On the Mechanism of a Mutated and Abnormally Functioning γ-Aminobutyric Acid (A) Receptor Linked to Epilepsy†

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ABSTRACT: A recent report indicates that a lysine-to-methionine mutation (K289M) in the γ2 subunit of a human γ-aminobutyric acid neurotransmitter receptor, the $GABA_A$ receptor, is linked to generalized epilepsy with febrile seizures [Baulac et al. (2001) *Nat. Genet. 28*, 46−48]. This mutation caused a decreased current response to GABA [Baulac et al. (2001) *Nat. Genet. 28*, 46−48]. Here we determine changes that occur in the mechanism of opening and closing of transmembrane channels formed by the $GABA_A$ receptor as a result of this mutation. The K289M mutation was introduced into the γ2L subunit of the rat $GABA_A$ receptor, and the mutated subunit was coexpressed with the α1 and β2 subunits in HEK293 cells. Transient kinetic techniques suitable for investigating reactions on cell surfaces with a microsecond-to-millisecond time resolution [Hess, G. P., and Grewer, C. (1998) *Methods Enzymol. 291*, 443−473] were used. They allow one to determine not only the channel-opening probability and rates of receptor desensitization but also the opening and closing rates of the mutated $GABA_A$ receptor channel. The channel-opening equilibrium constant of the mutated receptor was found to be 5-fold lower than that of the wild type. We calculated that this decrease in the channel-opening equilibrium accounts for the dysfunction of the mutated receptor. We discuss how a knowledge of the mechanism of the mutated receptor indicates an approach for alleviating this dysfunction.

Signal transmission between $\sim 10^{12}$ neurons in the nervous system is controlled by both excitatory and inhibitory neurotransmitter receptors (*1*). An imbalance in the action of the excitatory and inhibitory receptors is believed to cause excess activation of neuronal circuits and the convulsions observed in epilepsy (reviewed in ref *2*), a disease that affects ~50 million people worldwide (*3*). Many forms of epilepsy are inheritable (*4*). Reduced activity of the inhibitory γ-aminobutyric acid neurotransmitter ($GABA_A$) receptor has been implicated in epilepsy for many years (*5*). Recently, several different point mutations in the subunits of the $GABA_A$ receptor were linked to generalized epilepsy with febrile seizures plus (GEFS+),[1] childhood absence epilepsy, and juvenile myoclonic epilepsy (*6*−*8*). Some of these mutations were introduced into the rat $GABA_A$ receptor, and the mutated receptor was expressed in human embryonic kidney cells (HEK293) for investigation in greater detail (*8*, *9*). When human $GABA_A$ receptors with various point mutations were expressed in *Xenopus laevis* oocytes or HEK293 cells reduced activity was demonstrated (*6*, *8*, *9*). Specifically, on exposure to GABA the amount of current passing through the mutated receptor-channels was substantially reduced compared to the wild type.

In our experiments, we introduced the lysine-to-methionine mutation (*6*) at residue 289 (K289M) into the γ2L (long splice variant) subunit of the rat receptor and heterologously expressed it with the α1and β2 subunits in HEK293 cells. The sequences of the 428 amino acid residues in the rat and human γ2L subunits differ in only two residues; at position 81 the rat sequence contains a threonine and the human a methionine, and at position 142 there is a threonine in the rat and a serine in humans (*10*). The sequences adjacent to lysine residue 289 of the γ2L subunit are exactly the same in the rat and human receptors (Figure 1A). Lysine residue 289 is believed to occur in an extracellular loop that connects the M2 and M3 transmembrane segments of the γ2 subunit (*11*)**.** Experiments with the $\alpha 1\beta 3\gamma 2_{K289M}$ mutated receptor indicated that the mutated receptor channel can conduct the same amount of current as the wild-type receptor channel (*9*). The question we are asking is: What are the reasons for the decreased current response to GABA of the mutated receptor compared to the wild type? Because the channel-opening process and receptor desensitization are fast, transient kinetic techniques with microsecond-to-millisecond time resolutions, the laser-pulse photolysis (LaPP) (*12*, *13*) and the cell-flow (*14*) techniques, were used in our experiments to determine parameters pertaining to the channel-opening mechanism of the mutated receptor.

