On the Mechanism of Alleviation by Phenobarbital of the Malfunction of an Epilepsy-Linked $GABA_A$ Receptor†

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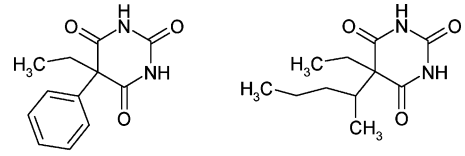
ABSTRACT: A mechanism for the alleviation of the malfunction of a mutated ($\gamma 2^{K289M}$) epilepsy-linked γ-aminobutyric acid (GABA) neurotransmitter receptor by phenobarbital is presented. Compared to the wild-type receptor, the GABA-induced current is considerably reduced in the mutated ($\alpha 1\beta 2\gamma 2^{K289M}$) epilepsy-linked $GABA_A$ receptor [Baulac, S., Huberfeld, G., Gurfinkel-An, I., Mitropoulou, G., Beranger, A., Prud'homme, J. F., Baulac, M., Brice, A., Bruzzone, R., and LeGuer, E. (2001) *Nat. Genet. 28*, 46−48]. This is due to an impaired GABA-induced equilibrium between the closed- and open-channel forms of the receptor [Ramakrishnan, L., and Hess, G. P. (2004) *Biochemistry 43*, 7534−7540]. We report that a barbiturate anticonvulsant, phenobarbital, alleviates the effect of this mutation. Transient kinetic techniques with a millisecond-to-microsecond time resolution and the wild-type and mutated receptors recombinantly expressed in mammalian HEK293T cells were used. The efficacy of phenobarbital in potentiating currents elicited by a saturating concentration of GABA is about 3 times higher for the mutated receptor than for the wild type. The results indicate that phenobarbital alleviates the malfunction of the mutated receptor by increasing its channel-opening equilibrium constant ($\Phi^{-1} = k_{op}/k_{cl}$) by about an order of magnitude. Phenobarbital changes the channel-opening rate constant (k_{op}) by less than 2-fold but decreases the channel-closing rate constant (k_{cl}) 8-fold. The dissociation constant of GABA is unaffected. The experiments also indicate that at saturating concentrations of GABA the mutated ($\gamma 2^{K289M}$) form of the $\alpha 1\beta 2\gamma 2$ $GABA_A$ receptor is well suited for a rapid and simple screening of positive allosteric modulators of the receptor.

Neurotransmitter-activated ion channels are integral membrane proteins that mediate signal transduction between $\sim 10^{12}$ neurons in the mammalian brain and at neuromuscular junctions (*1*, *2*). The type A γ-aminobutyric acid receptor ($GABA_A$ receptor)[1] is one of these neurotransmitter-activated ion channels. It mediates inhibitory neurotransmission in the mammalian central nervous system. The receptor is an oligomeric complex of five subunits (*3*), in which the Cl^--conducting channel is controlled by binding sites for GABA, benzodiazepines, barbiturates, and other ligands (reviewed in ref *4*). The most abundant subtype of the $GABA_A$ receptor in the mammalian brain is composed of two $\alpha 1$ subunits, two $\beta 2/3$ subunits, and one $\gamma 2$ subunit, although specific brain regions may contain different subtypes of subunits (reviewed in ref *5*).

The function of the $GABA_A$ receptor is positively modulated by a variety of anticonvulsants (e.g., benzodiazepines, barbiturates, and certain neurosteroids), anesthetics, and antianxiety drugs (reviewed in refs *4*, *6*, and *7*). An anticonvulsant barbiturate, phenobarbital (phenobarbitone) (**1**), is commonly used to treat epilepsy (*8*, *9*) and childhood febrile seizures (*10*, *11*). Phenobarbital and other barbiturates [e.g., pentobarbital (**2**)] have long been known to augment GABA-elicited currents and prolong inhibitory postsynaptic potentials (IPSPs) in nerve cells (*12*−*15*). Fluctuation noise analysis (*13*) and single-channel current-recording (*16*, *17*) studies revealed some important aspects of the modulation of the function of the $GABA_A$ receptor by phenobarbital. An increase in the mean open time of the channel and an increase in the mean duration of bursts of open channels were observed. Similar observations have been made with another barbiturate, pentobarbital (*17*−*21*), and with other classes of allosteric modulators of the $GABA_A$ receptor, such as neurosteroids (*22*−*24*). Arguments have been made that barbiturates reduce the channel-closing rate constant (*16*) or, alternatively, increase the relative rate for forming a long-duration open state (*17*, *18*). Changes in the kinetics of receptor desensitization were implicated as well (*21*).

Chart 1



phenobarbital **(1)** pentobarbital **(2)**

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[1] Abbreviations: cDNA, complementary DNA; αCNB, α-carboxy-*o*-nitrobenzyl; GABA, γ-aminobutyric acid; $GABA_A$ receptor, γ-aminobutyric acid receptor, type A; EC_{50}, concentration of a ligand at which 50% of the maximum effect is observed; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; IPSP, inhibitory postsynaptic potential; LaPP, laser-pulse photolysis; nAChR, nicotinic acetylcholine receptor; PhB, phenobarbital.

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Detailed single-channel measurements, conducted recently by Steinbach and co-workers (*20*, *24*), revealed that when coapplied with GABA, an anesthetic-type barbiturate, pentobarbital, and an anesthetic-type neurosteroid, (3α,5α,17*β*)-3-hydroxyandrostane-17-carbonitrile, each produce 2−5-fold increases in the mean open duration of the channel of the α1*β*2*γ*2L $GABA_A$ receptor. Additionally, these authors concluded from an analysis of the closed times between openings of single channels that the channel-opening rate is not changed by either pentobarbital or (3α,5α,17*β*)-3-hydroxyandrostane-17-carbonitrile. For the generally accepted channel-opening process of the receptor, the following mechanism has been proposed (*25*):

$$R + L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} RL \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} RL_2 \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} \overline{RL}_2 \text{ (open)}$$

