

ACCELERATED COMMUNICATION

Refinement of the Binding Site and Mode of Action of the Anticonvulsant Retigabine on KCNQ K⁺ Channels

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ABSTRACT

The discovery of retigabine has provided access to alternative anticonvulsant compounds with a novel mode of action. Acting as potassium channel opener, retigabine exclusively activates neuronal KCNQ-type K⁺ channels, mainly by shifting the voltage-dependence of channel activation to hyperpolarizing potentials. So far, only parts of the retigabine-binding site have been described, including Trp-265 and Gly-340 (according to KCNQ3 numbering) within transmembrane segments S5 and S6, respectively. Using a refined chimeric strategy, we additionally identified a Leu-314 within the pore region of KCNQ3 as crucial for the retigabine effect. Both Trp-265 and Leu-314 are likely to interact with the retigabine molecule, representing the upper and lower margins of the putative binding site. Guided by

a structural model of KCNQ3, which was constructed based on the Kv1.2 crystal structure, further residues affecting retigabine-binding could be proposed and were experimentally verified as mediators for the action of the compound. These results strongly suggest that, besides Trp-265 and Leu-314, it is highly likely that another S5 residue, Leu-272, which is conserved in all KCNQ subunits, contributes to the binding site in KCNQ3. More importantly, Leu-338, extending from S6 of the neighboring subunit is also apparently involved in lining the hydrophobic binding pocket for the drug. This pocket, which is formed at the interface of two adjacent subunits, may be present only in the open state of the channel, consistent with the idea that retigabine stabilizes an open-channel conformation.

Epilepsy, one of the most common neurological disorders, is defined by recurrent seizures that are caused by an electrical hyperexcitability in the central nervous system. Voltage-gated K⁺-channels are critical regulators of excitability and represent promising targets for therapeutical intervention. Pharmacological activation of these channels might have anticonvulsant effects, whereas a loss of K⁺-channel activity may favor neuronal hyperexcitability. Indeed, benign familial neonatal convulsions, an autosomal dominant epilepsy of infancy, is caused by mutations in *KCNQ2* or *KCNQ3*

K⁺-channel genes (Biervert et al., 1998; Charlier et al., 1998; Singh et al., 1998). Mutations in four of five human *KCNQ* genes lead to inherited diseases (Jentsch, 2000). In particular, *KCNQ1* mutations are associated with cardiac arrhythmias (Wang et al., 1996; Chen et al., 2003) and deafness (Neyroud et al., 1997), whereas mutations in *KCNQ4* underlie a form of progressive hearing loss (Kubisch et al., 1999).

KCNQ2 and KCNQ3 are mainly found in the nervous system and colocalize in several neuronal populations (Schroeder et al., 1998; Cooper et al., 2000). This suggests that they form heteromeric channels in a subset of neurons, which is further supported by heterologous expression (Schroeder et al., 1998; Yang et al., 1998) and coimmunoprecipitation studies (Cooper et al., 2000). However, the two proteins are not always colocalized in brain (Cooper et al., 2000), and evidence has been provided that KCNQ2 homomers are present in vivo

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ABBREVIATIONS: D-23129, *N*-(2-amino-4-[fluorobenzylamino]-phenyl)carbamic acid (retigabine); p loop, pore loop; sid, subunit interaction domain; TD, turret domain; ZnPy, zinc-pyrithione.

(Hadley et al., 2003; Devaux et al., 2004; Schwarz et al., 2006). In addition, the expression pattern of KCNQ5, which can also form heteromeric channels with KCNQ3, largely overlaps with that of KCNQ2 and KCNQ3 (Schroeder et al., 2000).

KCNQ2–5, and in particular KCNQ2/KCNQ3 heteromers, display properties of M-type K^+ -channels (Wang et al., 1998; Kubisch et al., 1999; Schroeder et al., 2000). These channels are characterized by slowly activating and deactivating K^+ -currents with distinct electrophysiological and pharmacological properties and are suppressed by the activation of muscarinic acetylcholine receptors (Brown and Adams, 1980). Because M-type K^+ -channels are already active at slightly depolarized potentials around the threshold of action potential firing, their activity modulates neuronal excitability. This suggested that activators of KCNQ2/KCNQ3 channels might be useful in treating epilepsy. Indeed it was found that retigabine [D-23129], which specifically activates these channels, exerts an anticonvulsant profile (Rundfeldt, 1997; Rundfeldt and Netzer, 2000).