MATERIALS AND METHODS

Site-Directed Mutagenesis. The QuickChange site-directed mutagenesis (Stratagene, La Jolla, CA) method was used to introduce the K289M mutation in the rat γ2L-subunit of the

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[1] Abbreviations: HEK293, human embryonic kidney cells; GEFS+, generalized epilepsy with febrile seizures plus; GABA, γ-aminobutyric acid; LaPP, laser-pulse photolysis; SELEX, systematic evolution of ligands by exponential enrichment; αCNB-caged GABA, *N*-(α-carboxy-2-nitrobenzyl) GABA; nAChR, nicotinic acetylcholine receptor.

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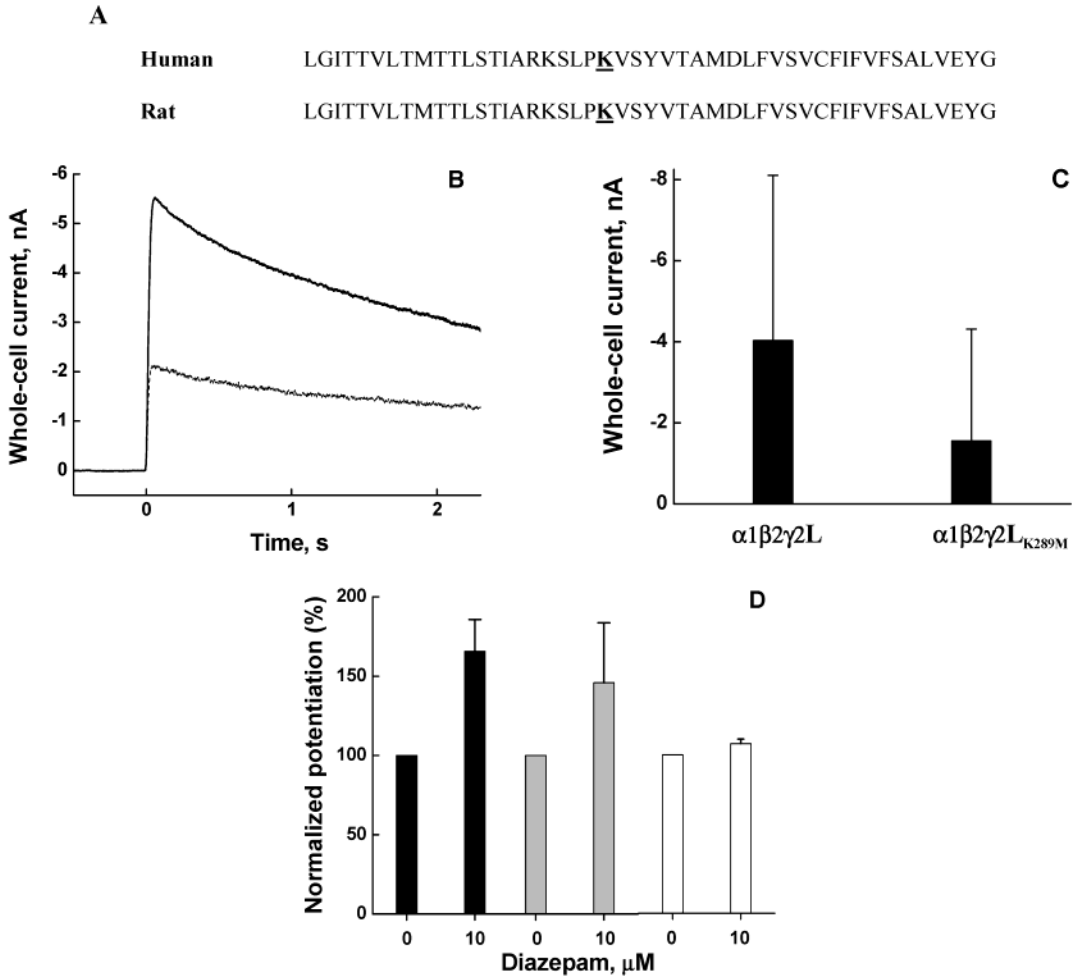

