An Asymmetric Contribution to \( \gamma \)-Aminobutyric Type A Receptor Function of a Conserved Lysine within TM2–3 of \( \alpha_1 \), \( \beta_2 \), and \( \gamma_2 \) Subunits

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Mutations that impair the expression and/or function of \( \gamma \)-aminobutyric acid type A (GABA\(_A\)) receptors can lead to epilepsy. The familial epilepsy \( \gamma_2(K289M) \) mutation affects a basic residue conserved in the TM2–3 linker of most GABA\(_A\) subunits. We investigated the effect on expression and function of the Lys \( \rightarrow \) Met mutation in mouse \( \alpha_1(K278M), \beta_2(K274M), \) and \( \gamma_2(K289M) \) subunits. Compared with cells expressing wild-type and \( \alpha_1\beta_2(K289M) \) receptors, cells expressing \( \alpha_1(K278M)\beta_2\gamma_2 \) and \( \alpha_1\beta_2(K274M)\gamma_2 \) receptors exhibited reduced agonist-evoked current density and reduced GABA potency, with no change in single channel conductance. The low current density of \( \alpha_1\beta_2(K274M) \) receptors coincided with reduced surface expression. By contrast the surface expression of \( \alpha_1(K278M)\beta_2\gamma_2 \) receptors was similar to wild-type and \( \alpha_1\beta_2(K289M) \) receptors suggesting that the \( \alpha_1(K278M) \) impairs function. In keeping with this interpretation GABA-activated channels mediated by \( \alpha_1(K278M)\beta_2\gamma_2 \) receptors had brief open times. To a lesser extent \( \gamma_2(K289M) \) also reduced mean open time, whereas \( \beta_2(K274M) \) had no effect. We used propofol as an alternative GABA\(_A\) receptor agonist to test whether the functional deficits of mutant subunits were specific to GABA activation. Propofol was less potent as an activator of \( \alpha_1(K278M)\beta_2\gamma_2 \) receptors. By contrast, neither \( \beta_2(K274M) \) nor \( \gamma_2(K289M) \) affected the potency of propofol. The \( \beta_2(K274M) \) construct was unique in that it reduced the efficacy of propofol activation relative to GABA. These data suggest that the \( \alpha_1 \) subunit Lys-278 residue plays a pivotal role in channel gating that is not dependent on occupancy of the GABA binding site. Moreover, the conserved TM2–3 loop lysine has an asymmetric function in different GABA\(_A\) subunits.

\( \gamma \)-Aminobutyric acid type A (GABA\(_A\)) receptors belong to the homologous Cys-loop superfamily of ion channels that includes the nicotinic acetylcholine, 5-hydroxytryptamine type-3 and glycine receptors, and the Zn\(^{2+}\)-activated ion channel (1, 2). GABA\(_A\) receptors are pentameric, composed of distinct subunit classes, including \( \alpha (1–6), \beta (1–3), \gamma (1–3), \delta, \epsilon, \theta, \) and \( \pi \).

GABA\(_A\) receptors mediate rapid synaptic inhibition. Impairment of GABAergic inhibition is associated with anxiety (3) and is the basis of several models of epilepsy (4). Several recently discovered GABA\(_A\) mutations that reduce inhibition accompany hereditary forms of epilepsy (5–14). To date, the most thoroughly investigated of these are \( \gamma_2(R43Q) \) and \( \gamma_2(K289M) \), both associated with febrile seizures. Two mechanisms could account for reduced inhibition caused by these mutations: impaired receptor expression and/or function. The \( \gamma_2(R43Q) \) mutation alters receptor kinetics (13, 14), and this may contribute to inhibitory deficits. The mutation also reduces receptor biogenesis (15–18). Likewise \( \gamma_2(K289M) \) also impairs receptor function and expression (5, 14, 18).

The N-terminal \( \gamma_2 \) subunit Arg-43 residue is conserved across GABA\(_A\) receptor subunits and systematic Arg \( \rightarrow \) Gln mutation in \( \alpha_1, \beta_2, \) and \( \gamma_2 \) uncovered a general role for the arginine in receptor assembly (17). Likewise the \( \gamma_2 \) subunit Lys-289 residue is conserved in the extracellular TM2–3 loops of GABA\(_A\) and glycine receptors, suggesting that information can be revealed about its role by a similar comparative mutagenesis approach.

Mutation of the equivalent glycine receptor \( \alpha_1 \) subunit lysine is associated with hereditary hyperekplexia (19). Moreover, equivalent and/or nearby residues within the TM2–3 loops of the \( \alpha_7 \) and the \( \beta_2 \) nicotinic acetylcholine receptor subunits (20–22), \( \alpha_1 \) (23) and \( \beta_2 \) (24) GABA\(_A\) receptor subunits, the glycine receptor \( \alpha_1 \) subunit (25, 26), and 5-hydroxytryptamine type 3A receptor (27), participate in channel gating.

