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# Mechanism of Potentiation of a Dysfunctional Epilepsy-Linked Mutated $GABA_A$ Receptor by a Neurosteroid (3α, 21-Dihydroxy-5α-pregnan-20-one): Transient Kinetic Investigations[†]

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ABSTRACT: The malfunction of a mutated $GABA_A$ receptor ($\alpha1\beta2\gamma2L^{K289M}$) in an inheritable form of epilepsy (GEFS+, generalized epilepsy with febrile seizures plus) in humans [Baulac, S., Huberfeld, G., Gourfinkel-An, I., Mitropoulou, G., Beranger, A., Prud'homme, J. F., Baulac, M., Brice, A., Bruzzone, R., and LeGuern, E. (2001) *Nat. Genet. 28*, 46−48] has been accounted for by a 5-fold decrease in the channel-opening equilibrium of the mutated receptor compared to the wild type [Ramakrishnan, L., and Hess, G. P. (2004) *Biochemistry 43*, 7534−7540]. Here we describe the mechanism by which the neurosteroid 3α, 21-dihydroxy-5α-pregnan-20-one (5α-THDOC) alleviates this malfunction of the mutated receptor transiently expressed in HEK293 cells. Two rapid reaction techniques, the cell-flow and the laser-pulse photolysis methods, were used in combination with whole-cell current recordings. 150-$\mu$M 5α-THDOC does not affect the rate constant for channel opening ($k_{op}$) of $\sim250\ s^{-1}$ but does decrease the rate constant for channel closing ($k_{cl}$) from $121 \pm 11\ s^{-1}$ to $56 \pm 21\ s^{-1}$. This results in an increase in the channel-opening equilibrium constant ($(\Phi^{-1} = k_{op}/k_{cl})$ by a factor of about 2, leading to about 50% alleviation of the malfunction of the inheritable mutated ($\alpha1\beta2\gamma2L^{K289M}$) $GABA_A$ receptor linked to GEFS+.

The mammalian inhibitory γ-aminobutyric acid type A ($GABA_A$)[1] receptor is one of the main membrane-bound receptors that regulate signal transmission between $\sim10^{12}$ neurons in the mammalian nervous system. Impaired function of this receptor can result in seizures such as those associated with epilepsy (*1*), a disease affecting ∼50 million people worldwide (*2*). Point mutations in $GABA_A$ receptor subunits play a significant role in epilepsy syndromes, such as generalized epilepsy with febrile seizures plus (GEFS+) (*3*), childhood absence epilepsy (*4*), and juvenile myoclonic epilepsy (*5*). Impaired receptor-mediated neurotransmission has been observed in the presence of such mutated $GABA_A$ receptors (*3*, *5*−*10*) and is considered to be due to changes in the mechanism of receptor-mediated reaction (*11*), changes in receptor expression (*12*, *13*), and changes in the sensitivity to allosteric modulators (*4*). The subunit composition of the $GABA_A$ receptor influences its pharmacology and kinetics. This paper presents results obtained with the wild-type $\alpha1\beta2\gamma2L$ and epilepsy-linked mutated $\alpha1\beta2\gamma2L^{K289M}$ $GABA_A$ receptors.

The mechanism of one mutated $GABA_A$ receptor ($\alpha1\beta2\gamma2L^{K289M}$) containing a lysine-to-methionine mutation at position 289 in the γ2 subunit (*3*) has been investigated (*11*) by use of transient kinetic techniques (reviewed in ref *14*). The mutation was first identified in a French family having the GEFS+ syndrome (*3*). It occurs in the short extracellular loop between transmembrane domains M2 and M3. The $\alpha1\beta2\gamma2L$ $GABA_A$ receptor occurs synaptically and has a phasic response to GABA (reviewed in ref *15*). Electrophysiological measurements indicated that the amount of chloride ions that pass through the $GABA_A$ receptor-channels upon activation of the mutated receptor by GABA is considerably decreased compared to its wild-type counterpart (*3*). By using a transient kinetic technique with a sub-millisecond time resolution (reviewed in ref *14*) and photolysis of caged neurotransmitters (*16*), we showed previously (*11*) that the channel-opening rate constant ($k_{op}$) of the mutated $GABA_A$ ($\alpha1\beta2\gamma2L^{K289M}$) receptor is reduced approximately 5-fold compared to that of the wild-type receptor, while the channel-closing rate constant ($k_{cl}$) remains essentially unaffected. This change leads to a 5-fold decrease in the channel-opening equilibrium constant ($\Phi^{-1} = k_{op}/k_{cl}$) and accounts for the decreased current flowing through the mutated receptor-channel. These mechanistic studies indicated that if we can find a molecule that binds with higher affinity for the allosteric site on the open-channel form than on the closed-channel form, it will increase the channel-opening equilibrium constant and, therefore, will be effective in alleviating the decreased current observed in the mutated receptor. In our search to find alleviatory molecules, the effects of phenobarbital on the mutated receptor were studied, and it was shown to partially reverse the decreased current of this mutated receptor (*17*).

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[1]Abbreviations: HEK293, human embryonic kidney cells; GABA, γ-aminobutyric acid; LaPP, laser-pulse photolysis; αCNB-caged GABA, *N*-(α-carboxy-2-nitrobenzyl) GABA; 5α-THDOC, 3α, 21-dihydroxy-5α-pregnan-20-one; GEFS+, generalized epilepsy with febrile seizures plus.

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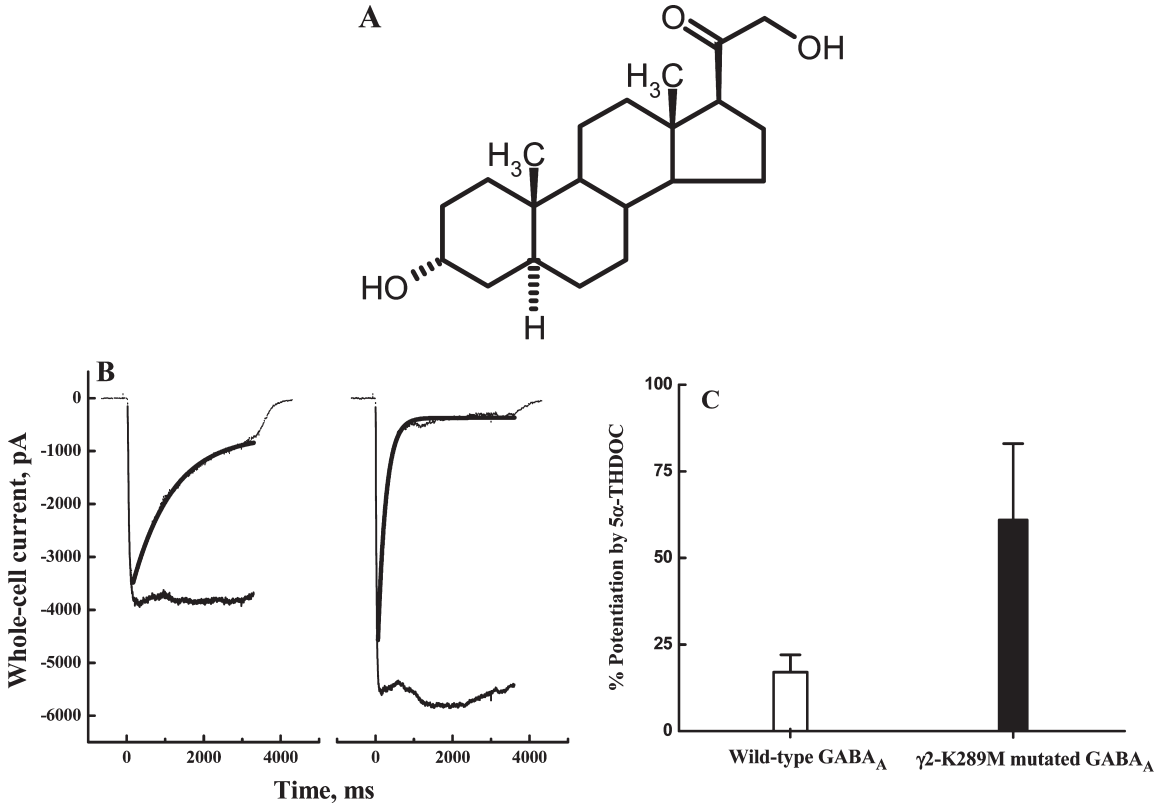