FIGURE 1: (A) Partial sequence of the γ2L subunit of the normal human (top) and rat (bottom) $GABA_A$ receptor showing the location of lysine residue 289 (K289) that is mutated to a methionine residue in the epilepsy-linked receptor. (B) Effect of the $\gamma2L_{K289M}$ mutation on GABA-induced whole-cell currents measured in HEK293 cells transiently expressing the $GABA_A$ receptor. Representative whole-cell current of the wild-type (upper line) and the mutated (lower line) $GABA_A$ receptors at −60 mV, ~22 °C, pH 7.4 and in the presence of 100 μM GABA. The cell-flow technique (*14*) with a 10-ms time resolution was used for these measurements. (C) Whole-cell current determined with the cell-flow technique was corrected for receptor desensitization as described (*14*). The experiments were conducted in the presence of 100 μM GABA at −60 mV, ~22 °C, and pH 7.4 using cells expressing either the wild-type (left) or the $\alpha1\beta2\gamma2L_{K289M}$ mutated (right) $GABA_A$ receptors. The results represent the mean ± SD of 42 measurements from 42 cells for the wild-type receptor and 30 measurements from 30 cells for the mutant receptor. The large variations in the measurements from different cells are believed to be due to variations in the number of receptors expressed per cells. (D) Diazepam potentiates the current induced by GABA in the $GABA_A$ receptor containing the γ2 subunit. 1 μM GABA was used in the experiments with the wild-type (gray symbols) and 10 μM GABA in the experiments with the mutated (black symbols) receptor and the receptor containing only the $\alpha1\beta2$ subunits (white symbols). Twenty cells were used in the experiments with the wild-type receptors, five cells in the experiments with the mutant receptors, and three cells in the experiment with the receptors containing only the $\alpha1\beta2$ subunits. The experiments were conducted at −60 mV, 22 °C, and pH 7.4.

$GABA_A$ receptor, using primers 5′ GGA AGT CTC TGC CCA TGG TCT CCT ATG TCA C 3′ (forward), and 5′ GTG ACA TAG GAG ACC ATG GGC AGA GAC TTC C 3′ (reverse) (BioResource Center, Cornell University, NY). The plasmid carrying the mutated cDNA was amplified in TOP10 *E. coli* (Invitrogen, Carlsbad, CA), and then the γ2L-subunit gene insert was sequenced (BioResource Center, Cornell University, NY) to confirm that the desired mutation was present, and to exclude any other variants that might have been introduced during PCR amplification.

Cell Culture and Transient Transfection. The cDNAs encoding the α1, β2, and γ2L subunits of the rat $GABA_A$ receptor, inserted in the pRK5 expression vector (BD Biosciences, San Jose, CA), were kindly provided by Professor P.H. Seeburg (Max Planck Institute for Medical Research, Heidelberg, Germany). Transient transfection of rat $GABA_A$ α1, β2, and γ2L cDNAs (in 1:1:3 ratio) in the HEK293 cells (American Type Cell Culture, Manassas, VA) was performed using the Effectene transfection reagent (Qiagen, Valencia, CA). Cells were cotransfected with cDNA encoding the green fluorescent protein (pGreenLantern plasmid, Life Technologies, Gaithersberg, MD) to detect transfected cells (*15*). The culture and transfection of the HEK293 cell line were as described previously (*16*). HEK293 cells were grown at 37°C in 50% CO_2 in 25-cm^2 canted-neck cell culture flasks (Corning, NY) containing the growth medium DMEM (Dulbecco's modified Eagle's medium, high glucose, Invitrogen GibcoBRL, Grand Island, NY) supplemented with 10% FBS (fetal bovine serum, Invitrogen GibcoBRL) and antibiotics (100 IU of penicillin, 100 μg/L streptomycin, both from Sigma, St. Louis, MO). Cells were passaged weekly (after reaching 80−90% confluence) and seeded with 2×10^5 cells in 35-mm Falcon dishes (Fisher Scientific) in 10% FBS and 90% DMEM containing 1%

penicillin/streptomycin. The transfection reaction mixture was added to the cells 24 h after they were passaged. After 6 h, the transfection mixture was removed from the cells, which were then washed once with 1× phosphate buffer saline (Invitrogen GibcoBRL) followed by the addition of 2 mL of 1% FBS in the DMEM growth medium. The cells were replated the following day in 35-mm Falcon dishes and were ready for electrophysiological measurements the following day. They could be used up to 48 h from the time of transfection.