The mechanism by which \( \gamma_2(K289M) \) reduces channel function is controversial. Baulac and colleagues (5) reported reduced current amplitude when compared with wild-type receptors upon expression of \( \alpha_1\beta_2\gamma_2(K289M) \) receptors in Xenopus oocytes. By contrast, Bianchi and colleagues (14) described faster GABA-evoked current deactivation without altered \( \alpha_1\beta_3\gamma_2(K289M) \)-mediated peak current or activation rate when compared with wild-type receptors expressed in human embryonic kidney (HEK293) cells. Using the same cells, examining the rate of current activation following laser initiated release of caged GABA onto \( \alpha_1\beta_2\gamma_2(K289M) \) and wild-type receptors, Ramakrishnan and Hess (28) concluded that the mutation reduced current activation rate. Homology modeling of the \( \alpha_1\beta_2\gamma_2 \) receptor onto the structural model of the Torpedo marmorata nicotinic acetylcholine receptor (29) revealed an additional possibility: that the K289M mutation may reduce single channel conductance (30).

The TM2–3 region may participate in the transduction of GABA binding to channel gating by coming into close proximity with N-terminal residues. Indeed an electrostatic interaction between the lysine

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\(^{3}\) The abbreviations used are: GABA\(_A\), \( \gamma \)-aminobutyric acid type A; PBS, phosphate-buffered saline; pA, picomolar(s); pF, picofarad(s); ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay.

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residue on the α1 subunit and acidic residues in loops 2 and 7 may be responsible for intramolecular transduction, coupling GABA binding to channel opening (23).

To address the role of Lys-289 and equivalent lysine residues in the most common (α1β2γ2) GABA<sub>A</sub> receptor, we explored the effects of α1(K278M), β2(K274M), or γ2L(K289M) on receptor-surface expression and function. Each of the subunits exhibited distinct phenotypes when mutated, indicating an important but asymmetric contribution of this site to GABA<sub>A</sub> receptor function.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—COS7 cells (ATCC CRL 1651) and HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 100 μg/ml streptomycin, and 100 units/ml penicillin in an atmosphere of 5% CO<sub>2</sub>. Exponentially growing cells were transfected by electroporation (400 V, infinity resistance, 125 mF, Bio-Rad Gene Electropulser II) in the case of COS7 cells and calcium phosphate precipitation, in the case of HEK293 cells (17). Cells were transfected with equimolar ratios of GABA<sub>A</sub> subunit cDNAs. Cells were analyzed 12–18 h and 24–96 h after transfection for biochemical and electrophysiological experiments, respectively.

DNA Constructions—Murine α1, β2, and γ2L subunit cDNAs containing the myc or FLAG tag (between amino acids 4 and 5 of the mature polypeptide) have been described previously (31) and shown to be functionally silent with respect to receptor pharmacology and physiology. The mutant expression constructs α1(K278M)Myc, β2(K274M)Myc and γ2L(K289M)Myc were generated by PCR. The fidelity of the final expression constructs was verified by DNA sequencing.

Antibodies—The 9E10 antibody was obtained from 9E10 hybridoma cells (32) and used directly as supernatant without purification. Antibodies to the FLAG epitope were purchased from Sigma. The secondary antibodies, goat anti-mouse Alexa Fluor 568 and goat anti-mouse Alexa Fluor 488, were purchased from Molecular Probes (UK), and goat anti-mouse horseradish peroxidase from Amersham Biosciences.

Immunofluorescence—COS7 cells were fixed in 3% paraformaldehyde (in PBS) and washed twice in 50 mM NH<sub>4</sub>Cl (in PBS) and blocked (10% fetal bovine serum, 0.5% bovine serum albumin in PBS) for 30 min. Subsequent washes and antibody dilutions were performed in PBS containing 10% fetal bovine serum and 0.5% bovine serum albumin. Following surface labeling, cells were permeabilized by the addition of 0.5% Triton X-100 (10 min), and the immunofluorescence protocol was repeated from the NH<sub>4</sub>Cl step. Cells were examined using a wide-field imaging system (Improvision).

Quantification of Cell-Surface Expression—COS7 cells were plated into 96-well dishes. Eight transfections were used per dish (12 wells per transfection, with nine determinants for each condition). Cells were fixed in 3% paraformaldehyde (in PBS). Cell-surface detection was performed in the absence of detergent, and total expression levels were determined following Triton X-100 (0.5%, 15 min) treatment. Cells were washed twice in 50 mM NH<sub>4</sub>Cl (in PBS) and blocked (10% fetal bovine serum, 0.5% bovine serum albumin in PBS) for 1 h. Subsequent washes were performed in block. Receptor expression was determined using an horseradish peroxidase-conjugated secondary antibody and assayed using 3,3′,5,5′-tetramethylenediamidine (Sigma) as the substrate, with detection at 450 nm after 30 min, following the addition of 0.5 M H<sub>2</sub>SO<sub>4</sub>. The reaction rate was determined to remain linear for up to 1 h.

