2L259S, γ2L274S). These mutants will be designated α_m, β_m, γ_m, and wild-type α1, β2, γ2 will be designated α, β, γ. cRNAs were mixed in the combinations $\alpha\beta\gamma$, $\alpha_m\beta\gamma$, $\alpha\beta_m\gamma$, $\alpha\beta\gamma_m$, $\alpha_m\beta_m\gamma$, $\alpha_m\beta\gamma_m$, $\alpha\beta_m\gamma_m$, and $\alpha_m\beta_m\gamma_m$ and injected into *Xenopus laevis* oocytes. Representative GABA-activated currents from these combinations are presented in Fig. 1 *A*, and the dose-response relationships are presented in Fig. 1 *B*. The EC_{50}s and Hill coefficients from a fit of the Hill equation to these data are provided in Table 1. All of the mutations increased the sensitivity of the receptors to GABA. The same symbols for the various receptor combinations used in Fig. 1 *B* are used throughout the manuscript.

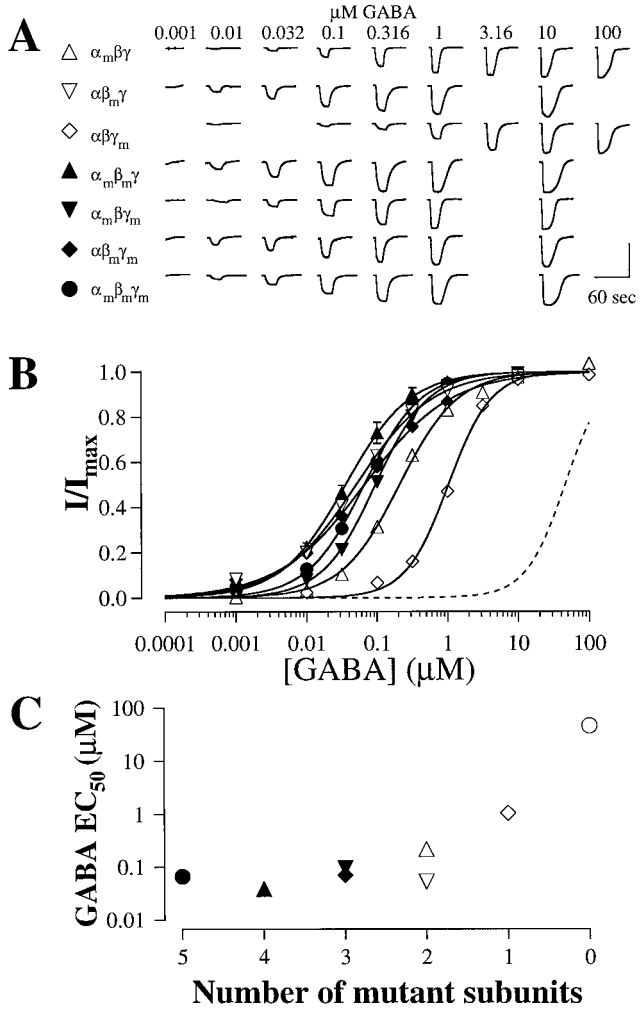

FIGURE 1 The conserved leucine mutation shifted the dose-response relationship for GABA to the left. (*A*) Representative GABA-activated currents in oocytes expressing differerent combinations of leucine mutations. Vertical scale bar: 225, 40, 190, 75, 190, 40, and 160 nA, from the top to bottom traces. (*B*) Plot of the GABA dose-response relationship for several oocytes of each combination. The solid lines are the best fit of the Hill equation (Eq. 1) to the data. The dashed line is the fit of the wild-type GABA dose-response relationship. Parameters from these fits are provided in Table 1. (*C*) Plot of the relationship between the number of mutant subunits in the pentamer and the EC_{50}, assuming a stoichiometry of two α subunits, two β subunits, and one γ subunit (Chang et al., 1996). Although the shift in EC_{50} depended on the number of mutant subunits, the shift exhibited a saturation when three or more subunits carried the leucine mutation. The open circle represents $\alpha\beta\gamma$.

Studies of muscle nACh receptors demonstrated that each additional subunit carrying a mutation at the homologous leucine residue imparted a ~10-fold increase in ACh sensitivity (Filatov and White, 1995; Labarca et al., 1995). Knowing the $\alpha\beta\gamma$ stoichiometry (two αs, two βs, and one γ; Chang et al., 1996), we can assess the shift in sensitivity as a function of the number of mutated subunits (Fig. 1 *C*). Although there was a correlation, in contrast to results from the nACh receptor, there was not a clear stepwise relation-

TABLE 1 EC_{50}, Hill coefficients, and I_{spont}/I_{GABA} for the various subunit combinations