Retigabine has two effects on KCNQ2–5 channels: The first and most prominent effect is a hyperpolarizing shift of the voltage of half-maximal activation ($V_{1/2}$), resulting in an increase of K^+ conductance near the neuronal resting potential. Consequently, retigabine accelerates activation and slows deactivation of these currents, typical hallmarks of open-state stabilization (Tatulian et al., 2001). Second, retigabine-treated KCNQ2/KCNQ3 channels display an increased open probability, which leads to an increase of macroscopic current amplitudes (Tatulian and Brown, 2003). Of particular clinical importance is the fact that retigabine acts selectively on KCNQ2–5 isoforms, whereas cardiac KCNQ1 channels are not affected (Tatulian et al., 2001).

Previous studies have used chimeric strategies to locate the retigabine agonist site, resulting in the identification of a crucial tryptophan residue (Trp-265 in KCNQ3) in the central part of the transmembrane segment S5 (Schenzer et al., 2005; Wuttke et al., 2005). Replacement of the leucine residue in homologous position of KCNQ1 by a tryptophan rendered the resulting KCNQ1(L266W) construct retigabine-sensitive, albeit with unusual characteristics. The effects of the compound were 1) a concentration-dependent reduction of current amplitudes, 2) a decrease of the maximal I/I_{\max} values at potentials more positive than -20 mV, and 3) a small shift of $V_{1/2}$ (Schenzer et al., 2005; Wuttke et al., 2005). Although these studies demonstrated the importance of the S5 tryptophan, they also suggested that additional residues are needed to define the full retigabine binding site. Wuttke et al. (2005) showed that the highly conserved gating hinge residue Gly-301 within the S6 segment of KCNQ2 (corresponding to Gly-340 in KCNQ3), which is generally crucial for KCNQ channel gating (Seeböhm et al., 2006), also contributes to retigabine sensitivity, whereas the flexibility of the S6 helix itself may allow access of retigabine to the specific binding site.

By refinement of our chimeric approach, we further identified a leucine residue (Leu-314) within the pore region of KCNQ3 as crucial for the retigabine effect. Guided by homology modeling to the crystal structure of the Kv1.2 channel protein, we show that an additional leucine (Leu-338), extending from the neighboring subunit, is involved in the interaction with retigabine. Our study defines the retigabine

interaction site on KCNQ channels and strongly suggests that residues from adjacent subunits contribute to each binding pocket.

Materials and Methods

cDNA Constructs. Starting from KCNQ cDNAs, which were subcloned into expression vector pTLN, the KCNQ1/KCNQ3 chimeras and point mutants were constructed by recombinant polymerase chain reaction and verified by sequencing. After linearization with HpaI, capped cRNA was synthesized using SP6 mMessage mMachine kit (Ambion, Austin, TX).

Expression in *Xenopus laevis* Oocytes. Individual stage V to VI oocytes were obtained from anesthetized frogs and isolated by collagenase treatment. 10 ng of total KCNQ cRNA were injected into oocytes (also for coinjection experiments, which contained a 1:1 cRNA mixture). After injection, oocytes were kept at 17°C in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4).

Electrophysiology. Three to 5 days after injection, two-electrode voltage clamp measurements were performed at room temperature in ND96 using a npI turbo tec05 amplifier (npI electronics, Tamm, Germany) and pClamp 8 software (Molecular Devices, Sunnyvale, CA). For experiments examining the effect of retigabine, 10 μ M retigabine was added to ND96 from a 100 mM retigabine stock solution, which was prepared in dimethyl sulfoxide and kept at 4°C in the dark.

Tail Current Analysis and Determination of I/I_{\max} Curves. The voltage protocols for current recordings were (unless stated otherwise): starting from a holding potential of -80 mV, cells were clamped for 3 s to test potentials between -140 mV and $+80$ mV in steps of 20 mV, followed by a 500-ms tail pulse to -30 mV. Tail current analysis was used to estimate I/I_{\max} . The initial tail current amplitudes at -30 mV were determined by extrapolating current traces to the time of the voltage step. Because channel gating is slow compared with the voltage jump, these currents are proportional to the number of channels that are open on average at the preceding test-pulse voltage. I/I_{\max} curves were obtained from a total number of oocytes as indicated in brackets in the figure legends. For each oocyte, values were normalized to the mean tail current amplitude at $+40$ mV measured in the absence of retigabine. Values are given as means \pm standard errors.

Quantitative Analyses of I/I_{\max} Curves. To determine the parameters for the voltage dependence of activation I/I_{\max} curves were fitted by a Boltzmann function

$$B = \frac{B_{\max} - B_{\min}}{1 + \exp\left(\frac{z_q \cdot F}{R \cdot T} (V - V_{1/2})\right)} + B_{\min} \quad (1)$$

in which B_{\min} and B_{\max} are the minimal or maximal I/I_{\max} values, respectively, R is the molar gas constant, z_q the slope factor (equivalent charge), F is the Faraday constant, T is the absolute temperature in K, V is the transmembrane potential, and $V_{1/2}$ is the potential of half-maximal activation.