Electrophysiology. For both the cell-flow (*14*) and the LaPP (*12*, *13*) experiments, the recording glass pipets 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 for whole-cell current measurements and the series resistance was 1−4 MΩ for whole-cell current measurements. Series resistance compensation of 60−70% was used in the whole-cell current-recording (*17*) 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 tetraethylammonium 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. 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 for 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. Data from each cell were normalized to the response measured with 100 μM GABA. All solutions used in the experiments were prepared on the day of measurements. GABA (5 mM) stock solution can be stored at −20 °C for several weeks. The αCNB-caged GABA stock solution (5 mM) was stored at −80 °C. During measurements, the caged GABA solution was kept on ice in brown tubes to minimize hydrolysis.

Cell-Flow and Laser-pulse Photolysis. The whole-cell current-recording technique developed by Hamill et al. (*17*) was used in conjunction with cell-flow and LaPP experiments. The cell-flow technique that allows rapid ligand application as well as a method to do desensitization correction of the observed whole-cell current has been described (*14*). Briefly, a cell (ca. 30−50 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). The flow rate of neurotransmitter solution emerging from the flow device was typically ∼1 cm/s. Dilutions of the 5 mM stock GABA solution were used to adjust the concentration of GABA in the solutions flowing over the cell. With 100 μM GABA (Sigma), the observed rise time of the whole-cell current to its maximum value, characteristic of the time for GABA to equilibrate with the cell surface receptors, was 60−100 ms. Receptor desensitization during this time can be significant. In whole-cell measurements, the current was, therefore, corrected, as described previously (*14*), for desensitization that occurs during the time it takes GABA to equilibrate with the receptors on the cell surface. 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, a time sufficient to guarantee full resensitization of the receptors (*18*).

The LaPP experiments were performed as described previously (*12*). In brief, a photolabile, biologically inactive precursor of a neurotransmitter (*12*, *19*), called a "caged neurotransmitter", was used. After equilibrating the receptors on the cell surface with the caged neurotransmitter using the cell-flow technique (*14*), free neurotransmitter was released by irradiation in the milliseond time region with a pulse of laser light. The resulting current, due to the binding of neurotransmitter leading to the opening of transmembrane channels, is measured. Several caging groups have been developed for the neurotransmitters (*12*, *19*). For the present work, the photolabile precursor, (*N*-(α-carboxy-2-nitrobenzyl) GABA (αCNB-caged GABA) (*20*), a gift from Molecular Probes (Eugene, OR) was used. It was used previously to investigate the mechanism of the normal rat hippocampal $GABA_A$ receptor (*21*). The cells were equilibrated with the caged compound for 400 ms before the laser irradiation. The concentration of caged compound ranged from 10 to 100 μM. Photocleavage of the caged GABA was initiated by a pulse of laser light from a nitrogen excimer laser ($\lambda = 337$ nm, pulse duration = 10 ns; COMPEX 101, Lambda Physik AG, Goettingen, Germany) coupled into a 300-μm core diameter optical fiber (Laser Components, Santa Rosa, CA), which delivered the laser light to the cell. The unattenuated laser energies were 100−500 μJ. 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 current corrected for receptor desensitization was recorded. After 2 min, the LaPP experiments were performed. 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 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 α-CNB caged GABA, the maximum current amplitude observed in the LaPP experiment was compared to that obtained in the cell-flow experiment performed with each cell used in the LaPP experiments. 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 (*13*, *21*, reviewed *12*). Cell-flow experiments were also conducted with the highest concentration of caged GABA (100 μM) used in the experiments

together with 25 μM GABA to determine whether the presence of αCNB-caged GABA changed the current amplitude measured in its absence. The maximum current amplitudes observed in these experiments were the same in the presence or absence of αCNB-caged GABA. The flash/flow system was controlled using the pClamp 8.0.1 software (Axon Instruments).