Combination	EC_{50} (μM) (fold shift)	Hill	N	I_{spont}/I_{GABA}	N	No. of mutants*
$\alpha\beta\gamma$	45.8 ± 1.6 (1)	1.57 ± 0.04	5	NA	NA	0
$\alpha\beta\gamma_m$	1.04 ± 0.052 (44)	1.43 ± 0.07	3	0.29 ± 0.07	15	1
$\alpha_m\beta\gamma$	0.22 ± 0.02 (208)	1.04 ± 0.06	4	0.22 ± 0.03	14	2
$\alpha\beta_m\gamma$	0.052 ± 0.005 (881)	0.81 ± 0.03	5	5.89 ± 0.58	15	2
$\alpha_m\beta\gamma_m$	0.095 ± 0.003 (482)	1.15 ± 0.03	4	1.10 ± 0.15	14	3
$\alpha\beta_m\gamma_m$	0.069 ± 0.01 (664)	0.71 ± 0.02	4	25.9 ± 8.7	15	3
$\alpha_m\beta_m\gamma$	0.038 ± 0.007 (1205)	0.97 ± 0.09	5	4.51 ± 0.60	17	4
$\alpha_m\beta_m\gamma_m$	0.066 ± 0.002 (694)	1.07 ± 0.02	5	2.48 ± 0.48	19	5

Values are mean ± SEM; N = number of oocytes.
*Number of mutant subunits within the pentamer, assuming a stoichiometry of 2:2:1 for $\alpha\beta\gamma$.

ship between the number of mutant subunits and the EC_{50}. For example, the EC_{50} for the all-mutant receptor ($\alpha_m\beta_m\gamma_m$) was shifted less than that of $\alpha\beta_m\gamma$ or $\alpha_m\beta_m\gamma$ (Table 1). These data indicate a subunit nonsymmetry in either the role these leucines play in activation or in the degree of perturbation imparted by the mutation.

Hydrophilic substitution of the conserved M2 leucine in the $\alpha1$, $\beta2$, or $\gamma2$ subunit-induced spontaneous openings of the GABA receptor

In addition to the shift in GABA sensitivity, oocytes expressing mutant subunits required a larger holding current to voltage clamp the membrane at −70 mV compared to oocytes expressing the wild-type receptor. This holding current was blocked by the GABA receptor antagonist picrotoxin, indicating that it was due to spontaneously opening GABA receptors (see next section). Fig. 2 *A* is a plot of the ratio of the holding current at −70 mV in the absence of GABA (I_{spont}) to the maximum GABA-activated current (I_{GABA}) for each subunit combination. These ratios are also provided in Table 1. Although Fig. 2 *B* shows that the degree of spontaneous opening (I_{spont}/I_{GABA}) increased as a function of the number of mutant subunits in the pentamer (*dashed line*), the ratio was highest when the β subunit carried the mutation.

The spontaneously opening channels were blocked by picrotoxin

The current traces in Fig. 3 *A* show picrotoxin-mediated block of the GABA-activated (10 μM) current for the wild-type receptor. The current traces in Fig. 3 *B* are examples of the picrotoxin blockage of the holding current in oocytes expressing $\alpha_m\beta_m\gamma_m$ GABA receptors. The holding current decreased in response to picrotoxin in a dose-dependent manner. Fig. 3 *C* shows the dose dependence of picrotoxin-mediated inhibition for all receptor combinations. The IC_{50}s and Hill coefficients determined from fitting Eq. 2 to these data (*continuous lines*) are provided in Table 2. The observation that picrotoxin blocked the holding current supports our conclusion about spontaneously opening mutant GABA receptors. Furthermore, the observation that these mutations shift the picrotoxin sensitivity indicates that this leucine residue may play a role in the picrotoxin-mediated antagonism. As shown in Fig. 3 *D*, there was a marked correlation between the IC_{50} and the number of mutant subunits in the pentamer, although a comparison of the single isoform mutants ($\alpha_m\beta\gamma$, $\alpha\beta_m\gamma$, and $\alpha\beta\gamma_m$) revealed that the γ subunit mutation had the most pronounced effect on picrotoxin sensitivity.

The spontaneously opening mutant channels were inhibited by bicuculline

According to the classical view, a purely competitive inhibitor should have no intrinsic activity; it would simply occupy the binding site and prevent agonist binding. Fig. 4 *A* shows the inhibition of I_{spont} by the presumably competitive inhibitor, bicuculline, in oocytes expressing $\alpha_m\beta_m\gamma_m$ subunits. Fig. 4 *B* is a plot of the relationship between the fraction of the current blocked and the bicuculline concentration. Equation 2 was fitted to these data and yielded an IC_{50} of 1.10 ± 0.06 μM and a slope factor of 1.20 ± 0.04 (n = 3). Note that the block by bicuculline was incomplete; only 0.41 ± 0.03 of I_{spont} was inhibited. We also examined the actions of the presumed competitive antagonist gabazine (SR95531) on $\alpha_m\beta_m\gamma_m$ receptors. The IC_{50} and slope factor were 0.15 ± 0.01 μM and 1.10 ± 0.06, respectively, with a fractional block of only 0.13 ± 0.02. Thus gabazine blocks less of I_{spont} than bicuculline. These data suggest that bicuculline and gabazine can stabilize the channel in the closed state and support the view that they may not be pure competitive antagonists of the $GABA_A$ receptor, but more likely are allosteric inhibitors, as has been proposed from the actions of these compounds on alphaxalone- and pentobarbital-activated currents (Ueno et al., 1997).