FIGURE 1: (A) Structure of the neurosteroid 5α-THDOC. (B) Whole-cell current traces recorded from the same HEK293 cell transiently expressing the $\alpha1\beta2\gamma2L^{K289M}$ $GABA_A$ receptor at pH 7.4, 22−24 °C, and −60 mV using the cell-flow technique (*29*). The observed current was recorded when a solution of 100 $\mu$M GABA (left trace) or 100 $\mu$M GABA and 200 $\mu$M 5α-THDOC (right trace) flowed over the cell from the U-tube flow device. The observed current was corrected for desensitization according to eq 1 and the corrected current is shown by the line parallel to the abscissa. The solid ascending line indicates the desensitization process. (C) The neurosteroid 5α-THDOC has a much larger effect on the $\alpha1\beta2\gamma2L^{K289M}$ mutated (filled bar) $GABA_A$ receptor than on the wild-type receptor (open bar), transiently expressed in HEK293 cells at −60 mV, 22 °C, and pH 7.4. The extent of potentiation was determined in the presence of 100 $\mu$M GABA and 200 $\mu$M 5α-THDOC. Each bar shown in Figure 1C is an average of 4−6 measurements made with 4−6 cells.

Here we report investigations of the mechanism by which a nontoxic anticonvulsant neurosteroid (*18*) affects the channel-opening and -closing kinetics of the mutated receptor and, therefore, the channel-opening equilibrium. Neurosteroids are known positive allosteric modulators of the $GABA_A$ receptor and are effective in seizure control (*19*). A synthetic neurosteroid analogue is in phase III clinical trials for treating epilepsy (*20*, *21*). We chose 3α, 21-dihydroxy-5α-pregnan-20-one (5α-THDOC) (Figure 1A) for this study. 5α-THDOC is a naturally occurring anticonvulsant that is effective against chemoconvulsant-induced seizures (*22*−*24*). Previous investigations on the action of this compound indicated that it altered the desensitization and deactivation kinetics of the wild-type $GABA_A$ receptor and increased the sensitivity of the receptor to GABA (*18*, *19*, *25*). In our investigations we used transient kinetic techniques with a microsecond-to-millisecond time resolution (reviewed in ref *14*) to understand how this compound affects the unfavorable channel-opening equilibrium observed with the mutated $\alpha1\beta2\gamma2L^{K289M}$ $GABA_A$ receptor.

## MATERIALS AND METHODS

GABA and 5α-THDOC were purchased from Sigma (St. Louis, MO). Plasmid cDNAs for the rat $GABA_A$ receptor subunit proteins were a gift from Professors P. H. Seeburg (Max Planck Institute for Medical Research, Heidelberg, Germany) and H. Lüddens (Johannes Gutenberg-Universität, Mainz, Germany). In these plasmids, the cDNAs encoding the α1, $\beta2$, and $\gamma2$L (long splice variant) rat $GABA_A$ subunit proteins are individually cloned into pRK-5 mammalian expression vectors (BD Biosciences, San Diego, CA). The QuickChange site-directed mutagenesis (Stratagene, La Jolla, CA) method was used to introduce the K289M mutation in the rat $\gamma2$L-subunit of the $GABA_A$ receptor (*26*) as reported previously (*11*). The primary amino acid sequence homology of the rat (UniGene ID: 1899245) and human $\gamma2$L (UniGene ID: 132064) subunits of the $GABA_A$ receptor protein is 99.6% as reported in the NCBI UniGene database (http://www.ncbi.nlm.nih.gov/UniGene/). Polyfect transfection reagent was purchased from Qiagen (Valencia, CA).

*Cell Culture and Transient Transfection*. The culture and transient transfection of the HEK293 cell line (American Type Cell Culture, Manassas, VA) were performed as described previously (*27*). HEK293 cells were grown in 25-$cm^2$ canted-neck cell culture flasks (Corning, NY) containing the growth medium DMEM (Dulbecco's modified Eagle's medium, high glucose, from Invitrogen Gibco BRL, Grand Island, NY) supplemented with 10% FBS (fetal bovine serum, from Invitrogen Gibco BRL) and antibiotics (100 IU of penicillin, 100 $\mu$g/L streptomycin, both from Sigma). Cells were passaged weekly (after reaching 70−80% confluence) and $\sim2 \times 10^5$ cells were seeded in 35-mm Falcon dishes (Fisher Scientific, Pittsburgh, PA) in 10% FBS and 90% DMEM containing 1% penicillin/streptomycin. HEK293 cells from passage numbers ranging from 3−12 were used for all experiments, with no noticeable difference in the desensitization behavior of the $GABA_A$ receptors between cells. Cells beyond 12 passages varied substantially in the expression of fast and slow

desensitizing receptor types, which resulted in multi-exponential desensitization kinetics. The HEK293 cells were transiently transfected with the rat $GABA_A$ α1, β2, and γ2L/γ2L$^{K289M}$ cDNAs in a 1:1:5 ratio using the Polyfect transfection reagent (Qiagen). The cells were cotransfected with cDNA encoding the green fluorescent protein (pGreenLantern plasmid, Life Technologies, Gaithersburg, MD) to detect transfected cells (*28*). The transfection reaction mixture was added to the cells 24 h after they were passaged. Twenty-four hours later the transfection mixture was removed from the cells, which were then washed once with 1× phosphate buffer saline solution, pH 7.4, (Invitrogen Gibco BRL) followed by the addition of 2 mL of 1% FBS in the DMEM growth medium. The cells were then replated into 35-mm Falcon dishes and were used for electrophysiological measurements up to 48 h from the time of transfection.