According to this mechanism, the closed time between openings of single channels of the $GABA_A$ receptor reflects the closed times of three different closed states of the receptor: the receptor without ligand bound (R) and the receptor with one (RL) and two (RL_2) ligand molecules bound. In addition to the channel-opening rate constant (k_{op}), four other rate constants, namely, k_{12}, k_{21}, k_{23}, and k_{32}, determine the closed times between openings of single channels (*26*). In contrast, transient kinetic investigations (reviewed in ref *27*) allow one to measure the first-order rate constant for the current rise, k_{obs}, determined by using whole-cell current recordings (*28*). k_{obs} is a measure of the rate constant for channel closing (k_{cl}) and channel opening (k_{op}) and depends on the concentration (L) and the dissociation constant (K_1) of the activating ligand. These constants can conveniently be evaluated from the ligand concentration dependence of k_{obs} (reviewed in ref *27*):

$$k_{obs} = k_{cl} + k_{op}[L/(L + K_1)]^2 \quad (1)$$

The mechanism of action of the anesthetic-type barbiturate pentobarbital has been the subject of many detailed kinetic studies using fluctuation noise analysis (*29*−*31*), single-channel current recording (*17*−*21*), IPSP recording (*32*), and rapid-mixing quench-flow $^{36}Cl^-$ influx measurements (*33*). However, much less attention has been paid to anticonvulsant-type barbiturates such as phenobarbital. The effect of phenobarbital on an epilepsy-linked mutated $GABA_A$ receptor has not previously been investigated.

A mutation of Lys^{289} to Met (K289M) in the *γ*2 subunit of the $GABA_A$ receptor is genetically linked to a form of idiopathic epilepsy, generalized epilepsy with febrile seizures plus (GEFS+) (*34*). This mutation greatly reduces currents elicited by GABA in the $\alpha1\beta2\gamma2^{K289M}$ $GABA_A$ receptor as compared to the normal (wild-type) receptor (*34*). A recent report (*35*) indicates that this mutation does not affect substantially either the single-channel conductance or surface expression of the receptor. Additional experiments indicated that the functional impairment is due to an unfavorable channel-opening equilibrium of the mutated receptor (*36*). Therefore, the mutated receptor, expressed in a mammalian cell line, presents a good system in which to determine the effects of positive allosteric modulators on the channel-opening equilibrium constant (k_{op}/k_{cl}). Compounds that increase only the affinity of the receptor for GABA are expected to potentiate currents elicited by low (subsaturating) concentrations of GABA but would not show an effect at high (saturating) concentrations of GABA. On the contrary, compounds that increase the channel-opening equilibrium constant are expected to potentiate the currents at both low and high concentrations of GABA.

Here we report investigations of the effects of phenobarbital on the kinetics of an epilepsy-linked mutated ($\gamma2L^{K289M}$) $GABA_A$ receptor (*34*), composed of two α1 subunits, two *β*2 subunits, and one *γ*2L subunit. Transient kinetic techniques with millisecond-to-microsecond time resolution, including the laser-pulse photolysis (LaPP) technique (*37*, *38*; reviewed in ref *27*) using α-carboxy-*o*-nitrobenzyl- (αCNB-) caged GABA (*39*) as the photolabile precursor of the activating ligand, in combination with whole-cell current recordings (*28*), were employed to resolve effects of phenobarbital on the affinity of the receptor for GABA, on receptor desensitization, and on the channel-opening equilibrium constant $\Phi^{-1} = k_{op}/k_{cl}$. A preliminary report of some of the experiments has appeared (*40*).

MATERIALS AND METHODS

Chemicals. Phenobarbital (sodium salt) and GABA were obtained from Sigma (St. Louis, MO). Other chemicals were of the highest purity available and were obtained from Sigma, Fisher Scientific, or EM Science. Cell culture media and supplements were purchased from Invitrogen/Gibco (Grand Island, NY); cell culture plastic ware was purchased from Corning Inc. (Corning, NY) and Becton Dickinson Labware (Bedford, MA). αCNB-caged GABA (*39*) was a kind gift from Dr. Kyle R. Gee (Molecular Probes, Inc., Eugene, OR).

Cell Culture, Recombinant DNA Manipulations, and Transfection. HEK293T cells were cultured at 37 °C in Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin G, and 0.1 mg/mL streptomycin, in a humidified atmosphere of 5% CO_2 and 95% air. Wild-type rat $GABA_A$ receptor cDNAs in the pRK-5 expression vector were kindly provided by Professors H. Lüddens (Johannes Gutenberg-Universität, Mainz, Germany) and P. H. Seeburg (Max-Plank-Institut für medizinische Forschung, Heidelberg, Germany). The pRK-5 expression vector for the *γ*2L subunit cDNA carrying the K289M mutation was previously prepared in this laboratory (*36*). The pGreen Lantern plasmid encoding the green fluorescent protein with a S65T mutation (GFP-S65T) was from Life Technologies, Inc. (Gaithersburg, MD). The S65T mutation improves the fluorescence properties of GFP (*41*). Amplification in *Escherichia coli* and purification of these vectors using a QIAfilter Plasmid Mega Kit (Qiagen Inc., Valencia, CA) was performed according to the manufacturer's recommendations. Transient transfection of HEK293T cells was performed using PolyFect transfection reagent (Qiagen Inc., Valencia, CA). Following Boileau et al. (*42*) and Baulac et al. (*34*), we used a 1:1:10 weight ratio of cDNAs for α1, *β*2, and *γ*2L subunits of the receptor. For our DNA vectors, the 1:1:10 weight ratio approximately corresponds to a 1:1:10 molar ratio. Usually, 2.0 or 3.4 *μ*g of total DNA and 20 *μ*L of PolyFect reagent were used, and transfection was carried out for 6 h. After this time, the PolyFect-containing medium was replaced with fresh medium. Cells were used for measurements 24−36 h after transfection.

The HEK293T cell line is a highly transfectable derivative of the HEK293 (human embryonic kidney) cell line (*43*). Using an activated-dendrimer transfection reagent, PolyFect, we routinely achieve a transfection efficiency of 80% with the HEK293T cells, as judged by the percentage of cells expressing green fluorescent protein (GFP-S65T). With the parent HEK293 cells, we obtain a transfection efficiency of only 30−40%.