The effects of a GABA binding site mutation and the conserved leucine mutation are independent

As shown in a previous study (Amin and Weiss, 1993), the binding site mutation β_2Y157S shifted the GABA dose-response curve to the right (952-fold), yielding an EC_{50} of

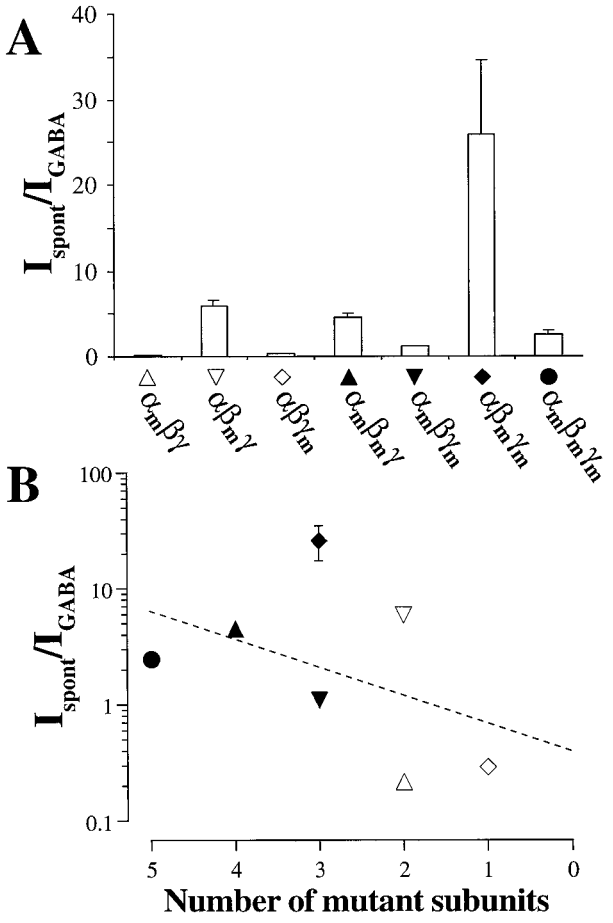

FIGURE 2 The leucine mutation created spontaneously opening GABA receptors. (*A*) cRNAs were mixed in a 1:1:1 ratio for all combinations and injected into oocytes. One day after injection, the oocytes were two-electrode voltage-clamped at −70 mV. The holding currents in the absence of GABA (I_{spont}) and the maximum GABA-activated currents (I_{GABA}) were measured and plotted as a ratio. (*B*) Plot of the relationship between the number of mutant subunits in the pentamer and I_{spont}/I_{GABA}, assuming a stoichiometry of two α subunits, two β subunits, and one γ subunit (Chang et al., 1996). The dashed line is from a linear regression to the data points. Although the degree of spontaneous opening appeared to increase as a function of the number of mutant subunits in the pentamer, the correlation was not statistically significant ($r = 0.12$, $p > 0.05$).