RESULTS

The maximum current amplitude observed in whole-cell current-recording experiments (*17*), using the cell-flow technique with a 10-ms time resolution (*14*), was considerably lower with the mutated receptor (Figure 1B, lower curve) than with the wild-type receptor (Figure 1B, upper curve). This result is in agreement with the results of Baulac et al. (*6*). Because the number of receptors expressed per cell varies, we made cell-flow measurements with 42 cells expressing the wild-type receptor and 30 cells expressing the mutated receptor. As can be seen (Figure 1C), in the presence of 100-μM GABA the maximum current amplitude of the mutated receptor is on average about half that of the wild type.

What could be the reasons for the lower activity of the mutated receptor? Was the mutated γ2L subunit expressed together with the α1 and β2 subunits in HEK 293 cells? Evidence for the presence of the γ2L subunit is presented in Figure 1D. Robust diazepam enhancement of the current response, an indication of the presence of the γ2L subunit (*10*, *22*), was observed in our experiments with both the wild-type (gray symbols) and mutated (black symbols) receptors (Figure 1D), in agreement with previous results with mutated $GABA_A$ receptors (*6*, *9*). It has been suggested that at the concentration (10 μM) of diazepam that we use, activation of the $GABA_A$ receptor may no longer be an indicator of the presence of the γ2 subunit (*23*). For this reason, we transfected the cells with only the α1 and β2 subunits of the $GABA_A$ receptor and showed that the GABA-induced current was not enhanced by the addition of 10 μM diazepam (Figure 1D, white symbols).

A possible reason for a decreased flow of ions through the channel, resulting in a decrease in the measured current, could be a decrease in the open-channel conductance. In previous experiments with the α1β3γ2 $GABA_A$ receptors, the $\gamma 2_{K289M}$ mutated receptors had the same single-channel conductance as the wild-type receptors (*9*). However, in those experiments, when the γ2 subunit containing the K289M mutation was expressed with the α1 and β3 subunits, rather than with the α1 and β2 subunits used by Baulac et al. (*6*) and in our experiments, the difference in response to GABA between the wild-type and mutated receptors was not observed (*9*). Subunit composition has previously been shown to influence the pharmacology, kinetics, and subcellular localization of $GABA_A$ receptors (*24*).

Seeking reasons for the dysfunction of the α1β2γ2L_{K289M} mutated $GABA_A$ receptor, we looked at the mechanism of the $GABA_A$ receptor-mediated reaction. The mechanism (Figure 2) is based on transient kinetic measurements made with the $GABA_A$ receptor in primary hippocampal neurons (*21*). The overall mechanism is essentially the same as that first proposed by Katz and Thesleff (*25*) for the muscle type of nAChR. In our mechanism, however, we specifically include the rate constants for channel opening and closing and the channel-opening equilibrium constant. The LaPP technique with its microsecond time resolution is uniquely suitable for determining k_{op} and, therefore, the channel-opening equilibrium constant, $\Phi^{-1} = k_{op}/k_{cl}$ (reviewed in ref *12*).

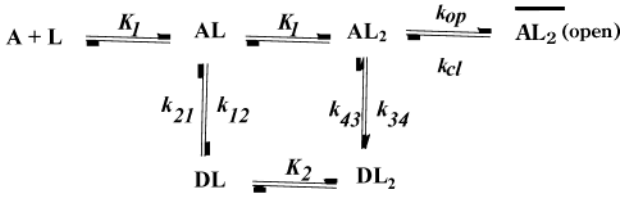

FIGURE 2: Minimum chemical mechanism for the $GABA_A$ receptor based on cell-flow (*14*) and quench-flow (*26*, *27*) measurements. A and D represent the active and inactive (desensitized) receptor forms, respectively, and K_1 and K_2 represent the receptor−neurotransmitter (L) complex dissociation constants for the A and D forms, respectively. Φ is the equilibrium constant between the closed and open forms of the receptor (*26*, *27*) and is equal to k_{cl}/k_{op}, where k_{cl} is the rate constant for channel closing and k_{op} is the rate constant for channel opening. k_{12} and k_{34} are the desensitization rate constants, and k_{21} and k_{43} the resensitization rate constants, for the AL and AL_2 forms, respectively. The same mechanism, with the exception of Φ, was first proposed for the nAChR by Katz and Thesleff (*25*).