Whole-Cell Current Recordings. The whole-cell currents were recorded with use of an Axopatch 200A integrating patch clamp amplifier and Clampex 9 data acquisition software (both from Axon Instruments, Foster City, CA) as described by Hamill et al. (*28*). Cells expressing the receptors were visualized by GFP fluorescence using an Olympus IX51 inverted microscope with a Xe light source. The composition of the intracellular buffer was 140 mM CsCl, 2 mM $MgCl_2$, 10 mM ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 mM tetraethylammonium chloride, and 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES)−CsOH, pH 7.4. The composition of the extracellular buffer was 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 10 mM HEPES−NaOH, pH 7.4. Borosilicate glass (World Precision Instruments, Inc., Sarasota, FL) pipets (electrodes) were pulled on a PIP5 two-stage vertical pipet puller (HEKA Elektronik, Lambrecht/Pfalz, Germany) and fire-polished on a MF830 microforge (Narishige, Tokyo, Japan); the electrode resistance was typically 2−3 MΩ. The series resistance was typically 3−5 MΩ and was up to 70% compensated electronically using the amplifier's circuit. All measurements were carried out at an ambient (22−24 °C) temperature and a transmembrane voltage of −60 mV.

Rapid Application of Ligand Solutions and Correction of Observed Current. The cell-flow method used for rapid ligand application has been described in detail (*44*). Briefly, while in the whole-cell current-recording mode (*28*), a single cell without processes was lifted off the surface of a cell culture dish and placed at a distance of *ca*. 200 μm from the porthole of a U-tube. The cell was perfused with neurotransmitter (GABA) solution in the presence or absence of phenobarbital. The U-tube flow device was connected to a peristaltic pump. The linear flow rate of solutions emerging from the U-tube porthole was typically ∼1.5 cm/s in cell-flow experiments and ∼0.75 cm/s in laser-pulse photolysis (LaPP) experiments. Cells were allowed to recover after each measurement for at least 2−3 min, a time sufficient for complete resensitization (reactivation) of the receptors. With each cell used in the measurements, the current maximum in the presence of 300 μM GABA was determined in order to be able to compare the results obtained with many different cells.

In cell-flow experiments, the observed current maximum was corrected for receptor desensitization that occurs during the observed rise time of the current as previously described (*44*). In laser-pulse photolysis experiments, a current correction was not necessary.

Laser-Pulse Photolysis of Caged GABA. These experiments were performed using an approach developed previously (*37*, *38*) for the muscle-type nicotinic acetylcholine receptor (nAChR), with certain modifications. The U-tube flow device was used to equilibrate the cell for 0.4 s with αCNB-caged GABA (*39*) in the presence or absence of 1 mM phenobarbital. Caged GABA (50 μM) was used to release low concentrations of free GABA, and 100 or 150 μM caged GABA was used to release high concentrations of free GABA. Under our experimental conditions, inhibition of the α1*β*2*γ*2L $GABA_A$ receptor by the αCNB-caged GABA is negligible. Free GABA was photolytically liberated by a pulse of UV light (*λ* 337 nm) of 10 ns duration and 10−13 mJ/mm^2 unattenuated energy produced by a COMPex 102 laser (Lambda Physik AG, Göttingen, Germany) operated in the N_2 mode. The pulse of laser light was delivered to the cell and an area around it *via* a fused-silica optical fiber (Superguide G, core diameter 200 μm; Fiberguide Industries, Stirling, NJ). The resulting current (filtered at 2 or 5 kHz using a low-pass four-pole Bessel filter and digitized at 10 kHz) was recorded for 100 or 200 ms. To prevent washout of the liberated GABA by the solution flowing from the U-tube, the peristaltic pump was stopped just before the laser pulse. The laser, the peristaltic pump, and the solenoid valves were controlled using a custom-built trigger interface and a Digidata 1322A data acquisition system. To determine that the cell had not been damaged by the UV light, a standard concentration of free GABA (300 μM) was applied before and after the laser pulse using the cell-flow technique (*44*), and the resulting current amplitude was measured. In all experiments presented in this paper, the observed currents before and after the laser pulse were about the same (±10%). Normalization of the currents obtained in LaPP experiments to a current elicited by an application of a standard concentration of free GABA (300 μM) was used to determine the concentration of GABA released in the LaPP experiment, as described before for LaPP experiments with the nAChR and αCNB-caged carbamoylcholine (*38*). For each data point recorded in Figure 4B, a different cell was used.

Fitting the Experimental Data to the Equations. All linear and nonlinear least squares regressions and plotting of the experimental results were performed using Origin 3.5 and Origin 7.0 data analysis software (OriginLab Corp., Northampton, MA) running on a Dell Optiplex GX240 personal computer (Dell Inc., Round Rock, TX).

RESULTS

Effects of Phenobarbital on Wild-Type and Mutated ($\gamma 2L^{K289M}$) $GABA_A$ Receptors. In the case of both the wild-type and the mutated receptor in the presence of a high (saturating) concentration of GABA (300 μM), 30−1000 μM phenobarbital produces concentration-dependent potentiation of the current (Figures 1 and 2A). The degree of potentiation by phenobarbital observed with the mutated receptor is about 3 times higher than with the wild-type receptor. As can be seen in Figure 1, phenobarbital substantially alleviates the functional impairment of the receptor caused by the epilepsy-linked $\gamma 2L^{K289M}$ mutation. The degree of potentiation of the wild-type recombinant α1*β*2*γ*2L $GABA_A$ receptor by phenobarbital in the presence of 300 μM GABA (15−17%, Figure 2A) compares well with previous results of about 20% (*45*) and 14% (*46*) obtained with wild-type native $GABA_A$ receptors at high (saturating) concentrations of GABA. Concentration-dependent potentiation by phenobarbital was also observed at a low (subsaturating) concentration of GABA (6 μM) (Figure 2B).

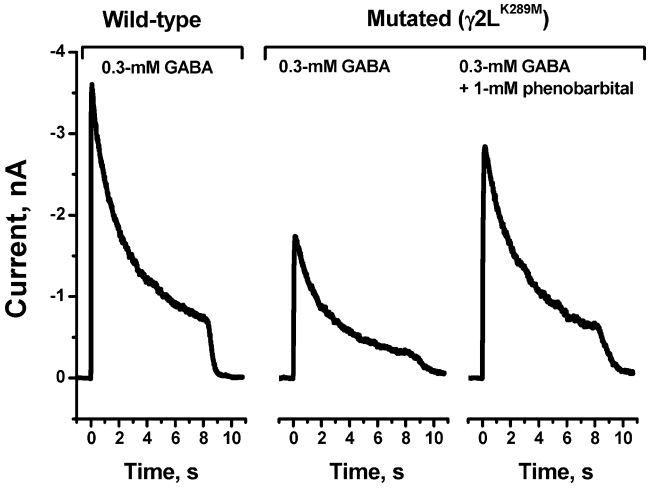

FIGURE 1: Alleviation by phenobarbital of the current reduction due to the epilepsy-linked $\gamma 2L^{K289M}$ mutation (*35*) of the $\alpha 1\beta 2\gamma 2L$ $GABA_A$ receptor. These representative current traces were obtained by the cell-flow technique (*44*) (pH 7.4, 22−24 °C, −60 mV).