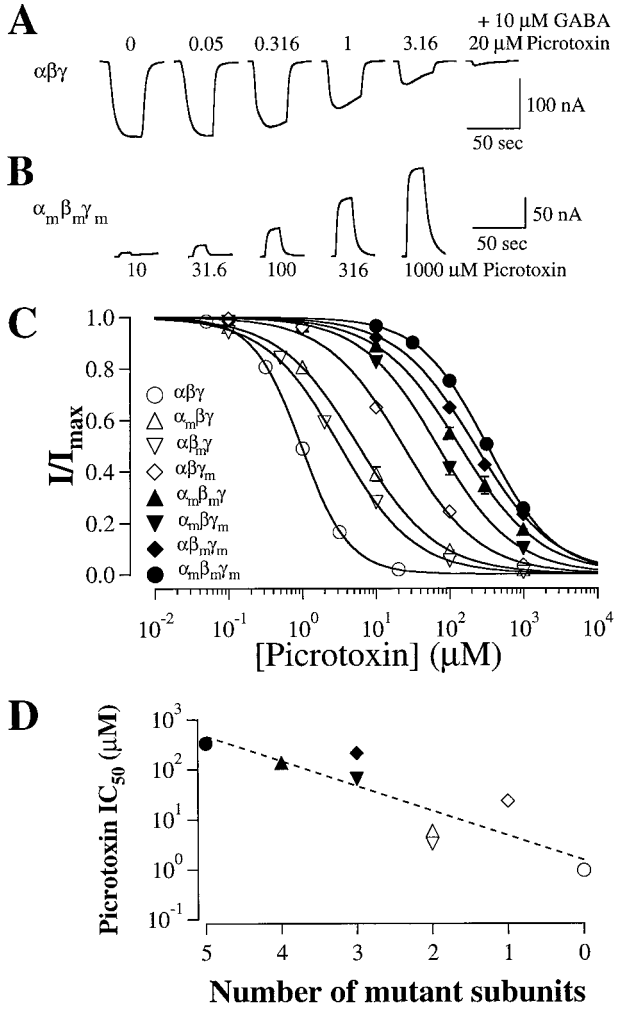

FIGURE 3 The leucine mutations impaired the picrotoxin-mediated antagonism. (*A*) GABA-activated currents (10 μM GABA) in the presence of increasing concentrations of picrotoxin. (*B*) Antagonism of the spontaneously opening $\alpha_m\beta_m\gamma_m$ GABA receptors by increasing concentrations of picrotoxin. (*C*) Average dose-response relationship for picrotoxin on all possible receptor combinations. For the wild-type receptor, the antagonism of the GABA-activated current (10 μM GABA) is plotted. For all others, the block of the current in the absence of GABA is plotted. The continuous line is the best fit of Eq. 2 to the data points. Parameters from the fits are presented in Table 2. (*D*) Plot of the picrotoxin IC_{50} as a function of the number of mutant subunits in the pentamer, assuming a stoichiometry of two α subunits, two β subunits, and one γ subunit (Chang et al., 1996). The IC_{50} increased with increasing number of mutant subunits. Also note that the γ mutation produced the largest impairment of picrotoxin sensitivity. The dashed line is from a linear regression to the data points ($r = 0.83$, $p < 0.01$).

43,580 μM. The β_2L259S mutation shifted the dose-response curve to the left (881-fold), yielding an EC_{50} of 0.052 μM (Table 1). If the effects of the two mutations were independent, the double mutant (β2Y157S + L259S) would have an EC_{50} intermediate of the two individual mutants; that is, ~47.6 μM. Fig. 5 *A* shows examples of currents in oocytes expressing $\alpha\beta$(Y157S + L259S)γ receptors in response to a range of GABA concentrations. The resting current of these oocytes was much higher than that of control oocytes, indicating that the receptors were opening spontaneously. Fig. 5 *B* plots the average fractional activation of the mutant receptor versus GABA concentration (*filled squares*). The continuous line is the best fit of the Hill equation to the data points, yielding an EC_{50} of 59.96 ± 1.39 μM and a Hill coefficient of 0.83 ± 0.07 ($n = 3$). The dashed lines are GABA dose-response relationships of $\alpha\beta$(L259S)γ receptors (*left*), $\alpha\beta$(Y157S)γ receptors (*right*), and the predicted relationship (47.6 μM), assuming an independent effect of the two mutations (*middle*). For the receptors containing both the βY157S and βL259S mutations, the observed EC_{50} of 59.96 ± 1.39 μM was very close to the predicted value of 47.6 μM, suggesting that the effects of the two mutations were independent.

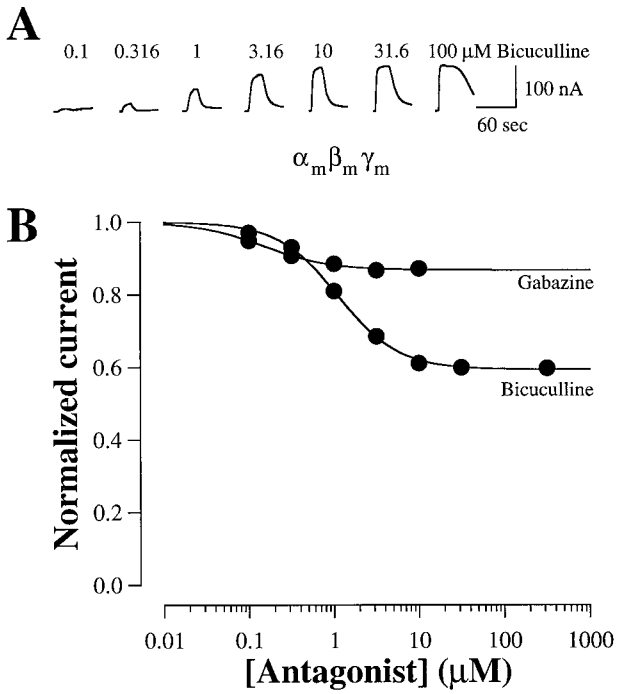

FIGURE 4 Bicuculline and gabazine inhibited the spontaneously opening GABA receptors. (*A*) Bicuculline blocked I_{spont} of $\alpha_m\beta_m\gamma_m$ in a dose-dependent manner. (*B*) The average fraction of inhibition is plotted as a function of bicuculline or gabazine concentration. The continuous line is the best fit of Eq. 2 to the data points, which gave an IC_{50} of 1.10 ± 0.06 μM and a Hill coefficient of 1.20 ± 0.04 for bicuculline (n = 3) and an IC_{50} of 0.15 ± 0.01 μM and a Hill coefficient of 1.10 ± 0.06 (n = 3) for gabazine. Bicuculline blocked 0.41 ± 0.03 of I_{spont}, whereas gabazine blocked only 0.13 ± 0.02.