Molecular Modeling. The KcsA, MthK, and Kv1.2 channel structures were retrieved from the Protein Data Bank (KcsA, 1BL8; MthK, 1LNQ; Kv1.2, 2A79). Three-dimensional structural models of the S5–S6 and S1–S6 domains of KCNQ3 were constructed based on the crystal structure of the corresponding domains of KcsA/MthK/Kv1.2. Initial KCNQ3 models were generated using a combination of SwissModel and de novo generation of an α -helical peptide chain of the S1–S6 encoding KCNQ3 sequence: NAKYRRIQTLLIYDALERP RGWALLYHALVFLIVLGLILAVLTTFKEYETVSGDWLLLETFALIFIGAEFALRIWAAGCCCRYKGWRGRLLKFKARKPLCMLDIFVLIASVPVAVGNQGNVLATSLRSLRFLQLRMLRMDRRGGTWKLLGSAICAHSKELITAWYIGFLTLLSSFLVYLVEKDVPEVD

AQGEEMKEEFETYADALWWGLITLATIGYGDKTPKWTWEGRLIAATFSLIGVSFFALPAGILGSLALKV.

The putative flexibility at glycines and prolines was used to allow kinks in the α -helical structure of the peptide to adopt a Kv1.2-similar folding. Subsequent structural optimizations were performed using Deep View Swiss PDB viewer version 3.7 (<http://www.expasy.ch/spdbv/text/getpc.htm>) and BALLView 1.1.1 (<http://www.ballview.org/>). Energy minimizations were carried out using force fields Amber and Gromos96 within BALLView 1.1.1 and Deep View Swiss PDB viewer, respectively. Docking of energy-optimized retigabine conformers to the KCNQ3 homology models was performed using a geometrical docking approach with the Global Range Molecular Matching program (Gramm v1.03; <http://www.bioinformatics.ku.edu/files/vakser/gramm/>) installed locally on the computer. The noncovalent bond finder19 and CASTp20 were used to further determine the interaction of the favored retigabine molecule with its putative binding site in KCNQ3. The model with the most likely binding conformation is represented here.

Results

Role of the Inner Pore Vestibule in Retigabine Sensitivity. In our previous study, we analyzed a chimera termed KCNQ3(TD;Q1), in which a part of the pore loop, the extracellular “turret” domain (TD), from KCNQ1 was inserted into the KCNQ3 backbone (Fig. 1A) (Schenzer et al., 2005). The retigabine sensitivity of the KCNQ3(TD;Q1) construct, as quantified by the negative shift of the voltage of half-maximal activation $V_{1/2}$ ($\Delta V_{1/2} = -45.5$ mV), was similar to that of wild-type KCNQ3 ($\Delta V_{1/2} = -49.4$ mV) (Schenzer et al., 2005). In contrast, a related chimera termed KCNQ3(P;Q1), which carried the complete pore loop of KCNQ1 (see scheme in Fig. 1A), showed a remarkably reduced retigabine sensitivity ($\Delta V_{1/2} = -17.4$ mV) (Schenzer et al., 2005), suggesting that amino acids within the inner pore vestibule might contribute to the retigabine binding site. It is noteworthy that the inner pore regions of KCNQ1 and KCNQ3 [and thus of the chimeras KCNQ3(P;Q1) and KCNQ3(TD;Q1)] vary in only six amino acid positions (Fig. 1B).

Thus, to identify amino acids of the inner pore vestibule that modulate retigabine sensitivity, we exchanged several of the respective amino acids within the KCNQ3(P;Q1) construct for the corresponding KCNQ3 residues. This resulted in two further subchimeras, in which the first four [KCNQ3(P;Q1)VV.VT-LI.LA] (bold letters indicate KCNQ1 and KCNQ3 residues, respectively) and the last two [KCNQ3(P;Q1)V.Q-T.K] differing amino acids were mutated back to those of KCNQ3. Upon expression in *X. laevis* oocytes, the resultant channels gave rise to large, fast-activating currents, which responded differently to retigabine (Fig. 1, D and E). Based on the strong hyperpolarizing shift in $V_{1/2}$, the KCNQ3(P;Q1)VV.VT-LI.LA construct could again be classified as retigabine-sensitive ($V_{1/2} = -44.1 \pm 1.3$ mV without retigabine, -78.9 ± 1.3 mV with $10 \mu\text{M}$ retigabine; $\Delta V_{1/2} = -34.8$ mV; Fig. 1F). In contrast, the KCNQ3(P;Q1)V.Q-T.K construct showed an even smaller $V_{1/2}$ shift than KCNQ3(P;Q1) ($V_{1/2} = -37.3 \pm 0.6$ mV without retigabine, -37.8 ± 1.3 mV with $10 \mu\text{M}$ retigabine; $\Delta V_{1/2} = -0.5$ mV; Fig. 1G). However, this result essentially means that one of the KCNQ3 residues LI.LA, which are exchanged to VV.VT of KCNQ1 in the KCNQ3(P;Q1)V.Q-T.K construct, are critical for the interaction with the drug. In addition, the KCNQ3(P;Q1)V.Q-T.K construct exhibited a slight increase of the I/I_{max}