At higher phenobarbital concentrations (e.g., 3 mM), the degree of potentiation of the mutated and wild-type receptors declines (Figure 2), and small transient inward currents (called "rebound" or "tail" currents) are observed at the end of GABA plus phenobarbital applications (data not shown). Such "rebound" currents have been observed previously (for example, see ref *19*) and are consistent with the rapid unbinding of phenobarbital from a low-affinity inhibitory site.

The relative current amplitudes (*N*) in Figure 2 are expressed as a fraction of the current elicited by a standard concentration of GABA (300 μM). The dependence of potentiation on the concentration of phenobarbital was evaluated using eq 2 (modified from ref *19*):

$$N = N_0 + \frac{N_{MAX} - N_0}{1 + (K_P/[PhB])^h} \quad (2)$$

N is the relative current amplitude that is observed at given concentrations of GABA and phenobarbital. N_0 is the relative current amplitude observed at a given concentration of GABA in the absence of phenobarbital (0.053 at 6 μM GABA and 1 at 300 μM GABA). N_{MAX} is the maximum relative current amplitude in the presence of phenobarbital. [PhB] is the concentration of phenobarbital, and K_P the apparent dissociation constant of phenobarbital for potentiation of GABA-elicited current. The power coefficient *h* is the Hill coefficient (*47*) and was about 1.5−1.9 in our experiments. Previous studies reported *h* values for various native and recombinant $GABA_A$ receptors of 1.5 (*19*) and 2.2 (*48*) for phenobarbital potentiation and 2 (*34*), 1.6 (*19*), 1.5 (*48*), and 1.9 (*20*) for pentobarbital potentiation.

Using the data in Figure 2A and eq 2, we determined that the value of the apparent dissociation constant for phenobarbital, K_P(app), at a high concentration of GABA (300 μM) is 0.23 ± 0.06 mM. It can be seen from the data in Figure 3 that 300 μM GABA is essentially a saturating concentration for the mutated receptor (Figure 2A). In the presence of a low concentration of GABA (6 μM), the value of K_P(app) for phenobarbital and the mutated receptor is 1.08 ± 0.32 mM, as determined from the measurements shown in Figure 2B and use of eq 2.

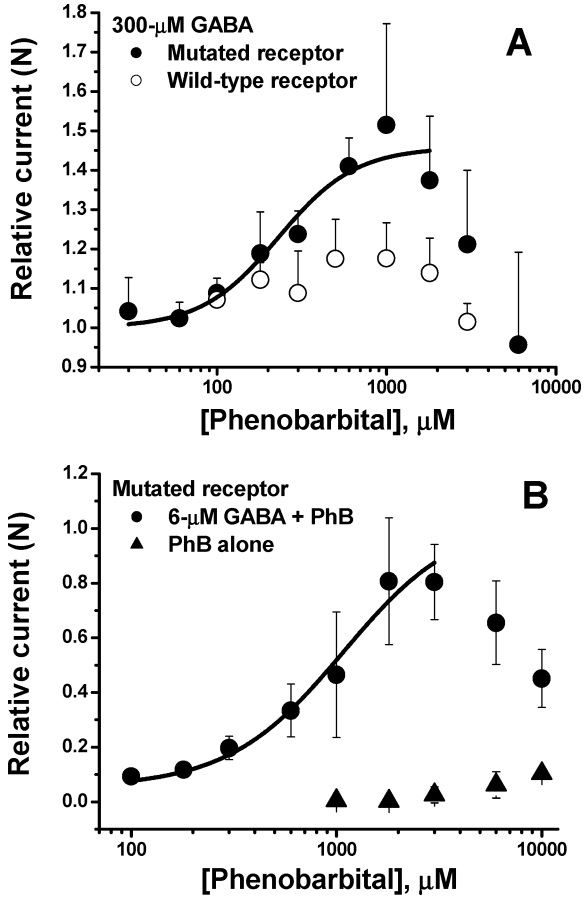

FIGURE 2: Potentiation of the $GABA_A$ receptor by phenobarbital (PhB) (pH 7.4, 22−24 °C, −60 mV). Currents were recorded using the cell-flow technique and corrected for receptor desensitization (*44*). Current amplitudes are expressed as fraction of current elicited by a standard (300 μM) concentration of GABA in the absence of phenobarbital. These normalized currents are designated as *N*. (A) Phenobarbital, at the indicated concentration, was coapplied with 300 μM GABA. For the mutated receptor (●), each data point represents three to six independent measurements. For the wild-type receptor (○), each data point represents two to three independent measurements. The solid line represents the fit of the data for the mutated receptor using eq 2. K_P(app) for the mutated receptor is 0.23 ± 0.06 mM, the maximum potentiation, N_{MAX}, is 1.45 ± 0.06, and the Hill coefficient, *h*, is 1.91 ± 0.75. (B) Phenobarbital, at the indicated concentration, was coapplied with a low (6 μM) GABA concentration (●). Each data point represents two to four independent measurements. K_P(app) is 1.08 ± 0.32 mM, the maximum potentiation, N_{MAX}, is 1.04 ± 0.17, and the Hill coefficient, *h*, is 1.55 ± 0.37. For comparison, currents elicited by phenobarbital alone are also plotted (▲; each point represents three to four independent measurements).