TABLE 2 Picrotoxin antagonism for the wild-type and mutant GABA receptors

Combination	IC_{50} (μM)	Hill	N	No. of mutants*
$\alpha\beta\gamma$	0.96 ± 0.05	1.33 ± 0.02	4	0
$\alpha\beta\gamma_m$	23.6 ± 2.46	0.84 ± 0.01	4	1
$\alpha_m\beta\gamma$	5.91 ± 0.80	0.82 ± 0.03	4	2
$\alpha\beta_m\gamma$	3.29 ± 0.18	0.87 ± 0.02	5	2
$\alpha_m\beta\gamma_m$	65.6 ± 9.5	0.82 ± 0.01	4	3
$\alpha\beta_m\gamma_m$	215 ± 24	0.79 ± 0.01	3	3
$\alpha_m\beta_m\gamma$	137 ± 15	0.77 ± 0.02	4	4
$\alpha_m\beta_m\gamma_m$	333 ± 11	0.92 ± 0.03	5	5

Values are mean ± SEM; N = number of oocytes.
*Number of mutant subunits within the pentamer, assuming a stoichiometry of 2:2:1 for $\alpha\beta\gamma$.

DISCUSSION

Comparison to other receptors

Serine substitution of the conserved leucine created spontaneously opening receptors in both heteromeric $\alpha1\beta2\gamma2$ and homomeric $\rho1$ GABA receptors (Chang and Weiss, 1998). Unlike $\alpha1\beta2\gamma2$ GABA receptors, however, homomeric $\rho1$ spontaneously opening mutant GABA receptors were closed by low concentrations of GABA and reopened by GABA concentrations greater than 1 μM (Chang and Weiss, 1998). This difference in the two GABA receptor classes suggests either a different contribution for the leucines in receptor activation or a difference in the degree of perturbation induced by the mutation in the different subunits.

Our results from $\alpha\beta\gamma$ GABA receptors demonstrated that agonist sensitivity increased with the hydrophilic substitution of the conserved M2 leucine. This is in agreement with studies in $\alpha7$ neuronal nACh receptors (Revah et al., 1991), 5-HT_3 receptors (Yakel et al., 1993), and heteromeric muscle nACh receptors (Akabas et al., 1992; Filatov and White, 1995; Labarca et al., 1995). Hydrophilic substitution of the conserved M2 leucine also created spontaneously opening channels, in agreement with observations in $\alpha1\beta1$ GABA receptors (Tierney et al., 1996) and the α subunit of muscle nicotinic acetylcholine receptors (Auerbach et al., 1996).

In the muscle nACh receptor, substitution of each additional subunit imparted an additional ~10-fold increase in agonist sensitivity (Filatov and White, 1995; Labarca et al., 1995). Thus, in terms of the shift in EC_{50}, the effects of the mutations were approximately symmetrical with respect to the five subunits. In a previous study (Chang et al., 1996) we observed that the effects of mutating the two α subunits or two β subunits in $GABA_A$ receptors were multiplicative in terms of the EC_{50} shift (additive in terms of the free energy), although the contributions of the α_m, β_m, and γ_m subunits were nonsymmterical. In the present study, when combinations of mutant subunit classes were coexpressed, we did not observe a strong relationship between the number of mutant subunits and the EC_{50} shift, as observed for the nACh receptor. For example, the EC_{50} of the combinations $\alpha_m\beta_m\gamma$ and $\alpha\beta_m\gamma_m$ were decreased more than the triple mutant $\alpha_m\beta_m\gamma_m$. One possibility is that the relationship between the number of mutant subunits and the EC_{50} (as well as I_{Spont}/I_{GABA}) depends upon whether the mutant subunits are neighbors within the pentamer; that is, the effects of the mutations on neighboring subunits were not completely independent.