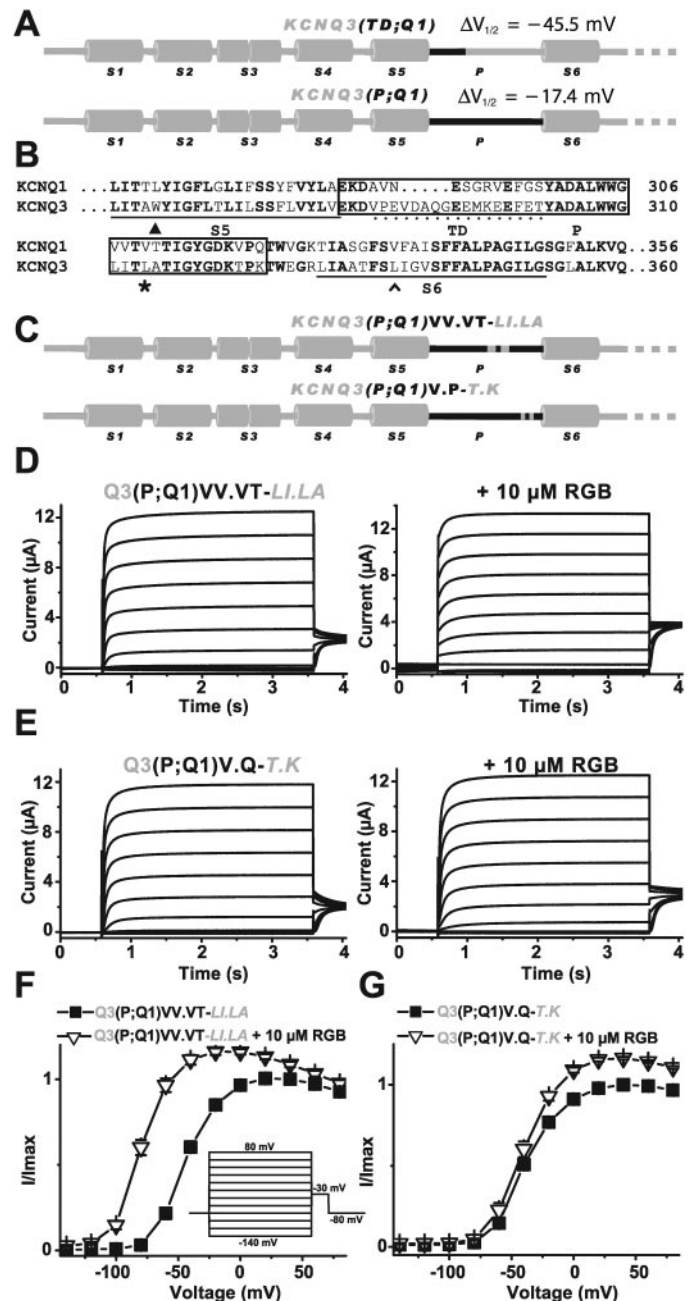


Fig. 1. Retigabine sensitivities of KCNQ3(P;Q1)VV.VT-LI.LA and KCNQ3(P;Q1)V.Q-T.K. A, schematic illustration of chimeras KCNQ3(TD;Q1) and KCNQ3(P;Q1). B, sequence alignment of human KCNQ1 and KCNQ3 in the pore region encompassing the α -helices S5 and S6 and the pore loop. Residues belonging to helices S5 and S6 are underlined. Boxed are the pore loops of KCNQ1 and KCNQ3, which consist of the extracellular “turret” domain (TD, dotted line), pore helix (P), and selectivity filter. The tryptophan residue in the S4-S5 linker, which is important for retigabine sensitivity, is marked with an arrowhead; the two critical leucine residues within the inner pore vestibule and S6 are marked with an asterisk (*) and a caret (^), respectively. C, schematic illustration of chimeras KCNQ3(P;Q1)VV.VT-LI.LA and KCNQ3(P;Q1)V.Q-T.K, respectively. Typical current traces recorded from *X. laevis* oocytes expressing KCNQ3(P;Q1)VV.VT-LI.LA (D) and KCNQ3(P;Q1)V.Q-T.K (E) are shown before (left) and during perfusion with $10 \mu\text{M}$ retigabine (right). Tail current analysis revealed voltage-dependent I/I_{max} curves for KCNQ3(P;Q1)VV.VT-LI.LA ($n = 20$) (F) and KCNQ3(P;Q1)V.Q-T.K ($n = 17$) (G), respectively, in the presence or absence of retigabine. The voltage protocol is shown as an inset in F. Voltages of half-maximal activation ($V_{1/2}$) and the respective shifts are given in Table 1.

curve at positive potentials (Fig. 1, E and G), as observed for other KCNQ constructs analyzed in our previous study, and for wild-type KCNQ3 (Schenzer et al., 2005).