If rapid reaction techniques are not used for the measurements, the decrease in the observed current amplitude could indicate that the mutated receptor desensitizes more rapidly than the wild type (*14*). The cell-flow measurements (Figure 1B) showed no significant difference in the desensitization rate coefficient between the wild-type and mutated receptors. At 100 μM, GABA the observed desensitization rate coefficients for the wild-type and mutated receptors were 0.6 and 0.4 s^{-1}, respectively.

In experiments with the human $GABA_A$ receptor, Baulac et al. (*6*) showed that the difference in the current amplitude between the wild-type and mutated receptors is not substantially affected by the concentration of GABA in the 10 μM to 1 mM range. The maximum current amplitudes obtained with the cell-flow technique and corrected for receptor desensitization (*14*) were plotted (Figure 3) according to eq 1 (appendix). The slopes of the lines, a measure of the dissociation constant of the neurotransmitter, indicate that the dissociation constants for the wild-type (solid symbols) and mutated (open symbols) receptors are similar: 37 ± 2 μM for the wild type and 36 ±1 μM for the mutated receptor. The values of the channel-closing equilibrium constant, Φ, are given by the square roots of the ordinate intercepts of the lines (Figure 3) (eq 1) and give indications that the value is higher for the wild-type than for the mutated receptor. The values of the channel-opening equilibrium constant, Φ^{-1} ($= k_{op}/k_{cl}$), were determined quantitatively in the experiments illustrated in Figure 4B.

The LaPP technique allows one to determine not only k_{op}, but also k_{cl} (reviewed in ref *12*). The data in Figure 4B are plotted according to eq 2 (appendix), where k_{obs} is the observed first-order rate coefficient for the current to reach its maximum value (see Figure 4A). The ordinate intercepts (corresponding to k_{cl}) of the two lines pertaining to the wild-type (closed symbols) and mutated (open symbols) receptors are essentially the same. The slopes of the lines, corresponding to k_{op}, are about 5-fold lower for the mutated receptor;

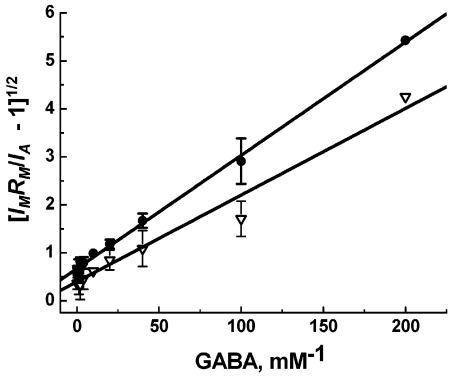

FIGURE 3: Linear fits of GABA-dose response data for the wild-type $\alpha1\beta2\gamma2L$ (●) and $\alpha1\beta2\gamma2L_{K289M}$ mutated receptor (▽). The experiments were conducted at −60 mV, 22 °C, and pH 7.4, and each data point is an average of 2−6 measurements from 2−10 different cells. The data were plotted according to the linear equation $(I_M R_M I_A^{-1} - 1)^{1/2} = \Phi^{1/2} + \Phi^{1/2} K_1 [L]^{-1}$ (eq 1, appendix). The dissociation constants evaluated from the slope of the lines are 37 ± 2 μM for the wild-type and 36 ± 1 μM for the mutated receptors, respectively. Equation 1 above was used for plotting the data and the values for $I_M R_M$ of 5.8 and 2.5 nA for the wild-type and mutated receptors, respectively, were calculated by using the observed maximum current values in the cell-flow experiments, 5.3 and 1.7 nA in the presence of 5000 μM GABA, and the values for the channel-opening equilibrium constant, Φ^{-1} of 10 and 2 obtained in the LaPP experiments shown in Figure 4B.