At concentrations of phenobarbital greater than 1 mM, inhibition (Figure 2) and some activation (Figure 2B) of the receptor by phenobarbital occur. Therefore, for our further experiments with the mutated $GABA_A$ receptor, we used 1 mM phenobarbital. This concentration is high enough to result in close to maximum potentiation of GABA-elicited currents (Figure 2) and has no observable inhibitory effect. Also, in the presence of 1 mM phenobarbital, direct activation of the receptor by phenobarbital is negligible (less than 1% of the current elicited by 300 μM GABA, Figure 2B). At lower concentrations of GABA, 1 mM is not a saturating concentration of phenobarbital, but for the reasons stated above we did not increase the concentration of phenobarbital. Concentrations of phenobarbital similar to those used here

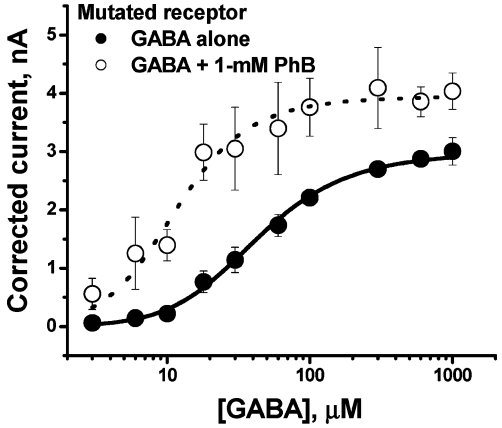

FIGURE 3: Dependence of the corrected current amplitudes, I_A, on the concentration of GABA in the absence (●) and presence (○) of 1 mM phenobarbital (pH 7.4, 22−24 °C, −60 mV). Currents were recorded using the cell-flow technique and corrected for receptor desensitization (*44*). Each data point represents 3−20 independent measurements. The lines represent the fit of the data to eq 3. The two data sets were fitted simultaneously, and K_1 and I_MR_M parameters were shared between the two data sets (see text for further details). Results of the fitting are as follows: K_1 is 48 ± 10 μM and I_MR_M is 4.10 ± 0.14 nA, in both the absence and presence of phenobarbital (PhB); Φ^{-1}, the channel-opening equilibrium constant, is 2.65 ± 0.56 in the absence of phenobarbital and 26 ± 10 in its presence.

have been employed in previous studies [0.5 mM (*17*); 0.3 mM (*46*)] in which allosteric potentiation of the wild-type native $GABA_A$ receptors was observed.

Because of the small potentiation of the wild-type receptor by phenobarbital, we did not determine the value of K_P for that protein.

Does Phenobarbital Change the Affinity of the Epileptic $GABA_A$ Receptor for GABA? The cell-flow technique (*44*) was used to determine the GABA concentration dependence of the $GABA_A$ receptor current, I_A, for the mutated receptor in the presence (○) and absence (●) of 1 mM phenobarbital (Figure 3). These data allow one to estimate the GABA dissociation constant for the site controlling receptor activation, K_1, using eq 3 (*27*):

$$I_A = I_M R_M L^2 [L^2 + \Phi(L + K_1)^2]^{-1} \qquad (3)$$

I_A is the current amplitude, L the concentration of activating ligand (GABA), I_M the current due to 1 mol of open receptor channels, and R_M the number of moles of the receptor in the membrane. K_1 is the GABA dissociation constant for the site controlling receptor activation, and Φ ($=k_{cl}/k_{op}$) is the channel-closing equilibrium constant (Figure 5). The value of I_MR_M can be obtained from the maximum observed current amplitude, I_{MAX}, at saturating activating ligand concentration, where $I_MR_M = I_{MAX}(1 + \Phi)$ (*49*).

The data in Figure 3, eq 3, and a computer program were used to determine the value of the dissociation constant of GABA and the value of Φ from the experiments performed in the absence (●) and presence (○) of 1 mM phenobarbital (Figure 3). The measurements indicated that phenobarbital does not significantly change the value of the dissociation constant of GABA, K_1, of the mutated receptor. Since (i) phenobarbital does not change the single-channel conductance (*13*, *16*, *17*) and (ii) the density of receptors is not changed by phenobarbital on the time scale of our experiments, I_MR_M is taken to be the same in the absence and in the presence of phenobarbital. To improve the precision and reliability of the estimated values of K_1, I_MR_M, and Φ, a global fitting procedure (reviewed in ref *50*) was used that allows one to fit all the data for the experiments obtained in both the absence and presence of phenobarbital (Figure 3) simultaneously. The K_1 and I_MR_M parameters of the mutated

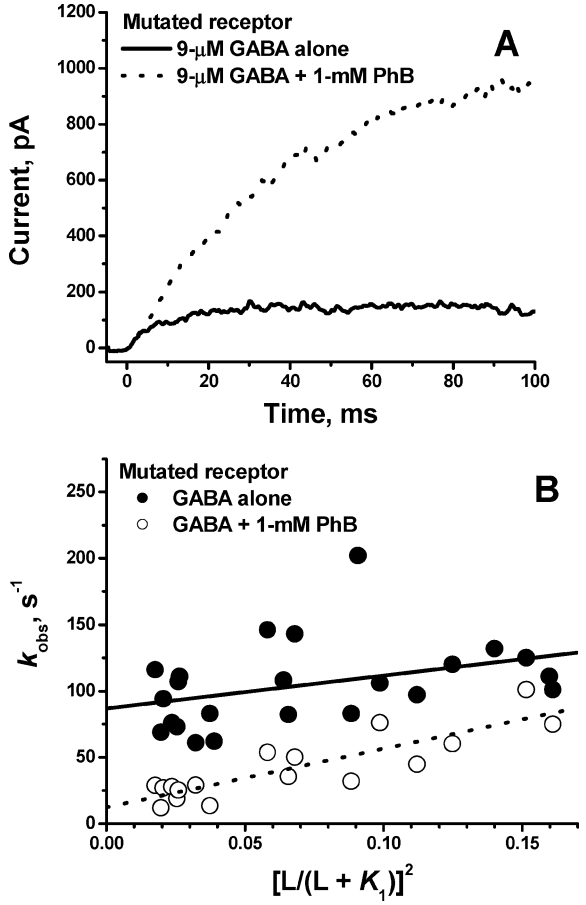

FIGURE 4: Laser-pulse photolysis experiments with the mutated $GABA_A$ receptor (pH 7.4, 22−24 °C, −60 mV). (A) Representative current traces obtained in a laser-pulse photolysis experiment (*38*) using αCNB-caged GABA (*39*). αCNB-caged GABA alone or in the presence of 1 mM phenobarbital (PhB) was equilibrated with the cell for 400 ms before the laser pulse initiated the release of free GABA. The current was recorded for 100 ms after the laser pulse. Current traces in the absence (solid line) and in the presence (dotted line) of phenobarbital were obtained sequentially with the same cell. The concentration of photolytically liberated GABA (determined as described in Materials and Methods) was 9 μM in this experiment. The current traces were fitted to the equation $I = A[1 - \exp(-k_{obs}t)]$ (*38*), where I is the observed current at time t, A the maximal current amplitude, and k_{obs} the observed rate constant for the current rise. In the absence of phenobarbital, the value of k_{obs} is 107 s^{-1}; and in its presence, 25 s^{-1}. (B) Laser-pulse photolysis (*38*) measurements as a function of released GABA concentration. αCNB-caged GABA alone (●) or in the presence of 1 mM phenobarbital (○) was equilibrated with the cell for 400 ms before the laser pulse initiated the release of free GABA. The current was recorded for 100 or 200 ms after the laser pulse. L is the concentration of the photolytically liberated neurotransmitter, GABA, and the value of K_1 was taken as 48 μM (see Figure 3). The observed rate constant for the current rise, k_{obs}, was obtained by fitting the experimental current traces as described in the legend to panel A. The lines represent the fitting of the data to eq 1. In the absence of phenobarbital (●), k_{op} is 248 ± 131 s^{-1} and k_{cl} is 87 ± 11 s^{-1} (solid line). In the presence of 1 mM phenobarbital (○), k_{op} is 441 ± 67 s^{-1} and k_{cl} is 12 ± 5 s^{-1} (dotted line).