*Electrophysiology*. For both cell-flow (*29*) and laser-pulse photolysis (LaPP) (reviewed in ref *14*) experiments, the recording pipettes were pulled from borosilicate glass (World Precision Instruments Inc., Sarasota, FL), using a two-stage puller (L/M 3 P-A, Adams & List, NY) and a flame polisher (MF-83, Narishige, Tokyo, Japan). Typical pipet resistances were 2−4 MΩ, and the series resistances were 1−4 MΩ for whole-cell current measurements (*30*). Series resistance compensation of 60−70% was used in the whole-cell current-recording (*30*) experiments. The bath buffer contained 145 mM sodium chloride, 5 mM potassium chloride, 2 mM calcium chloride, 1.5 mM magnesium chloride, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, and 10 mM glucose; the pH was adjusted to 7.4 using 5 N sodium hydroxide. The electrode solution contained 140 mM cesium chloride, 10 mM tetraethyl ammonium chloride, 2 mM magnesium chloride, 10 mM ethyleneglycol tetraacetic acid, and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; the pH was adjusted to 7.4 using cesium hydroxide (50 wt % solution in water). An Axopatch 200B amplifier (Axon Instruments, Union City, CA) with a 1−5 kHz low-pass filter was used for the current recordings. Signals were acquired using the pClamp 8.0.1 software packet (Axon Instruments). Whole-cell current-recording data were digitized at 0.5−2 kHz. LaPP data were digitized at 20−50 kHz using the Digidata 1320A (Axon Instruments). Data were analyzed offline on a PC and the time constants for the rising and decaying phases of the whole-cell current were obtained by using a nonlinear least-squares fitting program with Microcal Origin 3.5 software (Microcal, Northampton, MA). All measurements were carried out at pH 7.4, 22 °C, and a transmembrane voltage of −60 mV. Because of the variations in the number of receptors expressed per cell, the current response obtained in the presence of 100 μM GABA for each cell is used to normalize the currents obtained from different cells. All solutions used in the experiments were prepared on the day of measurements. The GABA (5 mM) stock solution can be stored at 4 °C for several weeks. The freshly prepared 5α-THDOC (50 mM) stock solution in dimethylsulfoxide (DMSO) was used for preparing different dilutions with solutions of GABA in the external bath buffer. The final concentration of DMSO in the test solutions was between 0.1−0.5%. Control electrophysiological measurements performed with 100 μM GABA and up to 0.5% DMSO showed that the whole-cell currents were essentially the same in the absence or in the presence of DMSO, indicating that DMSO had no effect on the cell current when used in these concentrations (data not shown). The αCNB-caged GABA [*N*-(α-carboxy-2-nitrobenzyl) GABA (*25*), Molecular Probes, Eugene, OR] stock solution (5 mM) was stored at −80 °C and protected from light. During measurements the caged GABA solution was kept on ice in brown tubes to minimize hydrolysis.

*Cell-Flow and Laser-Pulse Photolysis* (*LaPP*). The whole-cell current-recording technique developed by Hamill et al. (*30*) was used in conjunction with the cell-flow (*29*) and LaPP methods (reviewed in ref *14*). The cell-flow technique, which allows rapid ligand application as well as a method to make a correction for desensitization of the observed whole-cell current, has been described (*29*). Whole cells are used rather than excised patches because the receptor concentration in whole cells is, on average, 10-fold greater and, therefore, a much larger number of receptors are sampled in each measurement. In addition, the time resolution of laser-pulse photolysis with whole cells is good enough that excised patches do not have to be used. Briefly, a cell (ca. 10−20 μm diameter) in the whole-cell recording configuration was placed at a distance of ca. 100 μm from the porthole (diameter ca. 150 μm) of a U-tube made from stainless-steel high performance liquid chromatography (HPLC) tubing (Hamilton, Reno, NV). One advantage of the U-tube is that the orientation of the solution flowing from the U-tube porthole over the cell does not change during or between measurements. The flow rate of neurotransmitter solution emerging from the flow device was typically ∼1 cm/s. In cell-flow experiments with 100 μM GABA, the observed rise time of the whole-cell current to its maximum value, characteristic of the time for the flowing solution of GABA to equilibrate with the cell surface receptors, was 60−100 ms. Receptor desensitization during this time can be significant. In the cell-flow method, the observed current was, therefore, corrected for desensitization that occurs during the time it takes GABA to equilibrate with the receptors on the cell surface, by using eq 1 as described previously (*29*).

$$I_A = (e^{\alpha\Delta T} - 1)\sum_{i=1}^{n}(I_{obs})_{\Delta ti} + (I_{obs})_{\Delta tn} \quad (1)$$

$I_A$ denotes the current amplitude corrected for receptor desensitization. $(I_{obs})_{\Delta ti}$ is the observed current during the $i^{th}$ time interval and $(I_{obs})_{\Delta tn}$ the observed current in a time interval equal to or greater than the time needed to reach the maximum current. If a fraction of current remained after the desensitization reaction had gone to completion (usually less than 5% of the total current), it was subtracted from the observed current before correction. Cells were allowed to recover for 2 min after each experiment by flowing the bath buffer solution (composition given above), for a time sufficient to guarantee full resensitization of the receptors (*31*).

The LaPP experiments were performed as described previously (reviewed in ref *14*). In brief, a photolabile biologically inactive precursor of a neurotransmitter (*16*), called a "caged" compound (*32*), is used. After equilibrating the receptors on the cell surface with the caged neurotransmitter using the U-tube device (*29*), free neurotransmitter was released by irradiation in the μs time region with a pulse of laser light (reviewed in ref *14*). The resulting current, due to the binding of neurotransmitter leading to the opening of transmembrane channels, was measured. Several caging groups have been developed for neurotransmitters (reviewed in ref *16*). For the present work, the photolabile precursor αCNB-caged GABA (*33*) was used. It was used previously to investigate the mechanism of rat hippocampal $GABA_A$

receptors (*34*) and of recombinant rat wild-type (α1β2γ2L) and mutated (α1β2γ2L$^{K289M}$) $GABA_A$ receptors (*11*). The cell was equilibrated with either the caged compound alone or a mixture of the caged GABA and 5α-THDOC for 400 ms before the laser irradiation. Photocleavage of the caged GABA was initiated by a pulse of laser light from a nitrogen laser ($\lambda$ = 337 nm, pulse duration = 10 ns; COMPEX 101, Lambda Physik AG, Göttingen, Germany) coupled into a 300-μm core diameter optical fiber (Laser Components, Santa Rosa, CA), which delivered the laser light to the cell. The flash/flow system was controlled using the pClamp 8.0.1 software (Axon Instruments). The laser energy was ~200−500 μJ, which released free GABA from varying concentrations of caged GABA, with the maximum concentration of caged GABA used being 100 μM.

In a typical experiment, a cell in the whole-cell configuration was first rapidly perfused with 100 μM GABA using the U-tube and the induced current was recorded and corrected for receptor desensitization using eq 1. After 2 min, the LaPP experiments were performed. Before and after every LaPP experiment, a control cell-flow experiment was conducted with 100 μM GABA to monitor for possible changes in receptor activity or laser-induced damage of the receptors or the cell. Data obtained from cells with a significant change (> 20%) of the control current after photolysis were discarded. To determine the concentration of GABA released from the αCNB-caged GABA, the maximum current amplitude observed in the LaPP experiment was compared to that obtained in the cell-flow experiment performed with the same cell and a known concentration of GABA. This information, and the known relationship between GABA concentration and current amplitude obtained in the cell-flow experiments, was used to calculate the concentration of GABA released in LaPP experiments (reviewed in ref *14*).

Whenever laser-pulse photolysis measurements were made, cell-flow experiments were also conducted with the highest concentration of caged GABA (100 μM) used in the LaPP experiments together with 25 μM GABA to determine whether the presence of αCNB-caged GABA, *before* photolysis, changed the current amplitude measured in its absence. The maximum current amplitudes observed in these experiments were the same in either the presence or absence of αCNB-caged GABA (data not shown), indicating that the αCNB-caged GABA was biologically inert when concentrations up to 100 μM were used. When αCNB-caged GABA at a concentration greater than 100 μM was coapplied with 25 μM GABA, the caged compound inhibited the GABA-evoked whole-cell current. Inhibition of a $GABA_A$ receptor by αCNB-caged GABA (*33*) under certain circumstances has been reported previously (*35*).