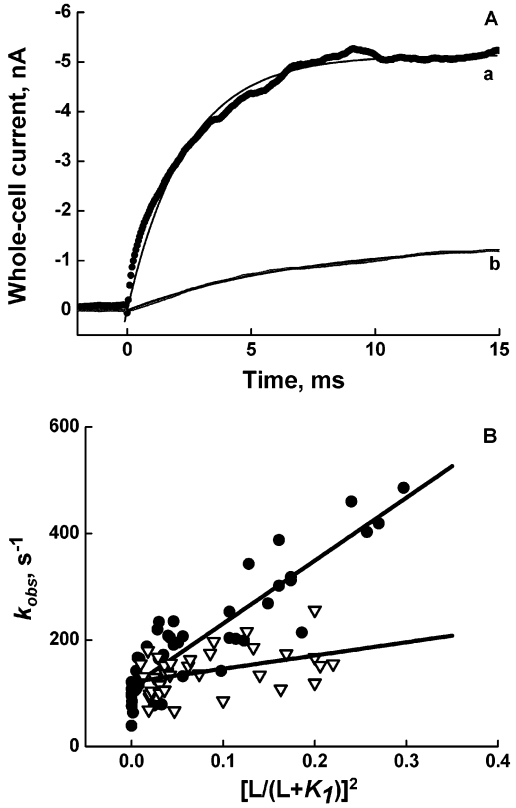

FIGURE 4: (A) Laser-pulse photolysis experiments with the wild-type (curve a) and mutated (curve b) $GABA_A$ receptors at −60 mV, 22 °C, and pH 7.4. The cells were preincubated with 100 μM caged GABA for 400 ms prior to photolysis at time 0. 35 μM free GABA was released in the case of the wild-type receptor experiment (curve a) and 33 μM in the case of the mutated receptor experiment (curve b). The rising phase of the current can be fitted by a single exponential (eq 2, appendix), and the observed rate coefficient, k_{obs}, for the current rise was determined to be 448 s^{-1} in case of the wild-type and 140 s^{-1} in the case of the mutated receptor. (B) The rate coefficient, k_{obs}, for the rising phase of the current obtained by the LaPP technique for the wild-type (●) and mutant (▽) $GABA_A$ receptors are plotted as a function of $(L/L + K_1)^2$, where L is the GABA concentration (eq 2, appendix). The parameters k_{op} and k_{cl} were evaluated by using the relationship $k_{obs} = k_{cl} + k_{op}[L/(L + K_1)]^2$. For the wild-type receptor, the total number of measurements included in the fit are 49 and 20 cells were used for collecting these data: $k_{cl} = 113 \pm 9$ s^{-1}, $k_{op} = 1183 \pm 82$ s^{-1}, $K_1 = 34 \pm 13$ μM. For the mutant receptor, the total number of measurements included in the fit are 33 and 15 cells were used for collecting these data: $k_{cl} = 121 \pm 11$ s^{-1}, $k_{op} = 249 \pm 99$ s^{-1}, and $K_1 = 39 \pm 8$ μM.

k_{op} for the mutated receptor is 249 ± 99 s^{-1} and for the wild type is 1183 ± 82 s^{-1}. These results correspond to a channel-opening equilibrium ($\Phi^{-1} = k_{op}/k_{cl}$) constant for the wild-type receptor of about 10 and the mutated receptor of about 2.

DISCUSSION

The LaPP technique was developed (*12*, *13*) to improve the time resolution of the measurements so that k_{op} as well as k_{cl}, and the other parameters pertaining to the receptor mechanism (see Figure 2), can be conveniently measured. Additionally, small cells in which a specific isoform of a neurotransmitter receptor can conveniently be expressed can be used in these measurements. The kinetic parameters we have determined for the wild-type $GABA_A$ receptor and for the $\alpha1\beta2\gamma2L_{K289M}$ mutated receptor linked to a form of epilepsy are compared in Table 1.

Do these values in Table 1 agree with the observed difference in the maximum current amplitudes of the two receptor types? The maximum observed current amplitude is given by eq 1 (appendix), which is based on the minimum kinetic mechanism of the $GABA_A$ receptor (*21*). The maximum mean observed current of the wild-type receptor in the presence of 100 μM GABA in the experiments shown in Figure 1C is 4 nA. By using eq 1 and the constants evaluated in the kinetic measurements, we calculated that for the mutated receptor in the presence of 100 μM GABA, the observed maximum current would be 2.2 nA. The maximum mean current amplitude recorded in the many measurements made with 100 μM GABA was 1.6 nA for the mutated receptor (Figure 1C).