If the hydrophobic interactions between the conserved M2 leucines were important for maintaining the receptor in the closed state, as has been proposed (Unwin, 1995), the weakening of this interaction by substitution with a less hydrophobic amino acid would reduce the energy barrier for channel opening. Our results show that substitution of the conserved M2 leucine with serine in the α, β, or γ subunit increases the GABA sensitivity and creates spontaneously opening GABA receptors. This is consistent with the hypothesis that the conserved M2 leucine in all five presumed subunits may be important for GABA receptor gating and the mutation either weakens the contacts that hold the channel closed or strengthen the contacts that hold the channel open. Mutation of a nearby threonine residue in the $\rho1$ M2 domain (Pan et al., 1997) or a nearby leucine in the nACh M2 domain (Akabas et al., 1992) could also produce constitutively open channels, suggesting that other M2 residues in addition to the conserved leucine may also play a

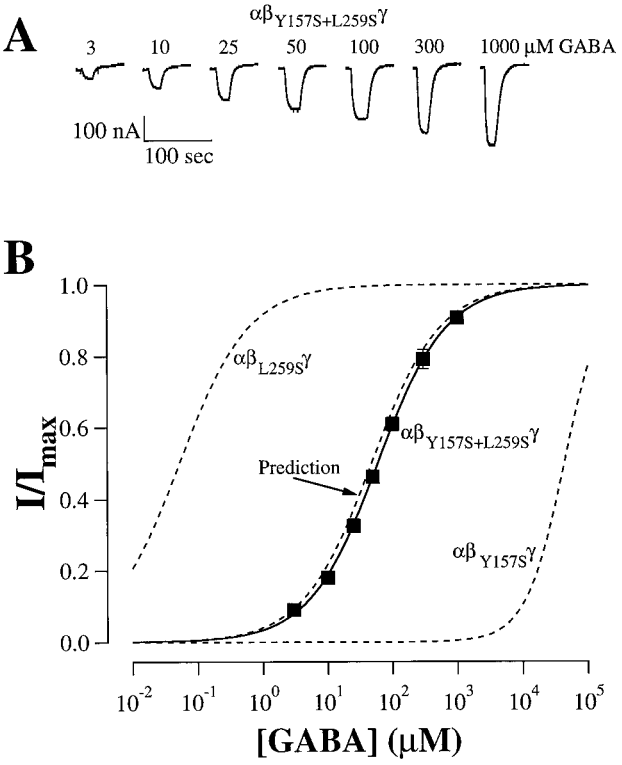

FIGURE 5 The effects of a binding site mutation and the conserved leucine mutation were independent. (*A*) Examples of GABA-induced currents in oocytes expressing $\alpha\beta$Y157S + L259Sγ GABA receptors. These receptors also demonstrated spontaneous opening. Application of GABA induced a greater inward current in a dose-dependent manner. (*B*) Plot of the average GABA dose-response relationship of the oocytes expressing $\alpha\beta$Y157S + L259Sγ GABA receptors (■). The continuous line is the best fit of the Hill equation to the data points with an EC_{50} of 59.96 ± 1.39 μM and a Hill coefficient of 0.83 ± 0.07 (n = 3). The leftmost dashed line is the dose-response relationship for $\alpha\beta_{L259S}\gamma$, and the rightmost dashed line is the dose-response relationship for the binding site mutant $\alpha\beta_{Y157S}\gamma$ (Amin and Weiss, 1993). The middle dashed line is the predicted dose-response relationship, assuming the effects of the binding site and leucine mutations were independent (47.6 μM).

role in receptor activation. Although this study is unable to assign the gate to the conserved M2 leucine as has been postulated (Unwin, 1995), our results suggest that this highly conserved leucine may play an important role in the gating of heteromeric $\alpha\beta\gamma$ GABA receptors.

Effects of antagonists

Mutation of this conserved leucine in any one of the three subunit isoforms impaired the antagonism by picrotoxin. In terms of the effects of the mutation in each of the three subunits, the rank order was different from that for the shift in GABA EC_{50} and spontaneous opening. It is not possible to equate this order with the degree of contribution of this leucine in the actions of picrotoxin, because the mutations could disrupt the structure in the three subunits to different degrees. For example, all subunits could contribute equally to the picrotoxin binding site, but the mutation may impart a greater structural change in the γ subunit. There was, however, a significant correlation between the number of subunits carrying the leucine mutation and the shift in picrotoxin sensitivity, although the contributions were not a product of the individual shifts. For example, the mutation in the γ subunit, of which there is only one copy in the pentamer (Chang et al., 1996), imparted a greater shift in picrotoxin sensitivity of the spontaneously opening receptors (IC_{50} = 23.6 ± 2.5 μM) than either α_m (IC_{50} = 5.91 ± 0.80 μM) or β_m (IC_{50} = 3.29 ± 0.18 μM), for which the pentamer contains two copies of each.