Electrophysiology—The whole cell patch clamp technique was used to record GABA-activated currents from HEK293 cells voltage-clamped at −60 mV. GABA (100 μM) was applied by local pressure ejection from low resistance micropipettes (33). In experiments investigating the modulation of GABA-evoked currents by bath applied flunitrazepam, GABA was applied for 1 s at −EC<sub>50</sub> concentrations. Data for concentration-response relationships were recorded by applying GABA or propofol for 4 s. The recording chamber was continuously perfused (5 ml/min) with an extracellular solution comprised of (in mM) NaCl, 140; KCl, 4.7; MgCl<sub>2</sub>, 1.2; CaCl<sub>2</sub>, 2.5; glucose, 10; and HEPES-NaOH, 10 (pH 7.4). The electrode solution contained (in mM): CsCl, 140; MgCl<sub>2</sub>, 2.0; EGTA, 11; ATP (Mg<sup>2+</sup>) salt) 3; and HEPES-CsOH, 10 (pH 7.4). Junction potentials were nullled with an open electrode in the recording chamber prior to each experiment. The liquid junction potential was trivial (−2 mV), and its inappropriate compensation was ignored. Experiments were performed at room temperature (20–24 °C). Macroscopic GABA-evoked currents were monitored by an Axopatch-200B amplifier, low pass filtered with a cut-off frequency of 2 KHz, and then recorded and digitized using a Digidata 1320A interface (Axon Instruments, Union City, CA) for acquisition at 10 kHz onto the hard drive of a personal computer. Currents were averaged and measured using pCLAMP 8.0 software (Axon Instruments).

Single Channel Recording—Single channel currents recorded from cell-attached and outside-out patches were low-pass filtered at 2 and 1 KHz, respectively (digitized at 10 KHz). Data were acquired as described previously (34). GABA was either applied to outside-out patches at 1 μM or 1 mM; there was no significant difference in the observed single channel conductances. GABA (1 mM) was applied to cell-attached patches through the recording electrode, which contained extracellular solution. Patches were voltage-clamped using electrode potentials provided in the figure legends. Sections of digitized data in which unitary events predominated were selected for analysis and were leak subtracted using Clampfit for the creation of all-points amplitude histograms and event lists using Fetchan (pCLAMP 8.0, Axon Instruments).

Analysis of Whole Cell Data—Graphs of GABA concentration-response relationships were fitted using the Hill equation as described previously (33). For fitting propofol concentration-response relationships (normalized to maximum GABA-evoked current) the Hill equation was modified as in Equation 1.

\[
\frac{I_{\text{Prop}}}{I_{\text{GMax}}} = \frac{I_{\text{Prop}}}{I_{\text{GMax}}} \left(1 + \frac{[\text{EC}_{50}]}{[\text{Prop}]} \right)^{-H}
\]

(Eq 1)

In this equation the whole cell current amplitude activated by propofol (\(I_{\text{Prop}}\)) is normalized to that activated by 10 mM GABA (\(I_{\text{GMax}}\)). \(I_{\text{GMax}}\) is the maximum amplitude of the propofol activated current relative to \(I_{\text{GMax}}\). \(\text{EC}_{50}\) is the concentration of propofol required to activate half of the maximum \(I_{\text{Prop}}\) and \(H\) is the slope factor of the concentration-response relationship.

Current density measurements were calculated from each cell by dividing the peak GABA- or propofol-activated current amplitude (measured in picocur (pA)) by the cell’s capacitance (measured in picofarads (pF)).

Analysis of Single Channel Data—All-points amplitude histograms for single channel recordings were fitted with multiple Gaussians (least squares minimization) to amplitude histograms using the Simplex method within pSTAT (pCLAMP 8.0). The amplitude of the single channel current recorded from each patch was determined from the difference between the mean current amplitudes determined from the Gaussians fitted to the closed- and unitary open-state currents. Single channel conductances are reported as the chord conductance derived as \(\gamma = i/(V_m - E_{rev})\), where \(i\) is unitary current amplitude, \(V_m\)
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The Role of Lysine at the Homologous Position in the α1 Subunit on Transport to the Cell Surface—To determine, qualitatively, the ability of the α1(K278M) subunit to access the cell surface, we examined its cellular distribution when expressed in COS7 cells. COS7 cells were used in these studies due to their clear definition of intracellular compartments (38). The existence of surface receptors was determined in the absence of detergent using anti-Myc antibodies and Alexa Fluor 488 secondary antibodies. Following permeabilization, cells were re-probed as above, using Alexa Fluor 568 secondary antibodies. As observed previously for wild-type α1<sup>Myc</sup> (31), the α1(K278M)<sup>Myc</sup> subunit could not access the cell surface when expressed alone (data not shown). When α1<sup>Myc</sup> was co-expressed with the β2 subunit, there was robust cell-surface staining (Fig. 2A, upper right panel). Likewise, the co-expression of α1(K278M)<sup>Myc</sup> with the β2 subunit produced robust cell-surface labeling (Fig. 2A, α<sub>β</sub>, lower right panel) as well as strong intracellular labeling. Identical results were observed when α1(K278M)<sup>Myc</sup> was co-expressed with β2 and γ2L<sup>FLAG</sup> subunits and immunofluorescence was performed via the FLAG epitope on the γ2L subunit (Fig. 2B). Because the γ2L subunit cannot access the surface in the absence of either the α1 or β2 subunits (31), its surface expression is a faithful indicator of the presence of α1β2γ2L receptors. From hereafter ‘γ2’ refers to the γ2L subunit, which was used throughout this study.