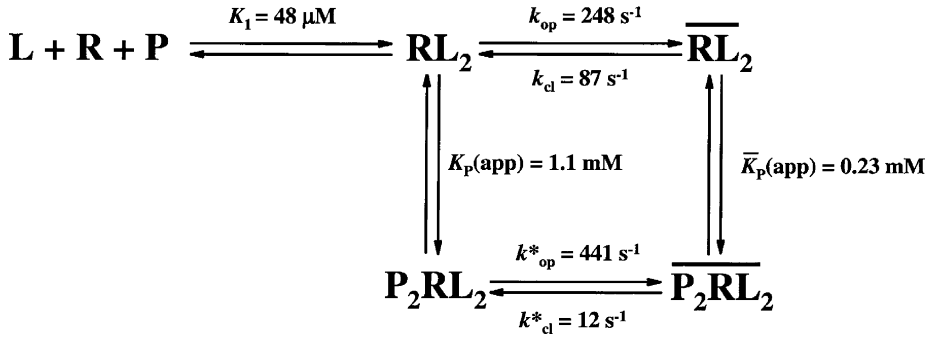

FIGURE 5: Proposed kinetic mechanism for the potentiation of the mutated ($\alpha1\beta2\gamma2^{K289M}$) $GABA_A$ receptor by phenobarbital. R represents the nondesensitized (active) receptor, L the neurotransmitter (GABA), and P the positive allosteric modulator, phenobarbital. $\overline{RL_2}$ and $\overline{P_2RL_2}$ are the open-channel forms of the receptor. K_1 is the receptor−GABA dissociation constant. $\bar{K}_P$(app) and K_P(app) are the apparent dissociation constants of phenobarbital from the open- and closed-channel forms, respectively (see text for details). k_{op} and k^*_{op} are the rate constants for channel opening in the absence and presence of phenobarbital, respectively. k_{cl} and k^*_{cl} are the rate constants for channel closing in the absence and presence of phenobarbital, respectively. The channel-opening equilibrium for the receptor in the absence of phenobarbital $\Phi^{-1} = k_{op}/k_{cl}$ and in the presence of phenobarbital $\Phi^{-1*} = k^*_{op}/k^*_{cl}$.

Table 1: GABA Activation of the Recombinantly Expressed $GABA_A$ Receptors

	1:1:10 cDNA ratio[a]	1:1:3 cDNA ratio[b]	
parameter	$\alpha1\beta2\gamma2L^{K289M}$	$\alpha1\beta2\gamma2L^{K289M}$	$\alpha1\beta2\gamma2L$
EC_{50} (μM)	45 ± 3	44 ± 6	23 ± 3
h	1.33 ± 0.09	1.05 ± 0.13	1.31 ± 0.23

[a] This study. [b] Calculated from data in Figure 3 of ref *36* (L. Ramakrishnan, personal communication). Data have been approximated by the empirical Hill equation $I_A = I_{max}[L^h/(L^h + EC_{50}{}^h)]$, where I_A is the amplitude of current corrected for desensitization, I_{max} is the maximum response, EC_{50} is concentration of GABA at which 50% of activation is observed, and h is the Hill coefficient (*47*).

receptor were taken to be the same in both the absence and presence of phenobarbital. In Figure 3, the solid (phenobarbital absent) and dotted (1 mM phenobarbital) lines represent the best fit of the data to eq 3. A K_1 value of 48 ± 10 μM was obtained for the binding of GABA to the receptor. This corresponds to an EC_{50} value of 45 ± 3 μM, the concentration at which the current amplitude is one-half that obtained at a saturating concentration of GABA (Table 1). The stoichiometry and pharmacological properties of hetero-oligomeric receptors transiently expressed in mammalian cells and in *Xenopus laevis* oocytes may be influenced by the ratio of DNA vectors used for transfection (*42*, *51*−*53*). To express the $GABA_A$ receptor, equal amounts of all cDNAs have been suggested for the transfection (*54*−*56*). However, it has been shown (*42*, *53*) that when equal amounts of cDNAs are used for transient expression in *X. laevis* oocytes and in mammalian cells, the resulting $GABA_A$ receptor population contains a mixture of $\alpha1\beta2\gamma2$ and $\alpha1\beta2$ receptors. A 10-fold excess of the $\gamma2$ subunit cDNA over the $\alpha1$ and $\beta2$ subunit cDNAs was found (*42*, *53*) to result in a homogeneous receptor population containing only the $\alpha1\beta2\gamma2$ receptor. It is of interest to note that the $\alpha1$:$\beta2$:$\gamma2$L cDNA ratio of 1:1:3 [used in previous studies with the wild-type and mutated receptor (*36*, *57*)] gives the same results as the 1:1:10 ratio used here (Table 1).