## RESULTS

Typical whole-cell current measurements from an HEK293 cell expressing the mutated α1β2γ2L$^{K289M}$ $GABA_A$ receptors in the presence of either 100 μM GABA alone (Figure 1B, left trace) or 100 μM GABA and 200 μM 5α-THDOC (Figure 1B, right trace), recorded at −60 mV, pH 7.4, and 22 °C, are shown. The allosteric modulator 5α-THDOC potentiated the current of the mutated $GABA_A$ receptor at high (100 μM) GABA concentrations [Figure 1B (right trace) and 1C (filled bar)]. 5α-THDOC also accelerated the desensitization kinetics of the mutated receptor (Figure 1B, right trace). It had little effect on the wild-type receptor (Figure 1C, open bar). The whole-cell currents induced by 100 μM GABA, recorded either in the absence (Figure 1B, left trace) or in the presence (Figure 1B, right trace) of 200 μM 5α-THDOC, were corrected for receptor desensitization by using eq 1 (*29*) (see Materials and Methods. The descending solid line in Figure 1B shows the change in receptor-mediated current. The ascending line shows the desensitization process, and the line parallel to the abscissa is the current corrected for desensitization that occurs before complete equilibration of the receptor with 100 μM GABA occurs. In the absence of 5α-THDOC, the desensitization process can be fitted with a single exponential process with a rate constant of 0.9 $s^{-1}$ (Figure 1B). In the presence of 5α-THDOC, the rate of desensitization increases, with a rate constant of 4.8 $s^{-1}$ (Figure 1B) and can be adequately fit with a single exponential. From the corrected whole-cell currents, one can see that 200 μM 5α-THDOC potentiated by ~50% the 100 μM GABA-evoked current of the mutated receptor (Figure 1C, filled bar). The same concentration of 5α-THDOC potentiated the wild-type $GABA_A$ receptor (Figure 1C, open bar) by only about 17%. Both experiments were performed in the presence of 100 μM GABA and 200 μM 5α-THDOC. The extent of potentiation by 5α-THDOC observed with the α1β2γ2L$^{K289M}$ $GABA_A$ receptor indicated that the impaired function of the mutated receptor can be partially restored by the neurosteroid.

We showed previously (*11*) that at 100 μM GABA the function of the α1β2γ2L$^{K289M}$ mutated receptor is reduced about 2-fold in comparison to the wild-type receptor. On the basis of transient kinetic investigations of the channel-opening mechanism of the $GABA_A$ receptor (*11*, *34*), the GABA concentration response curve is defined by eq 2:

$$I_A = \frac{I_{max} L^2}{(L+K_1)^2 \Phi + L^2} \quad (2)$$

$I_A$ is the observed current amplitude corrected for desensitization. $L$ denotes the concentration of the activating ligand, GABA, and $I_{max}$ is the maximum current that can be obtained from one cell when all the receptor channels are open. $K_1$ is the GABA-dissociation constant for the receptor, and $\Phi^{-1}$ is the channel-opening equilibrium constant. The following assumptions were made in deriving eq 2: The neurotransmitter concentration, $L$, is much larger than the concentration of ligand-binding sites, and these binding sites are characterized by a single dissociation constant, $K_1$ (*29*). Equation 2 can be linearized (*29*):

$$\left(\frac{I_{max}}{I_A} - 1\right)^{1/2} = \Phi^{1/2} + \frac{K_1}{L}\Phi^{1/2} \quad (2\text{-A})$$

The cell-flow method was used for determining the value of the GABA-dissociation constant ($K_1$) in the presence of 5α-THDOC. Figure 2A shows the GABA concentration−response curves for the α1β2γ2L$^{K289M}$ $GABA_A$ receptor in the presence of GABA alone (●) or in the presence of GABA and a constant concentration (150 μM) of 5α-THDOC (Δ). The solid lines are the best fits of the data to eq 2; the values of $I_{max}$, $K_1$, and the channel-opening equilibrium constant, $\Phi^{-1}$, were evaluated by nonlinear regression. In the presence of GABA alone (●) and using the value of 1.4 nA for the observed maximum whole-cell current ($I_{A,max}$) obtained in the cell-flow experiment, values of 2.2 ± 0.1 nA, 38 ± 5 μM, and 2 ± 0.6 were obtained for $I_{max}$, $K_1$, and $\Phi^{-1}$, respectively. In the presence of a constant concentration of 150 μM 5α-THDOC (Δ) and varying GABA concentrations, an experimentally observed value for $I_{A,max}$ of 1.7 nA

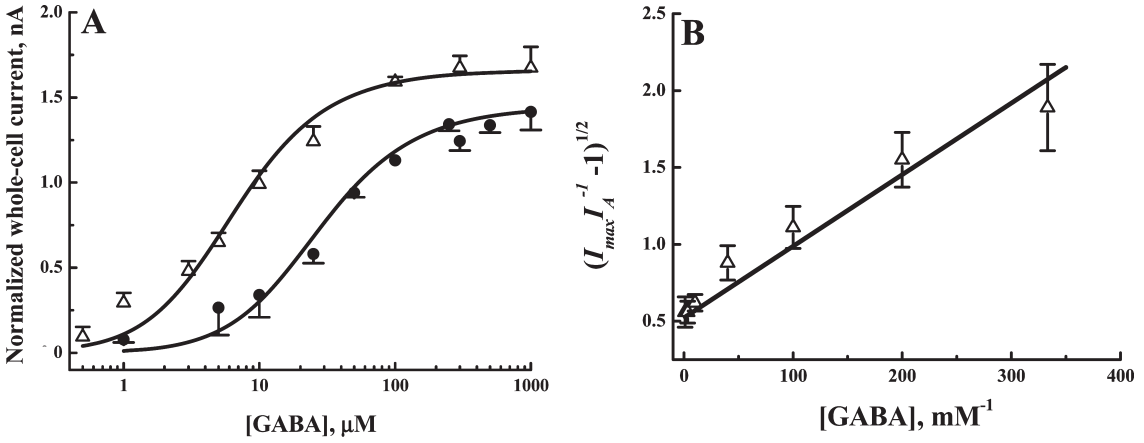


FIGURE 2: Effect of 150 $\mu$M 5α-THDOC on the GABA dose−response curve measured at −60 mV, pH 7.4, and 22 °C with HEK293 cells transiently expressing the mutated rat $\alpha1\beta2\gamma2L^{K289M}$ $GABA_A$ receptor. The data obtained in the presence of 100 $\mu$M GABA were normalized to the whole-cell current corrected for desensitization (*29*). Each data point shown in the figure is an average of 2−4 measurements made with 2−4 cells. (A) The values of the parameters used to compute the solid line (according to eq 2) for the closed symbols (●) representing the GABA concentration−response curve in the presence of GABA alone, $I_{max}$, $K_1$, and $\Phi^{-1}$, are 2.2 ± 0.1 nA, 38 ± 6 $\mu$M, and 2 ± 0.6, respectively. The values of the parameters used to compute the solid line for the open symbols (Δ) representing the GABA concentration−response curve in the presence of GABA and 150 $\mu$M 5α-THDOC, $I_{max}$, $K_1$, $\Phi_M^{-1}$, are 2.2 ± 0.1 nA, 11 ± 2 $\mu$M, 3.1 ± 0.3, respectively. (B) The GABA concentration−response curve in the presence of 150 $\mu$M 5α-THDOC (which was coapplied with GABA) presented in panel A (Δ symbols) is replotted here in a linear form according to eq 2-A. The solid line is the best fit of the data using eq 2-A. A value of 2.2 nA was used for $I_{max}$ (see panel A). The value of the channel-opening equilibrium constant $\Phi_M^{-1}$ was obtained from the ordinate intercept and is equal to 3.7 ± 1.8.