Other residues have been identified in the M2 domain that also impair the actions of picrotoxin in both GABA (Enz and Bormann, 1995; French-Constant et al., 1993; Gurley et al., 1995; Wang et al., 1995; Zhang et al., 1995; Zhang et al., 1994) and glycine (Pribilla et al., 1992) receptors. In addition, cysteine scanning mutagenesis demonstrated that picrotoxin protected $pCMBS^-$ modification of αVal^{257C} but not αThr^{261C} (Xu et al., 1995), the fourth and eighth residues from the presumed start of TM2. The conclusion was that picrotoxin was acting at the level of αVal^{257}, allowing access of the modifying reagent to the more extracellular αThr^{261}. The leucine residue we have mutated is even more extracellular than αVal^{257} and αThr^{261}, although all three residues are presumed to be exposed to the channel lumen (Xu and Akabas, 1996). Because our leucine mutation altered the gating kinetics of the receptor, it is possible that this perturbation had a secondary effect on the actions of picrotoxin, and therefore these data do not allow us to distinguish between an allosteric or pore-blocking mechanism for picrotoxin (see Discussion in Zhang et al., 1994).

According to the traditional view, a competitive antagonist should simply occupy the binding site for the agonist and have no intrinsic activity on its own. We therefore expected that if bicuculline were competitive it would have no effect on the spontaneously opening receptors. Surprisingly, the spontaneously opening mutant $\alpha\beta\gamma$ GABA receptors were inhibited by the $GABA_A$ receptor competitive antagonist bicuculline. Thus, in the strictest sense, bicuculline is not a pure competitive antagonist, but rather acts in an allosteric manner (Ueno et al., 1997). In this scenario, bicuculline would bind with greater affinity to the resting than the open state, thereby stabilizing the closed state of the channel.

Activation mechanism

We can begin to consider our results in terms of the following simple activation mechanism for the wild-type receptor (Del Castillo and Katz, 1957):

$$\begin{array}{ccccc} R & \leftrightarrow & AR & \leftrightarrow & A_2R \\ & & & & \updownarrow \\ & & & & A_2R^* \end{array} \qquad \text{(I)}$$

where the receptor (R) can bind an agonist molecule (A) to form the complex AR and bind a second agonist molecule to form the complex A_2R, from which it can undergo a confomational change and open (A_2R^*). For the mutant receptors that open in the absence of GABA, we must add a transition from the unbound closed state (R) to an unbound open state (R*):

$$\begin{array}{ccccc} R & \leftrightarrow & AR & \leftrightarrow & A_2R \\ \updownarrow & & & & \updownarrow \\ R^* & & & & A_2R^* \end{array} \qquad \text{(II)}$$

In the absence of agonist, the receptors are at equilibrium between R and R*. In the presence of agonist, the receptors would open by the normal activation pathway ($R \rightarrow AR \rightarrow A_2R \rightarrow A_2R^*$). For this mechanism to describe our data (e.g., increased agonist sensitivity), the mutations must also cause alterations in the binding affinity. This is not consistent with the results from $\alpha\beta$(Y157S + L259S)γ receptors, which suggested that the effects of the binding site and conserved leucine mutations were independent. Alternatively, we could consider the activation in terms of the following, more general, allosteric Monod-Wyman-Changeux activation mechanism (Changeux and Edelstein, 1998; Colquhoun, 1973; Edelstein and Changeux, 1996; Karlin, 1967; Monod et al., 1965):

$$\begin{array}{ccccc} R & \overset{K_R}{\longleftrightarrow} & AR & \overset{K_R}{\longleftrightarrow} & A_2R \\ {\scriptstyle L}\updownarrow & & {\scriptstyle Lc}\updownarrow & & {\scriptstyle Lc^2}\updownarrow \\ R^* & \underset{K_{R^*}}{\longleftrightarrow} & AR^* & \underset{K_{R^*}}{\longleftrightarrow} & A_2R^* \end{array} \qquad \text{(III)}$$

As for Scheme II, in the absence of GABA, the receptor is in equilibrium between R and R*. There is evidence that nACh receptors can open in the absence of agonist, giving further experimental credence to this allosteric activation mechanism (Jackson, 1984, 1986). K_R and K_R^* are the agonist binding affinities of the closed and open receptor, respectively; L is [R]/[R*] or the ratio of the equilibrium occupancies of the closed and open forms of the unbound receptor; and c is K_R^*/K_R. As originally proposed for this allosteric model, the open state has a higher affinity for agonist than the closed state ($K_R^* < K_R$), and therefore this model predicts that the mutant receptors with a lower L (greater degree of spontaneous opening) would be more sensitive to agonist (lower EC_{50}). As shown in Fig. 6 *A*, there was a strong correlation between L and the EC_{50} for the experimental data, supporting such an allosteric activation mechanism.