Identification of a Critical Residue for Retigabine Sensitivity in the Pore Loop. Based on the KCNQ3(P;Q1) construct, we individually replaced each of the four KCNQ1-derived residues (VV.VT) for the corresponding amino acids of KCNQ3 (LIL.A) and investigated their I/I_{\max} curves and pharmacological profiles. All mutants exhibited $V_{1/2}$ values shifted in a hyperpolarizing manner compared with KCNQ3(P;Q1) (Fig. 2, A–D, and Table 1). Upon application of 10 μ M retigabine, only mutant V314L (KCNQ3 numbering) displayed a larger $\Delta V_{1/2}$ (-24.5 ± 2.5 mV) compared with that of KCNQ3(P;Q1), indicating that the retigabine effect can partly be re-established upon introduction of Leu-314. For mutants V311L and V312L, the $V_{1/2}$ shifts were comparable with that of the KCNQ3(P;Q1) construct, and for T315A, the shift was significantly smaller. Again, this last result essentially implies that one of the KCNQ3 amino acids LIL(A)/T.K, which are changed to KCNQ1 VV.V(A)/V.Q, must be crucial. To summarize, these experiments strongly indicated that within the inner pore loop, Leu-314 is the most important residue for retigabine sensitivity.

We then tested for the direct effects of mutations within the inner pore loop of our initial reference construct KCNQ3(TD;Q1) and tested the point mutant KCNQ3(TD;Q1)-L314V, in which Leu-314 of KCNQ3 was replaced by the corresponding valine of KCNQ1 (see Fig. 1B). Compared with KCNQ3(TD;Q1), the mutation shifted the $V_{1/2}$ value to hyperpolarizing potentials (Fig. 2E), and application of 10 μ M retigabine shifted the $V_{1/2}$ value by only -7.6 mV (Table 1 and Fig. 2F), indicating a loss of retigabine sensitivity. As observed to some extent for the KCNQ3(TD;Q1)-W265L mutant described previously, the KCNQ3(TD;Q1)-L314V mutant exhibited a pronounced increase of the maximal I/I_{\max} values at more positive potentials than -40 mV (Fig. 2E, compare to Fig. 6I in Schenzer et al., 2005). This observation presumably reflects an effect of retigabine on the maximal open probability or single-channel conductance of KCNQ channels, which is different from the hyperpolarizing shift of the activation curve. However, we did not perform measurements of single-channel properties to answer this question. At this stage, we wanted to exclude the possibility that other amino acids within the inner pore vestibule might contribute to the retigabine binding site. Thus, we exchanged each of the remaining five amino acids in this region in which KCNQ3 [hence KCNQ3(TD;Q1)] and KCNQ1 differ. From the resultant constructs, mutant KCNQ3(TD;Q1)-I312V was nonfunctional, and the others [KCNQ3(TD;Q1)-L311V, -A315T, -T323V, and -K325Q] exhibited a retigabine sensitivity comparable with that of KCNQ3(TD;Q1) (Fig. 2F). Because only the already tested KCNQ3(TD;Q1)-L314V construct exhibited a largely reduced retigabine sensitivity (Fig. 2, E and F), within the inner pore vestibule, only Leu-314 was shown to be important for retigabine sensitivity of KCNQ3.

Replacement of the crucial S5 tryptophan for leucine in all KCNQ2–5 subunits leads to loss of the hyperpolarizing shift of the activation curve. In contrast, a vice versa KCNQ1 construct, in which the leucine in homologous position was replaced by a tryptophan (mutant KCNQ1-L266W) did not display retigabine sensitivity in terms of a large hyperpolarizing $V_{1/2}$ shift. However, a decrease in macroscopic current

amplitudes and of the maximal I/I_{\max} values at more positive potentials than -20 mV was observed (Schenzer et al., 2005; Wuttke et al., 2005), indicating that further residues are required for mediating the $V_{1/2}$ shift. Considering Val-310 (homologous to KCNQ3 Leu-314) as a candidate, we replaced this amino acid with a leucine within the KCNQ1-L266W backbone and tested whether simultaneous insertion of the two critical residues would be sufficient to render KCNQ1