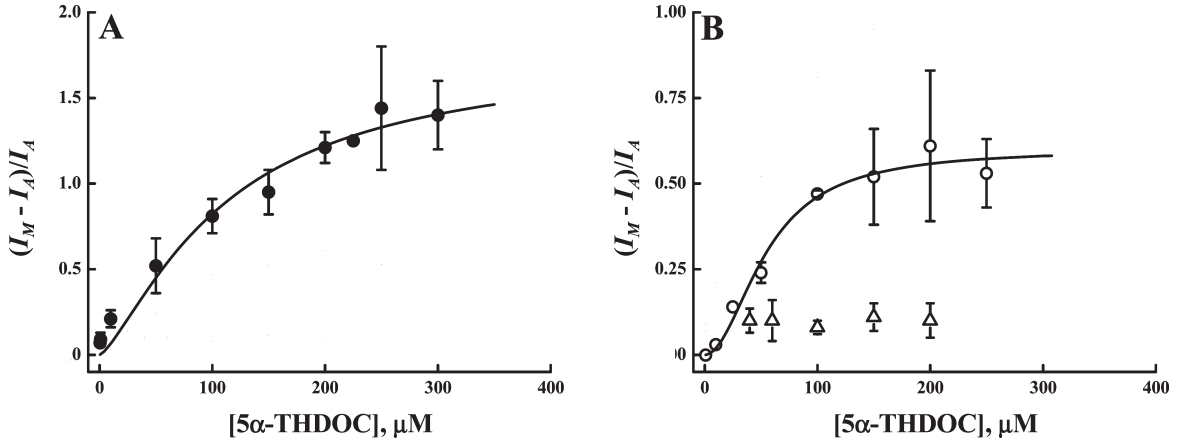


FIGURE 3: Potentiation by 5α-THDOC of the GABA-evoked current recorded from mutated $\alpha1\beta2\gamma2L^{K289M}$ $GABA_A$ receptors transiently transfected in HEK293 cells. The maximum normalized potentiation in the presence of the modulator 5α-THDOC was plotted as a function of the modulator concentration according to eq 3. Each data point is the mean of at least three measurements made with three different cells in the bath buffer (composition given in Materials and Methods) at −60 mV, pH 7.4, and 22 °C. Measurements were made with varying concentrations of the modulator 5α-THDOC, which was coapplied with 5 $\mu$M (●, Figure 3A) or 100 $\mu$M (○, Figure 3B) GABA, using a cell-flow U-tube device (*29*). The apparent dissociation constant values for 5α-THDOC of $K_M = 111 \pm 22$ $\mu$M (at 5 $\mu$M GABA) and $\overline{K_M} = 55 \pm 6$ $\mu$M (at 100 $\mu$M GABA) were determined from the best fit (solid line) of the data plotted according to eq 3. For comparison, the potentiation of 100 $\mu$M GABA-induced whole-cell currents from the wild-type $GABA_A$ receptor in the presence of varying concentrations of 5α-THDOC (Δ) is shown in Figure 3B.

(Figure 2A) was used to estimate the values for $I_{max}$, $K_1$, and $\Phi^{-1}$ of 2.2 ± 0.1 nA, 11 ± 2 $\mu$M, and 3.1 ± 0.3, respectively. In Figure 2B, a linear form of the GABA-concentration response curve in the presence of 150 $\mu$M 5α-THDOC, according to eq 2-A, is shown. From the intercept of the line the channel-opening equilibrium constant ($\Phi^{-1}$) was determined to be 3.7 ± 1.8. A value for $I_{max}$ of 2.2 nA was used in plotting the GABA-concentration response in the linear form using eq 2-A (Figure 2B).

The apparent dissociation constants of 5α-THDOC for the closed- ($K_M$) and open-channel ($\overline{K_M}$) forms of the mutated receptor in the presence of 5 $\mu$M (●, Figure 3A) and 100 $\mu$M (○, Figure 3B) GABA, respectively were determined. Whole-cell current measurements and the cell-flow method (*29*) were used. It can be calculated from the data in Figure 2B and eq 2 that at these two concentrations of GABA the fraction of receptors predominantly in the open-channel form is 0.026 and 0.51, respectively. The normalized potentiation observed in the presence of varying 5α-THDOC concentrations and either 5 $\mu$M (●, Figure 3A) or 100 $\mu$M (○, Figure 3B) GABA is plotted and the solid lines are the best fit of the data to eq 3.

$$\left(\frac{I_M - I_A}{I_A}\right) = \frac{(P_{M,max})}{1 + \left(\frac{K_M}{[M]}\right)^n} \quad (3)$$

$I_M$ is the whole-cell current recorded in the presence of either 5 or 100 $\mu$M GABA and varying concentrations of the allosteric modulator M, 5α-THDOC. $I_A$ is the whole-cell