To quantify our observations, we used the cell ELISA technique to compare surface and total expression levels. Cell-surface expression (in the absence of detergent) of the α1(K278M)<sup>Myc</sup> is presented as a percentage of total (in the presence of detergent) levels and normalized to wild-type controls (α1<sup>Myc</sup>) performed in parallel. Using this approach (Fig. 2C), the cell-surface level for α1(K278M)<sup>Myc</sup> β2 receptors was 66 ± 11%, compared with the normalized wild-type α1<sup>Myc</sup> β2 receptor level of 100 ± 11%. Similarly, the cell-surface level for α1(K278M)<sup>Myc</sup> β2γ2<sup>FLAG</sup> receptors was determined (via FLAG epitope) to be 87 ± 34%, compared with the normalized wild-type α1<sup>Myc</sup> β2γ2<sup>FLAG</sup> receptors at 100 ± 13%. Thus, the presence of K278M in the α1 subunit does not have a major impact on biogenesis or the surface transport of α1(K278M)β2γ2 receptors.

The Role of Lysine at the Homologous Position in the β2 Subunit on Transport to the Cell Surface—To determine, qualitatively, the ability of the β2(K274M) subunit to access the cell surface, we examined its cellular distribution when expressed in COS7 cells. As observed previously for wild-type β2<sup>Myc</sup> (31), the β2(K274M)<sup>Myc</sup> subunit could not access the cell surface when expressed alone (data not shown). When β2<sup>Myc</sup> was co-expressed with the α1 subunit, there was robust cell-surface staining (Fig. 3A, upper right panel). In contrast, when β2(K274M)<sup>Myc</sup> was co-expressed with the α1 subunit there was no cell-surface labeling (Fig. 3A, αβ<sub>2</sub>; lower right panel). Instead, strong intracellular labeling within the endoplasmic reticulum was observed, as evidenced by the characteristic reticular pattern, typically observed in COS7 cells (38). We obtained identical results when co-expressing β2(K274M)<sup>Myc</sup> with the α1 and γ2<sup>FLAG</sup> subunits, performing immunofluorescence via the FLAG epitope on the γ2 subunit to detect the presence of all three subunits (Fig. 3B). Quantification of these findings, using cell ELISA (Fig. 3C), revealed the cell-surface level for α1β2(K274M)<sup>Myc</sup> receptors to be 4.2 ± 18.9%, compared with the normalized wild-type α1β2<sup>Myc</sup> receptor at 100 ± 11%. Similarly, the cell-surface level of the α1β2(K274M)<sup>Myc</sup>γ2<sup>FLAG</sup> receptor was determined (via FLAG epitope)
to be 12.7 ± 12.6%, compared with the normalized wild-type α1βγβ2γ2FLAC receptor level of 100 ± 31%. Thus, the presence of K274M in the β2 subunit perturbs receptor distribution, preventing the significant expression of cell-surface α1β2(K274M)γ2 receptors.

The Role of Lysine in the γ2 Subunit on Transport to the Cell Surface—To determine, qualitatively, the ability of the γ2(K289M) to access the cell surface, we examined its cellular distribution when expressed in COS7 cells. As observed previously for wild-type γ2FLAC (31), the γ2(K289M)FLAC subunit could not access the cell surface when expressed alone, or in the presence of α1 or β2 subunits (data not shown). Co-expression of γ2FLAC with α1 and β2 subunits produced robust cell-surface staining (Fig. 4A, upper right panel). Likewise, co-expression of γ2(K289M)FLAC with α1 and β2 subunits also produced robust cell-surface labeling (Fig. 4A, αβγ; lower right panel) as well as strong intracellular labeling. To quantify these observations, we used the cell ELISA technique to compare surface and total expression levels. Cell-surface expression (in the absence of detergent) of the α1β2γ2(K289M)FLAC receptor was 108 ± 26%, compared with the normalized wild-type α1β2γ2FLAC receptor level of 100 ± 31%. Thus, the presence of the epilepsy mutation K289M in the β2 subunits perturbed receptor distribution, preventing the significant expression of cell-surface α1β2(K289M)γ2 receptors.

Asymmetric Effects of Mutant α1(K278M), β2(K274M), and γ2(K289M) Subunits on the GABA Concentration-Response Relationship—We transiently expressed wild-type or Lys → Met mutant α1, β2, and γ2...
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GABA<sub>A</sub> receptor subunits and used the whole cell patch clamp technique to characterize their functional properties in HEK293 cells voltage-clamped at −60 mV. Receptors containing the mutant γ2(K289M) subunit combined with wild-type α1 and β2 subunits exhibited a similar GABA concentration-response relationship compared with wild-type α1β2γ receptors (Fig. 5A). The EC<sub>50</sub> values were 10.5 ± 0.3 μM (n = 6) for the wild-type and 8.2 ± 1.0 μM (n = 5) for the α1β2γ(K289M) mutant receptor. Next we compared peak GABA-activated current densities (calculated by normalizing maximum current amplitudes to cell membrane capacitances) in HEK293 cells expressing α1β2γ(K289M) receptors. The EC<sub>50</sub> values were 10.5 ± 3 μM (n = 5) for the wild-type 2(K278M) channel current amplitude and voltage, with no difference in their equilibrium potentials (Fig. 6F). GABA-activated single channels mediated by α1β2γ(K289M) and wild-type α1β2γ receptors (Fig. 5C).