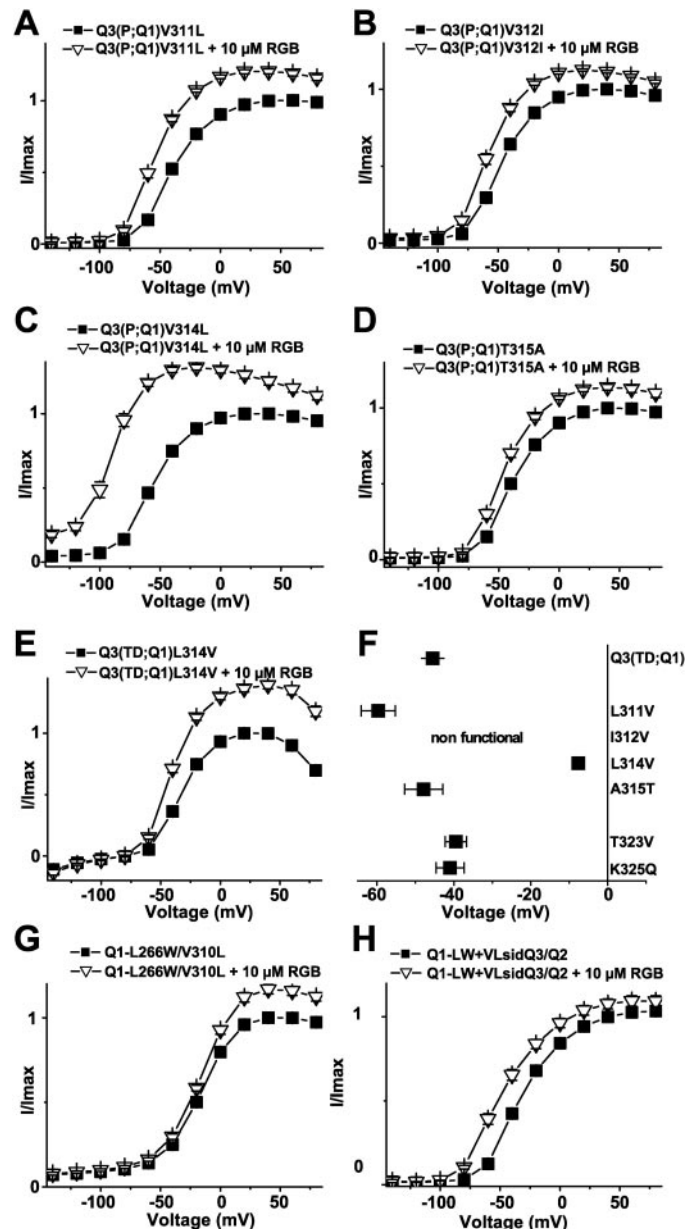


Fig. 2. Retigabine sensitivities of KCNQ3(P;Q1)-derived channels carrying point mutations within the pore region and retigabine sensitivities of KCNQ3(TD;Q1) pore loop mutants KCNQ1-(L266W/V310L)-*sid*_{Q3} and KCNQ1-(L266W/V310L)-*sid*_{Q3}/KCNQ2. I/I_{\max} curves of mutants KCNQ3(P;Q1)-V311L ($n = 18$) (A), KCNQ3(P;Q1)-V312L ($n = 18$) (B), KCNQ3(P;Q1)-V314L ($n = 18$) (C), and KCNQ3(P;Q1)-T315A ($n = 18$) (D) are shown as a function of voltage, as obtained from tail current analysis. The corresponding $V_{1/2}$ values and $V_{1/2}$ shifts are given in Table 1. Voltage-dependent I/I_{\max} curves of KCNQ3(TD;Q1)-L314V ($n = 14$) (E), KCNQ3(TD;Q1) pore loop mutants (L311V, $n = 9$; L314V, $n = 14$; A315T, $n = 10$; T323V, $n = 10$; K325Q, $n = 10$) (F), KCNQ1-L266W/V310L ($n = 20$) (G), and KCNQ1-(L266W/V310L)-*sid*_{Q3}/KCNQ2 ($n = 14$) (H). See Table 1 for $V_{1/2}$ values and $V_{1/2}$ shifts.

retigabine-sensitive. Whereas the $V_{1/2}$ shift of the KCNQ1-L266W/V310L double mutant channel again was not significant ($\Delta V_{1/2} = -0.8$ mV), the I/I_{\max} curve was significantly increased at voltages more positive than -20 mV (Fig. 2G). Thus, introduction of a critical leucine into the pore loop of the KCNQ1-L266W construct is sufficient to confer an important aspect of retigabine sensitivity to the mutant channel, suggesting that Leu-314, in concert with Trp-265 contributes to the retigabine-binding site in KCNQ3 channels.