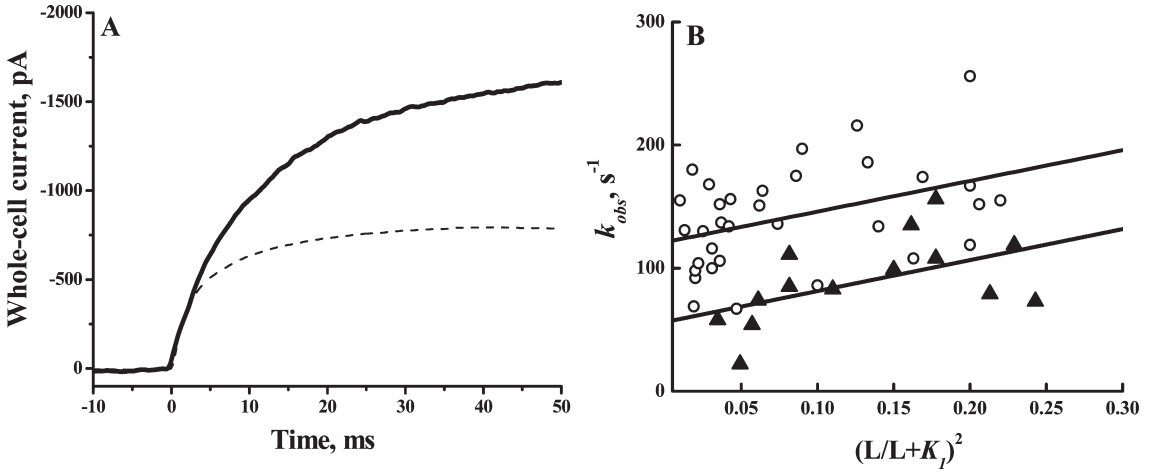


FIGURE 4: Whole-cell currents recorded from HEK293 cells transiently transfected with the mutated $\alpha1\beta2\gamma2L^{K289M}$ $GABA_A$ receptor at −60 mV, pH 7.4, and 22 °C in bath buffer, using laser-pulse photolysis of αCNB-caged GABA (*33*). (A) Currents induced by release of GABA from αCNB-caged GABA in the absence (lower dotted line) and presence (upper solid line) of 150 μM 5α-THDOC. The laser was fired at time 0 after the cells were preincubated with αCNB-caged GABA and 5α-THDOC for 400 ms. No current is observed during the preincubation, indicating the absence of free GABA before the laser is fired. The rising phase of the current is fitted by a single exponential using eq 4 (*37*). (B) Effect of 5α-THDOC on $k_{obs}$ for the current rise time determined using the LaPP technique (reviewed in ref *14*) at −60 mV, pH 7.4, and 22 °C in the bath buffer. Each measurement was made with a different cell. The observed first-order rate constant was obtained from the rising phase of the whole-cell current (eq 4) evoked by the free GABA released from αCNB-caged GABA by a pulse of laser light, either in the absence [(○), data previously reported (*11*)] or presence (▲) of 150 μM 5α-THDOC. The solid line represents the best fit of the measurements according to eq 4-A (*37*). In the presence of 5α-THDOC the values for $k_{cl(M)}$ and $k_{op(M)}$ were determined to be $56 \pm 21\ s^{-1}$ and $252 \pm 151\ s^{-1}$, respectively, while in the absence of the neurosteroid the values for $k_{cl}$ and $k_{op}$ of $121 \pm 11\ s^{-1}$ and $249 \pm 99\ s^{-1}$, respectively, were reported for the mutant receptor. The calculated *p*-values for the two data sets in panel B are 0.946 (for fitted slopes) and $< 0.0001$ (for fitted intercepts).

current measured in the presence of 5 or 100 μM GABA alone. $P_{M,max}$ is the maximum normalized potentiation observed with 150 μM 5α-THDOC in the presence of GABA. A value of 111 ± 22 μM for $K_M$ (in the presence of 5 μM GABA) or 55 ± 6 μM for $\overline{K_M}$ (in the presence of 100 μM GABA) was estimated, by using eq 3, from the data shown in Figure 3A (●, 5 μM GABA) or 3B (○, 100 μM GABA), respectively. $K_M$ and $\overline{K_M}$ are respectively the dissociation constants of the positive allosteric modulator, 5α-THDOC, from the closed- or open-channel form of the receptor (see Figure 5). In Figure 3B, the normalized potentiation (Δ) of the wild-type $GABA_A$ receptor observed with 5α-THDOC in presence of 100 μM GABA is shown for comparison. Figure 3B shows that 5α-THDOC significantly potentiates the mutated $GABA_A$ receptor (○) but not the wild-type receptor (Δ). Using eq 3, the maximum normalized potentiation ($P_{M,max}$) by 5α-THDOC of the mutated $GABA_A$ receptor at 100 μM GABA was determined to be 60 ± 3% (Figure 3B) and at 5 μM GABA was found to be 177 ± 16% (Figure 3A).

According to the mechanism of $GABA_A$ receptor activation in primary hippocampal neurons (*34*), in a LaPP experiment when [L] ≫ [A] (where [A] is the receptor concentration), the current rise follows a single exponential rate law and is described by the following equations (reviewed in ref *14*):

$$I_{(t)} = I_\infty[1 - \exp(-k_{obs}t)] \quad (4)$$

$$\text{where } k_{obs} = k_{cl} + k_{op}\left(\frac{L}{L+K_1}\right)^2 \quad (4\text{-A})$$

In eq 4, $I_\infty$ represents the current at $t = \infty$ (the maximum current in the absence of desensitization) and $k_{obs}$ is the apparent *pseudo* first-order rate constant of the current rise. The value of $k_{obs}$ is obtained from the rise time of the observed whole-cell current upon photolysis of the caged neurotransmitter in LaPP measurements. The rate constants for channel closing ($k_{cl}$) and channel opening ($k_{op}$) can be evaluated from the intercept and slope of the linear fit of eq 4A (*37*). In this equation $k_{obs}$ for the current rise is plotted versus $(L/L + K_1)^2$, where $L$ is the GABA concentration that was released from the αCNB-caged GABA by a laser-pulse and $K_1$ is the GABA-dissociation constant.

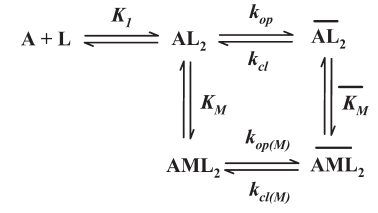


FIGURE 5: Minimum mechanism for the activation and allosteric modulation of the $GABA_A$ receptor, based on cell-flow (*29*) and LaPP (reviewed in ref *14*) measurements. A represents the active, nondesensitized receptor form, L the neurotransmitter GABA, and M the modulator, 5α-THDOC. $AML_2$ represents the complex in which the modulator is bound to the closed-channel form of the receptor. $\overline{AML_2}$ represents the modulator bound to the open-channel form of the receptor. $K_1$ is the dissociation constant of the receptor: neurotransmitter complex, and $K_M$ and $\overline{K_M}$ are the observed dissociation constants of the modulator from the closed- (A, AL, $AL_2$) and open-channel ($\overline{AL_2}$) forms of the receptor, respectively. The equilibrium constant ($\Phi^{-1}$) between the open- ($\overline{AL_2}$) and closed-channel ($AL_2$) forms is determined from $k_{op}/k_{cl}$ (*44*, *50*). $k_{op}$ is the rate constant for channel opening and $k_{cl}$ is the rate constant for channel closing. The corresponding channel-opening and channel-closing rate constants for receptor forms to which the modulator is bound are represented as $k_{op(M)}$ and $k_{cl(M)}$, respectively. It is assumed that the $K_1$ values for all the closed-channel forms (A, AL, $AL_2$) are the same. The transitions to desensitized receptor forms are not shown.

Upon photolysis of αCNB-caged GABA, whole-cell currents recorded from HEK293 cells transiently expressing the mutated $GABA_A$ receptors, in the absence (broken line) or presence (solid line) of 150 μM 5α-THDOC, are shown in Figure 4A. The rising phase of the current in the LaPP experiments (Figure 4A) follows

a single exponential for 85% of the reaction in all the measurements. The observed first-order rate constant for the current rise time, $k_{obs}$, was determined from experiments such as those shown in Figure 4A. The LaPP technique allows one to determine both $k_{op}$ and $k_{cl}$ (eq 4-A) (reviewed in ref *14*). The data in Figure 4B are plotted according to eq 4-A, where $k_{obs}$ is the observed first-order rate coefficient for the current to reach its maximum value (see Figure 4A). In absence of the neurosteroid (solid line, ○, Figure 4B), the ordinate intercept (corresponding to $k_{cl}$) has a value of 121 ± 11 $s^{-1}$ and the slope of the line (corresponding to $k_{op}$, the channel-opening rate constant) has a value of 249 ± 99 $s^{-1}$. These values correspond to a channel-opening equilibrium constant ($\Phi^{-1} = k_{op}/k_{cl}$) of 2.0 ± 0.6. In the presence of 150 $\mu$M 5α-THDOC (lower line ▲, Figure 4B) the values of the ordinate intercept (corresponding to $k_{cl(M)}$ in the presence of the modulator M), and the slope of the line (corresponding to $k_{op(M)}$ in the presence of the modulator M) are 56 ± 21 $s^{-1}$ and 252 ± 151 $s^{-1}$, respectively. These results correspond to a channel-opening equilibrium constant ($\Phi_M{}^{-1} = k_{op(M)}/k_{cl(M)}$) of 4.5 ± 3 for the mutated receptor in the presence of 150 $\mu$M 5α-THDOC, a value that is about 2-fold greater than that in the absence of 5α-THDOC.