We next examined the effect of the equivalent Lys → Met mutation in the α1 and β2 subunits. Unlike the γ2(K289M) mutant, both the α1(K278M) and β2(K274M) mutants caused a significant rightward shift in the GABA concentration-response relationships of α1(K278M)β2γ and α1β2(K274M)γ receptors, respectively (Fig. 5A). The EC<sub>50</sub> values were 42 ± 6 μM (n = 5) for the α1(K278M)β2γ receptor and 55 ± 3 μM (n = 5) for the α1β2(K274M)γ receptor. In both cases the mutant subunits caused a substantial decline in the current density, once again the β2(K274M) mutant had the most deleterious effect (Fig. 5B). Neither the α1(K278M) nor the β2(K274M) mutant subunit altered the potentiation of GABA-evoked currents by flunitrazepam (100 nM) (Fig. 5C).

α1(K278M), β2(K274M), and γ2(K289M) Mutations Have No Effect on Single Channel Conductance—Structural models of the α1β2γ GABA<sub>A</sub> receptor place the conserved TM2–3 loop lysine residue in the outer vestibule where it could contribute to the conduction pathway (30). Such a location could imply a role for the basic residue in ion conduction in which case its replacement by the unchanged methionine would be expected to cause a reduction in single channel conductance. Assuming a stoichiometry of 2α1β2γ1 and one would expect such an effect to be greatest when the Lys → Met mutation is present in either α or β subunits (39). We tested this hypothesis by recording single GABA-activated channels from outside-out patches excised from cells expressing α1β2γ, α1(K278M)β2γ, α1β2(K274M)γ, and α1β2γ(K289M) receptors. In all cases there was a linear relationship between single channel current amplitude and voltage, with no difference in their equilibrium potentials (Fig. 6F). In all cases GABA-activated single channels with similar amplitudes were recorded from patches at −60 mV (Fig. 6, A−D). The chord conductances for unitary events mediated by α1β2γ, α1(K278M)β2γ, α1β2(K274M)γ, and α1β2γ(K289M) receptors

\[ \text{Chord Conductance} \]
The effect on single channel conductance of mutating the conserved TM2–3 loop lysine to methionine. A–D, examples of channels activated by GABA (1 mM) applied transiently to outside-out patches excised from cells expressing α1β2γ2, α1(K278M)β2γ2, α1β2(K274M)γ2, and α1β2γ2(K289M) receptors, respectively. Patches were voltage-clamped at −60 mV. All-points amplitude histograms for sections of data containing predominantly single channel openings were fitted with the sum of two Gaussians, representing closed and open state current distributions. Current amplitudes in the examples shown in A–D were −1.6, −1.7, −1.8, and −1.5 pA, respectively. E, single channel currents mediated by α1β2γ2 (circles), α1(K278M)β2γ2 (triangles), α1β2(K274M)γ2 (squares), and α1β2γ2(K289M) (diamonds) receptors exhibited a linear relationship to voltage and reversed near the Cl⁻ equilibrium potential. F, cord conductances determined from data (n = 4), including those illustrated in A–D were not significantly different for any of the wild-type or mutant receptors. No significant difference (p > 0.05) was found using ANOVA with posthoc Tukey’s test.