We then determined the retigabine sensitivity of heteromeric channels assembled from retigabine-sensitive KCNQ2 subunits and constructs containing the transmembrane domains of the retigabine-insensitive KCNQ1 channel, as described previously (Schenzer et al., 2005). Because KCNQ1 wild-type does not assemble with KCNQ2, the KCNQ1-derived constructs were based on a chimera termed KCNQ1-*sid*_{Q3}, which contains the C-terminal subunit interaction domain (*sid*) from KCNQ3. It was shown previously that the KCNQ1-*sid*_{Q3} construct forms heteromeric channels with KCNQ2 in a way similar to that of KCNQ3 (Schwake et al., 2003), which are, importantly, retigabine-insensitive (Schenzer et al., 2005). Upon introduction of mutations L266W and V310L into the KCNQ1-*sid*_{Q3} chimera and coexpression of the resultant double mutant KCNQ1-(L266W/V310L)-*sid*_{Q3} with KCNQ2, heteromeric KCNQ1-(L266W/V310L)-*sid*_{Q3}/KCNQ2 channels were formed (Fig. 2H) that exhibited a clearly discernible shift of the activation curve ($\Delta V_{1/2} = -18.2 \pm 1.4$ mV). This effect was significantly more pronounced than in the case of the KCNQ1-(L266W)-*sid*_{Q3}/KCNQ2 coexpression scheme, for which only a $\Delta V_{1/2}$ of -7.5 mV in response to $10 \mu\text{M}$ retigabine was observed (Schenzer et al., 2005). Thus, whereas introduction of a Trp residue in position 266 and a Leu residue in position 310 within a channel homotetramer is not sufficient to confer a retigabine-dependent $V_{1/2}$ shift, apparently these two mutations are effective in heteromeric channels formed in concert with per se retigabine-sensitive subunits. These observations indicate that the per se retigabine-sensitive subunits contribute an extra property on their own and hint at the involvement of residues from adjacent subunits in defining the retigabine-binding site.

Location of the S5 Trp and Pore Loop Leu Residue within a Structural Homology Model of KCNQ3. To gain insight into the structural organization of the putative ago-

nist site, we constructed a three-dimensional structural model of KCNQ3 (Fig. 3) using the crystal structure of the *Shaker*-related mammalian voltage-gated potassium channel Kv1.2 (Long et al., 2005a,b) as a template. It is noteworthy that the docking studies with retigabine showed that the positioning of the agonist molecule is similar to the docking position within a former KCNQ2 model, which had been derived from the structure of the bacterial mechanosensitive MthK channel (Wuttke et al., 2005). As can be seen from Fig. 3, the Kv1.2-KCNQ3 model is supportive for a crucial involvement of residues Trp-265 and Leu-314 in delimiting the retigabine-binding pocket. Both residues are in contact with the retigabine molecule (Fig. 3) and represent the upper and lower margins of the putative binding site. Using these constraints, the Kv1.2-KCNQ3 model suggested that additional amino acids are involved in specific interactions with the retigabine molecule, such as the backbone of Thr-271 and the Leu-272 side chain, both located on the same subunit (blue in Fig. 3) as Trp-265, and Leu-338, which notably extends from the adjacent subunit (red in Fig. 3).

To validate these predictions, we substituted the aforementioned amino acids within KCNQ3(TD;Q1) for the corresponding KCNQ1 residues, resulting in mutants KCNQ3(TD;Q1)-T271G, -L272A, and -L338V. Whereas the mutants carrying the substitutions T271G and L272A were nonfunctional (Fig. 4A), the KCNQ3(TD;Q1)-L338V mutant could be functionally expressed and exhibited a reduced retigabine-sensitivity ($\Delta V_{1/2} = -18.4$ mV) (Fig. 4B), in agreement with our KCNQ3 model based on the Kv1.2 crystal structure. To further evaluate the role of KCNQ3 amino acids 271 and 272 in interaction with retigabine, we carried out several rather conservative amino acid exchanges, thus replacing Thr-271 by Ala, Cys, Phe, Val or Ser, and Leu-272 by Ala, Cys, Val, Ile, Gly, or Ser (Fig. 4A). From the Thr-271 mutants, which could be functionally expressed (only T271S was nonfunctional; see Fig. 4A), T271A, T271C, and T271V did not exhibit a $V_{1/2}$ shift different from that of KCNQ3(TD;Q1), suggesting that the individual side chain properties are less important, unless a more bulky side chain is introduced, as in the T271F mutant, which showed a largely reduced drug sensitivity. In contrast, most Leu-272 substitutions resulted in nonfunctional channels. The conservation of this S5-Leu in all KCNQ subunits suggests an important role of this residue for channel function. However, the Leu-272 mu-

TABLE 1

$V_{1/2}$ values of various chimeras and mutants in the absence or presence of retigabine.