Effects of Phenobarbital on the Channel-Opening (k_{op}) and Channel-Closing (k_{cl}) Rate Constants (Figure 5). These rate constants determine the equilibrium between the closed- and open-channel receptor conformations (*27*). The LaPP technique (*27*, *37*, *38*) was used to determine the observed rate coefficient for the current rise, k_{obs}, at a given GABA concentration. Figure 4A shows a representative LaPP experiment at a low concentration of released GABA (9 μM) in the presence and absence of phenobarbital. In the presence of 1 mM phenobarbital not only does the current amplitude increase, but also the observed rate constant for the current rise, k_{obs}, becomes much slower (107 s^{-1} in the absence of phenobarbital and 25 s^{-1} in the presence of phenobarbital). This effect can be reliably observed with the LaPP technique (100 μs time resolution) but not with the cell-flow technique (20 ms time resolution). LaPP measurements were performed over a concentration range of 7−33 μM released GABA (Figure 4B). The concentration dependency of k_{obs} allows one to determine both k_{op} and k_{cl} (*27*). When k_{obs} is plotted *versus* $[L/(L + K_1)]^2$ (eq 1), k_{op} is obtained from the slope and k_{cl} from the intercept of the graph in Figure 4B. Visual inspection of the data in Figure 4B reveals that 1 mM phenobarbital does not change the slope (k_{op}) significantly but dramatically decreases the intercept (k_{cl}). Fitting the data in Figure 4B to eq 1 yielded the numerical values for k_{op} and k_{cl}. In the absence of phenobarbital, the values of k_{op} (248 ± 131 s^{-1}) and k_{cl} (87 ± 11 s^{-1}) are essentially the same as those obtained previously (*36*) with HEK293T cells transfected with a 1:1:3 ratio of cDNAs for the $\alpha1$, $\beta2$, and $\gamma2$L subunit of the mutated $GABA_A$ receptor. The effect of phenobarbital on the channel-opening rate constant is small: In the presence of 1 mM phenobarbital the value of k_{op} is 441 ± 67 s^{-1}, and in the absence of the drug it is 248 ± 131 s^{-1}. On the other hand, as can be seen in Figure 4B, the value of k_{cl} in the absence of phenobarbital (87 ± 11 s^{-1}, ●) is much higher than in the presence of 1 mM phenobarbital (12 ± 5 s^{-1}, ○). One can conclude that the main action of phenobarbital on the $GABA_A$ receptor is to increase the channel-opening equilibrium constant Φ^{-1} ($=k_{op}/k_{cl}$), by more than an order of magnitude, by decreasing k_{cl} by a factor of ∼7 with much less of an effect on k_{op}.

The pertinent kinetic constants for the proposed kinetic mechanism for allosteric modulation of the mutated $GABA_A$ receptor by phenobarbital are summarized in Table 2 and Figure 5. While the true stoichiometry of the barbiturate−$GABA_A$ receptor complex is unknown, previous studies as well as our own results (see above) indicate that the Hill coefficient (apparent cooperativity) for barbiturate potentiation of GABA-elicited currents is more than 1. Therefore,

Table 2: Kinetic Parameters Pertinent to the Allosteric Modulation of the Epileptic ($\gamma 2L^{K289M}$) $GABA_A$ Receptor by Phenobarbital

technique	kinetic parameter	GABA alone	GABA + 1 mM PhB
cell flow (eq 3, Figure 3)	$I_M R_M$ (nA)	4.10 ± 0.14	
	K_1 (μM)	48 ± 10	
	Φ^{-1}	2.7 ± 0.6	26 ± 10^a
LaPP (eq 1, Figure 4B)	k_{op} (s^{-1})	248 ± 131	441 ± 67^a
	k_{cl} (s^{-1})	87 ± 11	12 ± 5^a
	Φ^{-1}	2.8 ± 1.9	35 ± 21^a

[a] These are apparent values, since 1 mM is not a saturating concentration of phenobarbital at lower concentrations of the neurotransmitter, GABA, and therefore a mixture of phenobarbital-bound and free receptors will be present. Concentrations of phenobarbital higher than 1 mM may lead to receptor inhibition (see Figure 2).

the mechanism we propose (Figure 5) entails two binding sites for phenobarbital.

Phenobarbital does not change the dissociation constant of GABA from the mutated receptor, as determined from the data in Figure 3 and the use of eq 3. Phenobarbital increases the channel-opening equilibrium constant, Φ^{-1}, by about 10-fold. As can be seen in Table 2, the values obtained from laser-pulse photolysis and cell-flow experiments are in excellent agreement.

A mechanism for the potentiation of the $GABA_A$ receptor by phenobarbital can be deduced from the results presented. The mechanism shown in Figure 5 is based on transient kinetic measurements that indicate that (i) phenobarbital binding to the receptor increases the channel-opening equilibrium constant Φ^{-1} by an order of magnitude and (ii) phenobarbital binds with higher affinity at high concentrations of GABA, where the receptor is mainly in the open-channel conformation, than at low concentrations of GABA, where the closed-channel conformation predominates. For the proposed mechanism (Figure 5), the principle of detailed balance (*58*) requires that the ratio of the dissociation constants of phenobarbital from the closed- (K_P) and open-channel ($\bar{K}_P$) receptor conformations has the same value as the ratio of the channel-opening equilibrium constant of the receptor in the presence of phenobarbital, $\Phi^{-1}*$, to that in its absence, Φ^{-1} (Figure 5). The data indicate the ratio of K_P(app)/$\bar{K}_P$(app) is only about 5 (4.7 ± 2.6). It should be pointed out that the value of $\bar{K}_P$, the dissociation constant of phenobarbital from the open-channel receptor form, cannot be obtained directly from the measurements. The apparent value of K_P pertaining to the closed-channel receptor conformation obtained in the presence of 6 μM GABA is expected to closely reflect its true (intrinsic) value. From the data in Figure 3, and by using eq 3, one can calculate that about 97% of the receptors are in the closed-channel conformation under the conditions at which these measurements were made. Because of the unfavorable channel-opening equilibrium of the mutated receptor, in the presence of nearly saturating concentrations of GABA only about 66% of the mutated receptors are in the open-channel conformation. Under these conditions, the dissociation constant of phenobarbital from the closed-channel conformation, which is larger than that of the open-channel conformation, is expected to contribute to the value of the measured dissociation constant of phenobarbital from the open-channel conformation, $\bar{K}_P$(app). Therefore, the actual value of $\bar{K}_P$ for phenobarbital from the open channel is expected to be lower than the value observed. This would make the value of $K_P/\bar{K}_P$ larger than the one observed and more compatible with the values of $\Phi^{-1}*/\Phi^{-1}$, as required by the mechanism in Figure 5.