Asymmetric Effects of Mutant α1(K278M), β2(K274M), and γ2(K289M) Subunits on Channel Open Time—The Lys → Met mutation in the γ2 subunit increases the deactivation rate of macroscopic GABA-activated currents and shortens single channel mean open time in outside-out patch recordings (14). We examined the effect of the Lys → Met mutation in α1, β2, and γ2 subunits on open time of single channels activated by GABA (1 mM) in cell-attached patches (Fig. 7). This approach ensures steady-state exposure of receptors to a saturating concentration of GABA. Stretches of data were analyzed in which unitary events occurred >90% of the time. Three methods for comparing channel open time of wild-type and mutant receptors revealed that both α1(K278M) and γ2(K289M) made openings briefer, whereas β2(K274M) had no effect (Fig. 7). The open time histogram generated from events mediated by wild-type α1β2γ2 receptors, when fitted with the sum of three exponentials (14, 37), revealed three time constants (τ1–3) representing open times of brief, medium duration, and long lasting events (Fig. 7A). The average open time of α1β2γ2 receptors was 3.9 ± 0.3 ms (n = 4) corresponding to the midpoint of the cumulative distribution curve (Fig. 7, E and F) a less conventional method of displaying the data that nevertheless provides a convenient comparison of the full data range (35). By contrast to wild-type receptors, α1(K278M)β2γ2 receptors exhibited brief openings with only a minor contribution of long lasting events represented by the τ3 component (Fig. 7B). The mean open time of α1(K278M)β2γ2 receptors (1.4 ± 0.4 ms, n = 7) was significantly briefer (p < 0.001) than the mean open times of both wild-type α1β2γ2 receptors and mutant α1β2(K274M)γ2 receptors (3.5 ± 0.4 ms, n = 5) (Fig. 7F). The open times of α1β2(K274M)γ2 receptors were similar to those of wild-type receptors suggesting that the conserved lysine in the β2 subunit does not participate in gating kinetics. By contrast, but in agreement with a previous report (14), α1β2γ2(K289M) receptors have disrupted gating kinetics (Fig. 7D) with a briefer mean open time (2.4 ± 0.2 ms, n = 8) compared with both wild-type α1β2γ2 receptors and...
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FIGURE 8. The effect on direct activation by propofol of mutating the conserved TM2–3 loop lysine to methionine. A, currents activated by propofol (3, 10, 30, 100, and 300 μM) applied to HEK293 cells expressing α1β2γ2 (top) and α1(K278M)β2γ2 receptors (bottom). Arrows indicate surge currents seen upon cessation of propofol (300 μM) administration. B, concentration-dependent activation and block of α1β2γ2 (filled circles) and α1(K278M)β2γ2 (open circles) receptors by propofol normalized to GABA (10 mM)-evoked current amplitudes recorded from the same cells. Data points (excluding 300 μM) were fitted with the Hill equation (“Experimental Procedures”) and values of EC<sub>50</sub>, slope, and efficacy relative to GABA are provided in the text. C, concentration-dependent activation and block of α1β2(K274M)γ2 (filled triangles) and α1β2γ2(K289M) receptors by propofol normalized to GABA (10 mM)-evoked current amplitudes recorded from the same cells. The gray line represents the Hill equation fit to the wild-type α1β2γ2 data in B, provided here for comparison. Data were fitted as described in B, and values are provided in the text. D, maximal propofol-activated current densities expressed as current amplitude (picoamps (pA)) normalized to cell membrane capacitance (picofarads (pF)). Statistical analyses by ANOVA with posthoc Tukey’s test: ∗, current density significantly below that of the wild-type α1β2γ2 (p < 0.05).

mutant α1β2(K274M)γ2 receptors (p < 0.05, Fig. 7F). These data demonstrate that the Lys → Met mutation in either the α1 or γ2 subunits disrupts gating kinetics culminating in a reduction in mean GABA<sub>A</sub>-gated channel open time.

Asymmetric Effects of Mutant α1(K278M), β2(K274M), and γ2(K289M) Subunits on the Propofol Concentration-Response Relationship—Mutation of Lys-278 and Lys-274 residues in α1 and β2 subunits, respectively, may disrupt the intramolecular transduction of GABA binding to channel gating (23). If this is the case, such mutations may not affect the ability of anesthetics, such as propofol, to directly activate the GABA<sub>A</sub> receptor (40) through sites distinct from the GABA binding site (41). We examined the ability of propofol relative to GABA to activate α1β2γ2, α1(K278M)β2γ2, α1β2(K274M)γ2, and α1β2γ2(K289M) receptors. Propofol activates GABA<sub>A</sub> receptors at concentrations between 3 and 100 μM. The blocking effect of propofol (33, 40, 41) reduces the amplitude of the propofol (300 μM)-activated current and is associated with a pronounced surge current upon cessation of application (Fig. 8A). The blocking effect complicates attempts to determine the EC<sub>50</sub> of propofol (Fig. 8, B and C). Surge currents were negligible following the application of 100 μM propofol suggesting that there is minimal blockade with this concentration. Therefore we fitted data points between 3 and 100 μM propofol with the modified Hill equation to obtain estimates of EC<sub>50</sub> values and the efficacy of propofol relative to GABA as an activator of current. We excluded the final data point obtained with 300 μM propofol due to blockade associated with this concentration. Using this approach the EC<sub>50</sub> value for propofol as an activator of α1β2γ2 receptors was 32 ± 3 μM (slope factor = 2.0 ± 0.3) (Fig. 8B). The apparent maximum efficacy of propofol, relative to GABA (10 mM), was 56 ± 3%. The mutant α1(K278M) subunit reduced the apparent potency of propofol, shifting the concentration-response relationship to the right (Fig. 8B) without altering the maximum current amplitude activated by propofol (100 μM) relative to GABA (10 mM). Assuming an unaltered maximum efficacy of propofol relative to GABA (56%) the fit to the propofol concentration-response relationship provided an estimate of the EC<sub>50</sub> of propofol as an agonist of the α1(K278M)β2γ2 receptor of 68 ± 2 μM (slope factor = 3.7 ± 0.2).