## DISCUSSION

Previous studies of the potentiation of the wild-type $GABA_A$ receptor by neurosteroids have, by and large, focused on the potentiation of current responses at submaximal (< 1 mM) concentrations of GABA (*36*, *38*−*40*). Depending on the subunit composition of the $GABA_A$ receptor used, those studies showed that 5α-THDOC potentiates less at high GABA concentrations than at low concentrations (*40*) and can even inhibit the receptor at maximal concentrations of GABA (*39*). In the case of the wild-type α1β2γ2L $GABA_A$ receptor used in this study, at 100 $\mu$M GABA 5α-THDOC does not significantly modulate the GABA-induced current (Figures 1C and 3B). However, in the mutated (α1β2γ2$L^{K289M}$) form of this receptor, which has an unfavorable channel-opening equilibrium constant (*11*), 5α-THDOC significantly potentiates the current induced by high (100 $\mu$M) GABA concentrations (Figures 1C and 3B). 100 $\mu$M GABA elicits 83% of the current response obtained in the presence of the maximal concentration of 1 mM GABA (Figure 2A).

In the mutated $GABA_A$ receptor, a channel-opening rate constant much smaller than that observed with the wild-type counterpart (*11*), shifts the channel-opening equilibrium ($\Phi$) toward the closed-channel form of the receptor (Figure 5). At the same GABA concentration of 100 $\mu$M, the whole-cell current is approximately 2-fold lower for the mutated $GABA_A$ receptor than for the wild-type receptor (*11*). As calculated by using eq 2, at 100 $\mu$M GABA the channel-opening equilibrium constant of the mutated receptor is 5-fold smaller than that of the wild-type receptor (*11*). Thus, in the presence of an activating ligand, the fraction of receptors in the closed-channel form increases from 0.15 for the wild type to 0.49 for the mutated receptor. It is assumed that in the absence of an activating ligand the receptor-channels are closed. Investigating the single-channel burst characteristics at saturating concentrations of GABA (10 mM) yields information regarding gating kinetics of the fully liganded ($RL_2$) channels. Keramidas and Harrison (*51*) performed single-channel current measurements in the presence of saturating concentrations of GABA (10 mM) using outside-out membrane patches expressing recombinant α1β2γ2S $GABA_A$ receptors. Ten millimolar GABA induced long clusters of activity and they noted basically three distinct bursting patterns. For the high and medium burst modes, they determined the $P_0$ (intraburst or maximum open probability) to be 0.87 ± 0.02 (five patches) and 0.69 ± 0.02 (11 patches), respectively. Keramidas and Harrison (*52*) also performed GABA application to the macropatches using a piezoelectric transducer and they estimated the channel open probability ($P_0$) value to be 0.56.

The results shown in Figures 1C and 3B indicate that 5α-THDOC is able to partially restore the function of the mutated receptor. However, the significant increase in whole-cell current induced by 100 $\mu$M GABA in the presence of 5α-THDOC could be due to a higher affinity of the receptor for GABA in the presence of the neurosteroid. The GABA-dissociation constant, $K_1$, decreased from 38 ± 5 $\mu$M in the absence of 150 $\mu$M 5α-THDOC to 11 ± 2 $\mu$M in its presence (Figure 2A). The GABA-dissociation constant ($K_1$) determined in the presence of 5α-THDOC is ~3.5 times smaller than the value obtained in its absence (Figure 2A). 5α-THDOC does increase the affinity of the receptor for the neurotransmitter GABA, as reported previously for similar neurosteroids (*41*, *42*). If 5α-THDOC increased only the affinity of the receptor for GABA then it would potentiate currents elicited by low (subsaturating) concentrations of GABA but would not show an effect at high (saturating) concentrations of GABA. Increasing the receptor affinity for GABA would not lead to potentiation of the observed current at nearly saturating concentrations of the activating ligand (Figure 2A). The results described here, however, indicate that 5α-THDOC potentiation of the mutated receptor at high GABA concentration is mainly due to an increase in the channel-opening equilibrium constant.

The GABA concentration−response curve can be replotted in a linear form according to eq 2-A (Figure 2B) to obtain an estimate of the channel-opening equilibrium constant ($\Phi^{-1}$). From the ordinate intercept in cell-flow experiments, a value of 3.7 ± 1.8 was obtained for $\Phi_M{}^{-1}$, the channel-opening equilibrium constant in the presence of the neurosteroid. Apart from increasing the affinity of the receptor for GABA, 5α-THDOC increases about 2-fold the channel-opening equilibrium of the mutated receptor by shifting it toward the open-channel form of the receptor.

The results in Figure 3 indicate that the affinity of the open-channel receptor form for 5α-THDOC is about 2-fold higher ($\overline{K_M}$ = 55 ± 6 $\mu$M) than that of the closed-channel form ($K_M$ = 111 ± 22 $\mu$M). Correspondingly, the value of the channel-opening equilibrium constant, $\Phi_M{}^{-1}$, of the receptor in the presence of 150 $\mu$M 5α-THDOC is 4.5 ± 3. This is 2.2 times higher than the value obtained in the absence of 5α-THDOC (*11*). The evidence presented here indicates that the channel-opening equilibrium, which plays a central role in the noncompetitive inhibition of the excitatory nAChR and its alleviation (*43*−*45*), also plays a central role in the potentiation of the inhibitory $GABA_A$ receptor.

The results indicate that the LaPP technique (reviewed in ref *14*) is useful for determining the individual rate constants associated with steps in the opening and closing of a neurotransmitter receptor-channel. Using this technique with the mutated α1β2γ2$L^{K289M}$ $GABA_A$ receptor, the rate constants for channel opening ($k_{op}$ = 249 ± 99 $s^{-1}$) and closing ($k_{cl}$ = 121 ± 11 $s^{-1}$) were previously determined (*11*). They correspond to a channel-opening equilibrium constant ($\Phi^{-1} = k_{op}/k_{cl}$) of 2 ± 0.6, which is 5-fold lower than the value ($\Phi^{-1}$ = 10 ± 1) obtained for the

Table 1: Comparison of Kinetic Parameters Determining the Formation of Open Channels by the Wild-Type $\alpha1\beta2\gamma2L$ $GABA_A$ Receptor, and by a Mutated ($\alpha1\beta2\gamma2L^{K289M}$) Epilepsy-Linked $GABA_A$ Receptor in the Absence and Presence of 5α-THDOC[a]

| parameter | wild-type ($\alpha1\beta2\gamma2L$) $GABA_A$ receptor | mutant ($\alpha1\beta2\gamma2L^{K289M}$) $GABA_A$ receptor | |
|---|---|---|---|
| | | GABA | GABA + 150 $\mu$M 5α-THDOC |
| $K_1$ ($\mu$M) GABA-dissociation constant | $37 \pm 2$[b] | $38 \pm 5$[b] | $11 \pm 2$[b] |
| $\Phi^{-1}$ or $\Phi_M{}^{-1}$ channel-opening equilibrium constant | $10 \pm 1$[c] | $2 \pm 0.6$[c] | $4.5 \pm 3$[c] |
| $k_{cl}$ or $k_{cl(M)}$ ($s^{-1}$) channel-closing rate constant | $113 \pm 9$[c] | $121 \pm 11$[c] | $56 \pm 21$[c] |
| $k_{op}$ or $k_{op(M)}$ ($s^{-1}$) channel-opening rate constant | $1183 \pm 82$[c] | $249 \pm 99$[c] | $252 \pm 151$[c] |