The symbols in Fig. 6 *B* replot the dose-response relationships for the mutant receptors (as in Fig. 1 *B*), but in this case the plot takes into account the spontaneous opening; that is, the intercept of the ordinate is the fraction of receptors that are open in the absence of GABA. These wild-type and mutant dose-response relationships were simultaneously fitted with Eq. 3 (based on Scheme III) to derive K_R, K_R^*, and wild-type L. The L values for the mutant receptors were experimentally determined. The thick solid lines in Fig. 6 *B* represent the binding curves of the open and closed states, respectively, and the dashed lines are the

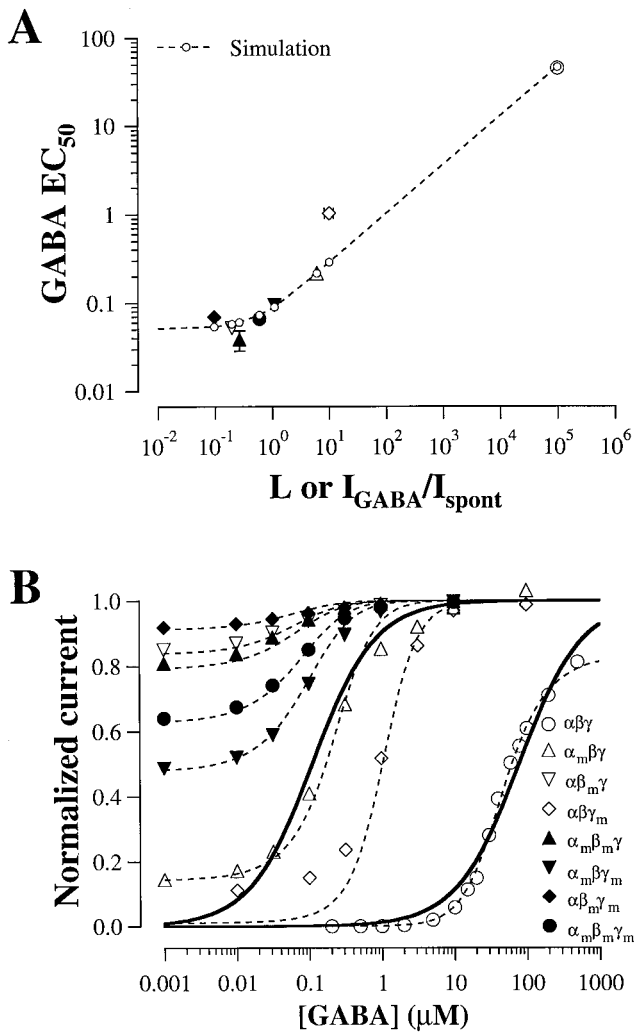

FIGURE 6 An allosteric mechanism can describe the activation of the wild-type and mutant receptors. (*A*) The large symbols are a plot of L (I_{GABA}/I_{spont}) versus the EC_{50} of the various receptor combinations. The small open circles are the prediction for Scheme III with $K_R = 78.5\ \mu M$, $K_R^* = 0.12\ \mu M$. The dashed line connects the small open circles. (*B*) The symbols are a plot of the dose-response relationship for the various mutant receptor combinations (as in Fig. 1 *B*), but in this case taking into account the spontaneous opening; that is, the intercept of the ordinate is the fraction of receptors that are open in the absence of GABA. The two thick solid lines represent the binding functions of the open (*left*) and closed (*right*) states with affinities of 0.12 μM and 78.5 μM, respectively. The dashed lines are the predicted dose-response relationships for Scheme III, using parameters determined from the simultaneous fit of Eq. 3 to these data. The allosteric activation mechanism gave an excellent description of the activation of the wild-type and mutant receptors, except for $\alpha\beta\gamma_m$, which exhibited a greater I_{spont} than that predicted from the shift in EC_{50} and was therefore not included in the fitting process (see Materials and Methods). For the purpose of display, the dashed line through the $\alpha\beta\gamma_m$ data points was generated using an L value (94.4) that was adjusted to describe the experimentally observed $\alpha\beta\gamma_m$ dose-response curve.

predictions of Scheme III. This allosteric mechanism, with constant K_R and K_R^*, gave an excellent description of the activation of the mutant GABA receptors. This further supports a role for this leucine residue in receptor gating. For the wild-type receptor, Scheme III and the estimated values of K_R, K_R^*, and L predict a P_{open} of 9.9×10^{-6}, 0.007, and 0.84 from the R, AR, and A_2R states, respectively. Therefore, entry into R* and AR* is negligible, and wild-type receptors essentially activate via Scheme I. For the spontaneously opening mutant receptors, however, L is significantly lower than in the wild-type receptor, and thus the channel readily enters states R* and AR*.

Based on these data, we would conclude that an allosteric mechanism such as that in Scheme III is a reasonable working hypothesis for the activation of the $\alpha 1\gamma 2\beta 2$ GABA receptor. Normally, spontaneous openings in the absence of GABA are rare, and the wild-type receptor exhibits a linear mechanism of activation (Scheme I). It is the mutation-induced destablization of the closed state that revealed the underlying allosteric activation mechanism. It is worth testing whether such an allosteric mechanism for the $GABA_A$ receptor, via alterations in L, might account for the actions of select GABA receptor modulators.

This research was supported by National Institutes of Health grants NS36195 and NS35291.

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