The EC<sub>50</sub> values for propofol as an activator of α1β2(K274M)γ2 and α1β2γ2(K289M) receptors were similar to that of the wild-type receptor: 31 ± 4 (slope factor = 3 ± 1) and 31 ± 2 μM (slope factor = 3 ± 1), respectively (Fig. 8C). The fits yielded the following values for the efficacy of propofol relative to GABA as an agonist at α1β2(K274M)γ2 and α1β2γ2(K289M) receptors: 25 ± 2% and 62 ± 4%, respectively. Interestingly, although the Lys → Met mutation in the β2 subunit had no effect on the potency of propofol as an agonist, it caused a significant reduction in the efficacy of propofol (100 μM) as an agonist relative to GABA (10 mM) compared with the wild-type receptor (p < 0.05).

We determined the propofol current density by expressing peak propofol-activated current amplitudes as a function of cell capacitance (Fig. 8D). The pattern of propofol current densities for wild-type and mutant receptors resembles that seen for GABA current densities (Fig. 5B).

DISCUSSION

We examined the impact of replacing a TM2–3 loop lysine residue by methionine in α1(K278M), β2(K274M), and γ2(K289M) subunits on GABA<sub>A</sub> receptor surface expression and function. The γ2(K289M) mutation is associated with hereditary epilepsy characterized by febrile seizures (5). By introducing the Lys → Met mutation into each of the three major GABA<sub>A</sub> subunits expressed in the brain we examined whether the role of the homologous lysine was similar at each position in the heteropentamer.

We assayed surface expression of epitope-tagged receptors qualitatively and quantitatively using epifluorescence microscopy and ELISA approaches, respectively. Surface expression was compared with functional receptor expression by measuring GABA-activated current density. We used outside-out and cell-attached patch recording configurations to evaluate the effects of the Lys → Met mutation on GABA-
activated single channel conductance and open times, respectively. Finally we examined whether deficits in channel function associated with mutant subunits were specific to GABA activation or generalized to activation by propofol, a GABA<sub>α</sub> receptor agonist that acts through a site distinct from that of GABA (41).

Using these varied approaches we found that the conserved lysine within the extracellular TM2–3 loop plays an asymmetric role in GABA<sub>α</sub> receptor function and expression that is dependent on the subunit in which it is located.

The α1(K278M) and γ2(K289M) constructs had no effect on the level of receptor surface expression, whereas β2(K274M) caused a marked reduction in cell-surface receptors. A recent report demonstrates that γ2S(K289M) has a temperature-dependent effect on GABA<sub>α</sub> receptor expression. Elevating the temperature of HEK293 cells from 37 to 40 °C reduced cell-surface expression of α1β2γ2S receptors, an effect that was more pronounced in the presence of γ2S(K289M). This temperature-dependent effect may participate in the deficit in inhibitory signaling underlying febrile seizures in individuals harboring the mutation (18).

Our functional studies were performed at room temperature, and cell-surface expression assays were performed on cells cultured at 37 °C prior to fixation. Furthermore it is worth noting that we used the γ2L subunit variant, which may have different trafficking properties compared with those of γ2S.

Unaltered surface expression of α1β2γ2(K289M) receptors compared with wild-type receptors coincided with unaltered GABA-evoked current density. By contrast, despite having no effect on surface expression, α1(K278M) reduced GABA-evoked current density. Two mechanisms contribute to current density: expression levels of cell-surface receptors and GABA<sub>α</sub> receptor function. Because there was no difference in the surface expression of α1β2γ2S receptors compared with wild-type receptors, reduced current density must result from a functional deficit. Consistent with this assertion GABA had a substantially reduced apparent potency as an activator of α1(K278M)β2γ2 receptors compared with wild-type receptors; the GABA concentration–response relationship was shifted to the right, reflecting a 4-fold increase in the GABA EC<sub>50</sub>. Such a reduction in the potency of GABA could be caused by reduced binding affinity, impaired transduction of GABA-binding to channel activation, or both (42). Analysis of single channels activated in cell-attached patches identified a deficit in channel function caused by α1(K278M): the durations of channel openings were substantially reduced compared with those of wild-type receptors. This functional deficit presumably reduces the efficacy of GABA leading to a diminished current density.

In contrast to wild-type α1β2γ2, α1β2γ2(K289M), and α1(K278M)-β2γ2 receptors, α1β2(K274M)γ2γ2 receptors had substantially reduced surface expression, suggesting that the β2 TM2–3 region may be important in receptor biogenesis and/or transport. The consequence of reduced surface expression of α1β2(K274M)γ2γ2 receptors is a dramatic reduction in current density. GABA-activated channels mediated by α1β2(K274M)γ2γ2 receptors were indistinguishable from those mediated by wild-type receptors in terms of their open times, suggesting a lack of a role for the β2 Lys-274 in gating by GABA. However, the GABA concentration–response relationship was shifted to the right by β2(K274M) consistent with a reduced GABA potency. It seems likely that this shift is caused by a reduction in GABA affinity for the mutant receptor.