DISCUSSION

Ionotropic GABA receptors are allosterically modulated by a variety of biologically active substances, such as antiepileptic drugs, anesthetics, and insecticides (*4*, *6*). Benzodiazepines and barbiturates are well-known classes of positive allosteric modulators of the $GABA_A$ receptor. Benzodiazepines (e.g., diazepam, chlorodiazepoxide) potentiate the function of native and recombinant $GABA_A$ receptors by shifting the GABA dose−response curve to lower concentrations of GABA without increasing the maximal current (*46*, *59*, *60*). This is consistent with a change in the intrinsic affinity of the $GABA_A$ receptor for GABA (*16*, *61*). On the other hand, phenobarbital potentiates the function of the wild-type native $GABA_A$ receptors natively expressed in mammalian neurons by both shifting the GABA dose−response curve to lower concentrations of GABA and potentiating the maximal GABA-elicited current by a few percent (14−20%) (*45*, *46*). Such potentiation of the maximal current elicited by saturating concentrations of GABA is consistent with an increase in the channel-gating efficiency (*16*), which, for the mutated receptor, we determined to be due to an approximately 10-fold increase in the channel-opening equilibrium constant. Regarding the dependency of the potentiation on phenobarbital concentration, previous studies examined potentiation of currents elicited only by subsaturating concentrations of GABA. At such low concentrations of GABA, potentiation due to an increase in the channel-opening equilibrium cannot be distinguished from potentiation due to an increase in the affinity of the receptor for GABA.

Several previous studies (see the introduction) suggested that an increase in the duration of the mean open-channel time [a measure of the channel-closing rate (*62*)] contributes to enhanced gating of the $GABA_A$ receptor in the presence of barbiturates and similarly acting compounds (e.g., neurosteroids), but it was not known whether the channel-opening rate is affected as well. The channel-opening rate, however, can have a drastic effect on the channel-opening equilibrium constant, which is determined by the channel-opening rate constant, k_{op}, divided by the channel-closing rate constant, k_{cl}. Regarding the effect of barbiturates and neurosteroids on the wild-type $GABA_A$ receptor, studies by Steinbach and co-workers (*20*, *24*) argue against changes in the channel-opening rate, while Macdonald with collaborators (*17*, *19*) suggested that an increase in the relative opening rate for the long-duration open state might be involved. By using the cell-flow technique with a 20 ms time resolution and laser-pulse photolysis of αCNB-caged GABA with a 100 μs time resolution (reviewed in ref *27*), we have directly demonstrated that the primary action of phenobarbital on a mutated $GABA_A$ receptor is to increase the channel-opening equilibrium constant by decreasing the channel-closing rate constant, without a large effect on the channel-opening rate constant.

To examine the effects of phenobarbital specifically on the channel-opening equilibrium constant, experiments were

performed in which phenobarbital, at different concentrations, was coapplied with a nearly saturating concentration (300 μM) of GABA. Over 90% of the observed maximum current response for either the wild-type (*36*) or the mutated (Figure 3) receptor is observed at this concentration of GABA. Potentiation due to an increase in the affinity of the receptor for GABA would be negligible at this GABA concentration.

The K_P(app) values (0.23 mM at high and 1.1 mM at low concentration of GABA) for phenobarbital obtained in our study are similar to the K_P(app) values previously reported for the effect of phenobarbital on currents elicited by application of subsaturating concentrations of GABA to the native $GABA_A$ receptors: 0.4 mM in frog motor neurons (*15*), 0.29 mM in mouse brain membrane vesicles (*63*), ~0.3 mM in frog dorsal root ganglia neurons (estimated by us from Figure 2B in ref *64*), 0.89 mM in rat hippocampal neurons (*19*), and 0.41 mM in a NT2-N clonal cell line (*48*). Two reports cite substantially lower K_P values: 75 μM in mouse spinal cord neurons (*65*) and 12 μM in guinea pig hippocampal CA1 neurons (*66*). A quantitative comparison of these values is precluded by the fact that different concentrations of GABA were used. As we have shown here, the value of K_P depends on the GABA concentration used in the experiments.

This is the first report of an allosteric ligand that discriminates between the wild-type and mutated ($\gamma 2L^{K289M}$) $GABA_A$ receptors. Earlier, it was shown that benzodiazepines exert the same degree of potentiation of both the wild-type and the mutated ($\gamma 2L^{K289M}$) $GABA_A$ receptors (*34*−*36*, *67*).

The enhanced potentiation of the $\alpha 1\beta 2\gamma 2L^{K289M}$ $GABA_A$ receptor by phenobarbital allowed us to resolve effects of this drug on ligand binding and on channel opening. With the wild-type receptor, such resolution is problematic, since the effect of phenobarbital on ligand binding can easily obscure its relatively small effect on the channel-opening equilibrium. It is interesting to note that phenobarbital is commonly used to treat febrile seizures (*10*), and the $\gamma 2^{K289M}$ mutation is genetically linked to generalized epilepsy with febrile seizures plus (GEFS+) (*34*). Is the increased allosteric potency of phenobarbital at the $\alpha 1\beta 2\gamma 2L^{K289M}$ $GABA_A$ receptor of pharmacological significance? In vivo studies in animal models and/or human clinical trials are necessary to clarify this.

It should be mentioned that when phenobarbital is administered to epileptic humans and mammals, its blood concentration typically reaches levels of 50−200 μM and on some occasions can exceed 300 μM (*68*−*70*). Therefore, the allosteric modulation of the $\alpha 1\beta 2\gamma 2L$ $GABA_A$ receptor by phenobarbital observed in our study occurs at clinically relevant concentrations of this anticonvulsant. Sedation is a common side effect of higher doses of phenobarbital, and it is unclear at this point to what extent the allosteric modulation of the $GABA_A$ receptor is related to its anticonvulsant or its sedative action.

The results presented also indicate that a simple test system, based on whole-cell current recording, is capable of differentiating between the positive allosteric modulators that affect only the intrinsic affinity of a receptor for its neurotransmitter and those that affect the channel-opening equilibrium constant. The former class of compounds will potentiate currents at subsaturating, but not saturating, concentrations of neurotransmitter. The latter class of compounds will potentiate currents at both subsaturating and saturating concentrations of neurotransmitter.

A change in the channel-opening equilibrium constant that accounts for the mechanism of phenobarbital potentiation of the mutated receptor (Figure 5) has also been shown to account for a number of important changes in the function of various neurotransmitter receptors. Among these are the noncompetitive inhibition of the nicotinic acetylcholine receptor (reviewed in refs *27* and *71*) and the dysfunction of a mutated ($\gamma 2^{K289M}$) epilepsy-linked $GABA_A$ receptor (*36*). Other mutated $GABA_A$ receptors linked to epilepsy have been discovered (e.g., refs *72* and *73*). The channel-opening mechanism of those receptors is still to be fully determined.

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