The importance of the channel-opening equilibrium constant was first recognized in quench-flow transient kinetic experiments with nAChR-rich membrane vesicles (*26*). It was shown that a difference in the channel-opening equilibrium constant could account for the observation that different activating ligands, such as acetylcholine and carbamoylcholine, are associated with different maximum receptor-controlled flux rates of inorganic ions. The channel-opening equilibrium was consequently added as a specific step to the mechanism of the nAChR-mediated reaction (*26*, *27*) first proposed by Katz and Thesleff (*25*).

A comparison of the measurements in Table 1 with those made by using other techniques is not available at the moment. In previous experiments, excellent agreement between the results obtained with the transient kinetic techniques used here and those obtained using the single-channel current-recording technique (*28*) were measured under conditions in which slow equilibration of the ligand with the receptor did not interfere with the measurements (reviewed in ref *12*).

Table 1: Comparison of Kinetic Parameters Determining the Formation of Open Channels in the Wild-Type and Mutant $GABA_A$ Receptors Transiently Expressed in HEK293 Cells at −60 mV, pH 7.4, 22 °C[a]

parameter	wild type	mutant
K_1 (μM), GABA dissociation constant	37 ± 2[b] 34 ± 13[c]	36 ± 1[b] 39 ± 8[c]
Φ^{-1}, channel-opening equilibrium constant	10 ± 1[c]	2 ± 0.8[c]
desensitization rate constant (s^{-1}) at 100 μM GABA	0.6 ± 0.2[b]	0.4 ± 0.2[b]
k_{cl} (s^{-1})	113 ± 9[c]	121 ± 11[c]
k_{op} (s^{-1})	1183 ± 82[c]	249 ± 99[c]

[a] The number of measurements made to determine each parameter listed in this table are specified in the legends pertaining to the figures illustrating the experiments used to evaluate the parameters. [b] Determined from cell-flow measurements (*14*). [c] Determined from laser-pulse photolysis measurements (*12*, *13*).

Can we alleviate the dysfunction of the mutated $GABA_A$ receptor linked to a form of epilepsy? We have previously used transient kinetic techniques to show that noncompetitive inhibitors, such as cocaine, inhibit the nAChR by shifting the channel-opening equilibrium toward the closed-channel form (*29*−*31*). We reported (*32*) that by using the SELEX technique (*33*, *34*), the noncompetitive inhibitors can be used to isolate combinatorially synthesized RNA polymers (aptamers) with specific properties. Aptamers can be found that bind with higher affinity to the closed-channel form of the receptor and like inhibitors inhibit the receptor (*32*). However, the same procedure also results in aptamers that bind to the same site as do the noncompetitive inhibitors but, in contrast to the inhibitors, with higher affinity for the site on the open-channel form of the receptor (*35*). These aptamers prevent receptor inhibition (*35*) by shifting the channel-opening equilibrium toward the open-channel form. Analogously, one should be able to find a ligand that affects the function of the $GABA_A$ receptor, by changing its channel-opening equilibrium. In analogy with the experiments with the nAChR (*32*, *35*), such a ligand is expected to yield RNA aptamers that bind with higher affinity to an allosteric site on the open-channel form of the $GABA_A$ receptor. These aptamers are expected to alleviate the dysfunction caused by a mutation in the $GABA_A$ receptor that decreases the channel-opening equilibrium.

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APPENDIX

$$I_A = \frac{I_M R_M L^2}{(L + K_1)^2 \Phi + L^2} \quad (1)$$

and in linear form $(I_M R_M I_A^{-1} - 1)^{1/2} = \Phi^{1/2} + \Phi^{1/2} K_1 [L]^{-1}$. I_A is the current due to open receptor-channels corrected for desensitization (*14*). I_M is the current due to 1 mol of open channels, and R_M represents the number of moles of receptors in the cell membrane. L represents the molar concentration of the neurotransmitter, and K_1 is its apparent dissociation constant (*12*).

$$I_t = I_{max}[1 - \exp(-k_{obs} t)] \quad (2)$$

where $k_{obs} = k_{cl} + k_{op}[L/(L + K_1)]^2$. I_t is the measured current at time t, and I_{max} is the maximum observed current in the LaPP experiment. k_{obs} is the observed first-order rate constant for the current rise (*12*). All other constants are defined in Figure 2.

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