[a]The number of measurements made to determine each parameter listed in this table is specified in the legends pertaining to the figures illustrating the experiments used to evaluate the parameters. The values shown for the wild-type receptor in the absence of 5α-THDOC were reported previously (*11*). The values shown for the mutated receptor in the absence of 5α-THDOC are similar to those reported previously (*11*). The receptors were transiently expressed in HEK293 cells. Measurements were made at −60 mV, pH 7.4, and 22 °C. [b]Determined from cell-flow measurements (*29*). [c]Determined from laser-pulse measurements (reviewed in *14*). We reported the value of $1183 \pm 82$ $s^{-1}$ for the channel-opening rate constant of the wild-type $\alpha1\beta2\gamma2L$ $GABA_A$ receptor (Ramakrishnan and Hess, 2004) based on transient kinetic investigations using the caged neurotransmitter. Keramidas et al. (*51*) determined the channel-opening rate constant ($k_{op}$) to be $1489 \pm 217$ $s^{-1}$ for the recombinant $\alpha1\beta2\gamma2S$ subtype of the $GABA_A$ receptor using a piezoelectric transducer driven rapid solution changer device with complete drug exchange time of 100−200 ms and solution exchange time of 250 $\mu$s. They also reported the channel closing rate constant ($k_{cl}$) to be $96 \pm 25$ $s^{-1}$ which agrees well with our result of $113 \pm 9$ $s^{-1}$ for the wild-type $GABA_A$ receptor (*11*).

wild-type $\alpha1\beta2\gamma2L$ $GABA_A$ receptor (*11*). LaPP data collected with the mutated receptor in the presence of GABA and 150 $\mu$M 5α-THDOC (▲, Figure 4B) were analyzed using eq 4-A to obtain values for $k_{cl}$ and $k_{op}$, $56 \pm 21$ $s^{-1}$ and $252 \pm 151$ $s^{-1}$, respectively (Table 1). 5α-THDOC decreases the channel-closing rate constant without significantly affecting the opening rate constant. Previous studies have indicated that neurosteroids selectively decrease the channel-closing rate constant without affecting the channel-opening rate constant (*46*, *47*). In the case of the K289M mutated receptor, this decrease in $k_{cl}$ without a change in $k_{op}$ corresponds to an increase in the channel-opening equilibrium constant ($\Phi_M{}^{-1}$) of $4.5 \pm 3$, which is 2.2 times higher than the $\Phi^{-1}$ value obtained for the mutated receptor in the absence of 5α-THDOC (Table 1). Thus, in the presence of GABA the receptor is shifted to the open-channel conformation to a greater extent when 5α-THDOC is present than in its absence. From these results it is also evident that the allosteric modulator of the $GABA_A$ receptor, 5α-THDOC, is able to partially restore the function of the mutated $GABA_A$ receptor identified in an inheritable form of human epilepsy (*3*). Mutation of equivalent lysine residues in the TM2-3 loop of α1 and β2 subunits of $GABA_A$ receptor produced asymmetric phenotypes (*53*) and the mutated $\alpha1^{K278M}\beta2\gamma2$ $GABA_A$ receptor had substantial reduction in channel open times (*53*). Investigating the mechanism of action of $GABA_A$ positive allosteric modulators such as, 5α-THDOC on such dysfunctional receptors using transient kinetic techniques will help us in finding molecules that might completely restore the function of the mutated receptors.

According to the Principle of Microscopic Reversibility (see ref *48*), for the cyclic mechanism shown in Figure 5, the ratio of the modulator's affinity constant for the closed-channel form to that of the open-channel form ($K_M/\overline{K_M}$) is equal to the ratio of the channel-opening equilibrium constant determined in the presence of the modulatory compound to the value obtained in its absence ($\Phi_M{}^{-1}/\Phi^{-1}$). The results obtained are in good agreement with this expectation. From Figure 3, the value of ($K_M/\overline{K_M}$) was found to be 2. Correspondingly, the value for $\Phi_M{}^{-1}/\Phi^{-1}$ of 2.2 obtained from the LaPP experiments (Figure 4B) is in agreement with the Principle of Microscopic Reversibility (see ref *48*).

The desensitization kinetics of the recombinant $\alpha1\beta2\gamma2L$ $GABA_A$ are very slow at low GABA (0.5−10 $\mu$M) and are represented by a single exponential (0.9 $s^{-1}$) at high GABA (25−1000 $\mu$M) concentrations. 5α-THDOC increased the receptor desensitization kinetics, as reported previously (*18*, *49*). At 100 $\mu$M GABA, fitting the falling phase of the whole-cell current recording (Figure 1B, left trace) with a single exponential function gave a desensitization rate constant of 0.9 $s^{-1}$, while 200 $\mu$M 5α-THDOC in the presence of the same GABA concentration (100 $\mu$M) increased the desensitization rate constant to 4.9 $s^{-1}$ (Figure 1B, right trace). In the presence of THDOC, the rate of desensitization does increase, as reported, and we tried to fit the falling phase of the current amplitude with one, two, or three exponentials. We determined that the current desensitization could be adequately fitted using a single exponential (4.9 $s^{-1}$).

## CONCLUSIONS

The results indicate that a change in the channel-opening equilibrium constant accounts for the mechanism by which the neurosteroid 5α-THDOC specifically alleviates the dysfunction of the mutant $\gamma2L^{K289M}$ $GABA_A$ receptor linked to an inheritable form of epilepsy (*3*, *11*). Using the cell-flow method (*29*) for whole-cell current measurements (*30*), we had shown previously that at a saturating concentration of 100 $\mu$M GABA, in HEK293 cells the ability of the mutated receptor to pass current through its channels is reduced by a factor of ∼2 in comparison to the wild-type receptor (*11*). In the present study, using the LaPP technique with a $\mu$s-ms time resolution (reviewed ref *14*), we have shown that the positive allosteric modulator of the $GABA_A$ receptor, 5α-THDOC, can restore up to ∼50% of the mutated receptor's current.

The question remains as to whether a molecule exists that can completely restore the function of the mutated $GABA_A$ receptor. This and previous (*11*, *17*, *43*−*48*) studies indicate that in cases when the channel-opening equilibrium is unfavorably affected, a simple test system for finding compounds that alleviate this dysfunction is to determine the binding affinities of the compounds to the open- and closed-channel forms of the affected receptor. The higher the binding affinities of the compound for the open channel compared to the closed-channel receptor form, the more efficient is the compound in alleviating the dysfunction of the receptor due to an unfavorable channel-opening equilibrium (*44*, *45*). Determining the affinity of potential compounds for the closed- and open-channel conformations of the receptor is a relatively simple experiment, and in the case of the nAChR led to compounds that completely alleviate the

inhibition of that receptor by noncompetitive inhibitors, such as cocaine (*44*, *45*).

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