Replacement of the conserved TM2–3 loop lysine by either alanine (43) or aspartate (23, 24) in either the α1 or the β2 subunit also increases the GABA EC<sub>50</sub>. In the case of the human α1(K279D) subunit (equivalent to mouse α1(K278)) the deficit in receptor function could be rescued by simultaneously introducing either D57K or D149K mutations into the subunit’s N-terminal domain loops 2 and 7, respectively (23). These data support the hypothesis that, upon activation of wild-type receptors by GABA, there is an interaction between acidic residues in the N-terminal domain of the α1 subunit and the lysine in the TM2–3 loop. This mechanism appears to be unique to the α1 subunit of the GABA<sub>α</sub> receptor, because similar simultaneous charge reversals in the β2 subunit fail to recover the reduced GABA potency induced by the β2(K274D) mutation (24). This is an intriguing difference that again demonstrates asymmetry in the role of the conserved TM2–3 lysine in GABA<sub>α</sub> receptor function. The failure of charge reversal to recover the potency of activation of α1β2(K274D)γ2 receptors for GABA may also point to a role of β2(K274) in GABA binding affinity rather than receptor activation.

The γ2(K289M) mutant reduced GABA-gated channel open time albeit to a lesser extent than did α1(K278M). The attenuation of open time induced by γ2(K289M) was not sufficient to significantly impact current density. Our data examining α1β2γ2(K289M) receptors in cell-attached patches agree with those previously reported for α1β3γ2(K289M) receptors in outside-out patches (14) suggesting that decreased inhibition underlying epilepsy associated with the mutation could be caused in part by briefer channel openings.

Interestingly, none of the mutant subunits significantly altered potentiation by flunitrazepam. Similarly there was no difference in the potentiation by a maximal concentration of diazepam of GABA-evoked currents mediated by wild-type α1β3γ2 and mutant α1β3γ2(K289M) receptors (14). These data suggest that the conserved lysine is not responsible for transducing the potentiating effects of benzodiazepines. However, this needs to be tested over a broader range of benzodiazepine concentrations in future studies.

Febrile seizures, the most common of childhood seizures, are linked to several environmental factors and a host of mutations, several of which affect genes encoding ion channels (44). A more complete understanding of the molecular mechanisms underlying each ion channel deficit associated with familial febrile seizures may lead to the development of individualized pharmacotherapies. The observation that α1β2γ2(K289M) and α1β3γ2(K289M) receptors (14) have reduced GABA-evoked channel open times, compared with their respective wild-type receptors, suggests that drugs that prolong open time may help rectify the functional deficit. Benzodiazepines increase the amplitude of sub-maximal GABA-evoked currents by increasing the frequency of GABA-gated channel bursts (45). This contrasts with the mechanism of enhancement by barbiturates and other general anesthetics that predominantly increase channel burst duration (45, 46). It is possible that the anesthetic potentiation of GABA-evoked responses mediated by mutant α1(K278M)β2γ2 and α1β2γ2(K289M) receptors will be enhanced through “correction” of the functional deficit of brief open time. This hypothesis will be tested in future single channel studies.

Deficits in the function of GABA<sub>α</sub> receptors containing α1(K278M) or γ2(K289M) may indicate a role for the conserved lysine residue in the transduction of GABA binding to channel gating. Indeed this residue appears ideally situated for such a mechanism. If so, this intramolecular transduction mechanism specific to GABA binding or can it be generalized to agonists that act through different sites on the GABA<sub>α</sub> receptor? Several general anesthetics, including the intravenous agent propofol, directly activate GABA<sub>α</sub> receptors when applied in the mid to high micromolar concentration range in the absence of GABA (40). Propofol also potentiates GABA-evoked currents starting in the low micromolar range and at concentrations &gt;100 μM causes a concentration-dependent receptor blockade (33). Direct activation of
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\( \text{GABA}_A \) receptors by propofol occurs through a distinct mechanism from that of GABA-evoked activation as demonstrated by the observation that propofol activates homomeric \( \beta_3 \) receptors that are resistant to activation by GABA (41). Furthermore, activation of recombinant \( \alpha_1\beta_2\gamma_2 \) receptors\(^4\) by propofol is resistant to blockade by the competitive GABA antagonist SR95531 (gabazine). Despite having different sites on the \( \text{GABA}_A \) receptor, activation by both propofol and GABA is adversely affected by the \( \alpha_1(K278M) \) mutation. The disruption of propofol activation by \( \alpha_1(K278M) \) demonstrates that intramolecular transduction of binding to gating by this residue is not specific to GABA, propofol without altering surface expression. Furthermore, the mutation reduces the apparent potency of gating by both GABA and propofol as an agonist relative to GABA. In the previous report (14). There is likely to be only one 4 M. R. McCartney, T. Z. Deeb, and T. G. Hales, unpublished observation.

Our data suggest that conserved TM2–3 lysine residues in the \( \alpha_1 \) and \( \gamma_2 \) subunits stabilize the open state of the receptor perhaps through a previously described electrostatic interaction with N-terminal acidic residues (23). The Lys-278 residue in the \( \alpha_1 \) subunit appears to be required for normal activation by both GABA and propofol suggesting that its putative role in initiating gating is not dependent on GABA binding.

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