Construct	$V_{1/2}$	$V_{1/2}$	$\Delta V_{1/2}$
	mV	(10 μM RGB)	(10 μM RGB)
KCNQ3	-50 ± 0.9	-89.4 ± 1.9	-39.4 ± 2.1
KCNQ3(TD;Q1)	-28.2 ± 1.4	-73.7 ± 0.8	-45.5 ± 1.6
KCNQ3(P;Q1)	-36.8 ± 1.2	-54.2 ± 1.2	-17.4 ± 1.7
KCNQ3(P;Q1)V.VT-L.LLA	-44.1 ± 1.3	-78.9 ± 1.3	-34.8 ± 1.8
KCNQ3(P;Q1)V.Q-T.K	-37.3 ± 0.6	-37.8 ± 1.3	-0.5 ± 1.4
KCNQ3(P;Q1)V311L	-37.7 ± 0.6	-52.3 ± 0.8	-14.6 ± 1.0
KCNQ3(P;Q1)V312I	-47.7 ± 1.2	-55.8 ± 1.0	-8.1 ± 1.6
KCNQ3(P;Q1)V314L	-56.1 ± 1.2	-80.6 ± 2.2	-24.5 ± 2.5
KCNQ3(P;Q1)T315A	-39.5 ± 1.4	-43.9 ± 1.0	-4.4 ± 1.7
KCNQ3(TD;Q1)L314V	-32.9 ± 0.5	-40.5 ± 1.0	-7.6 ± 1.1
KCNQ1-L266W+V310L	-15.6 ± 0.9	-16.3 ± 1.4	-0.7 ± 1.7
KCNQ1-L266W+V310L- <i>sid</i> _{Q3} /KCNQ2	-27.8 ± 0.5	-46.0 ± 1.3	-18.2 ± 1.4
KCNQ3(TD;Q1)L338V	-24.9 ± 1.5	-43.3 ± 1.2	-18.4 ± 1.5
KCNQ1-L266W+V310L + V334L <i>sid</i> _{Q3}	21.8 ± 0.9	9.7 ± 1.9	-12.1 ± 1.1
KCNQ1-L266W+V310L + V334L <i>sid</i> _{Q3} /KCNQ2	-17.5 ± 0.6	-23.7 ± 0.5	-6.2 ± 1.1

tants L272C and L272V, which could be functionally expressed, both were virtually retigabine-insensitive (Fig. 4A). This is supportive of an involvement of Leu-272 in interaction with the retigabine molecule, as suggested by the model structure.

After this, we determined the retigabine sensitivity of heteromeric channels assembled from KCNQ2 subunits and a KCNQ1-*sid*_{Q3}-derived triple mutant termed KCNQ1-(L266W/V310L/V334L)-*sid*_{Q3}. This KCNQ1-(L266W/V310L/V334L)-*sid*_{Q3} mutant was functional as a homotetramer and also in a heteromeric expression scheme with KCNQ2 (Fig. 4, C and D). Homo- and heteromeric channels exhibited significant retigabine sensitivity in terms of a hyperpolarizing $V_{1/2}$ shift (homomeric KCNQ1-(L266W/V310L/V334L)-*sid*_{Q3}: $\Delta V_{1/2} = -12.1$ mV, heteromers with KCNQ2: $\Delta V_{1/2} = -6.2$ mV). In addition, for KCNQ1-(L266W/V310L/V334L)-*sid*_{Q3}/KCNQ2 channels increased maximal I/I_{\max} values were measured, which were significantly more pronounced than for the KCNQ1-(L266W)-*sid*_{Q3}/KCNQ2 and KCNQ1-(L266W/V310L)-*sid*_{Q3}/KCNQ2 coexpression scheme. These results, which are supported by the model structure, strongly suggest that two amino acids, Trp-265 in S5 and Leu-314 in the inner pore vestibule from one subunit, and a second leucine (Leu-338) in S6 from the adjacent subunit are delimiting the retigabine-binding site on KCNQ3 channels.

To further validate the contribution of Leu-314 in the pore loop and Leu-338 in S6 to the retigabine binding site in heteromeric assemblies with other KCNQ subunits, we mutated the homologous residues in KCNQ2 and coexpressed the resulting KCNQ2-L275V and -L299V (Fig. 5A) mutants with the triple-mutant KCNQ1-(L266W/V310L/V334L)-*sid*_{Q3}. Heteromeric KCNQ1-(L266W/V310L/V334L)-*sid*_{Q3}/KCNQ2-L275V channels displayed a reduced shift of $V_{1/2}$ ($\Delta V_{1/2} = +3.6$ mV compared with the KCNQ1-(L266W/V310L/V334L)-*sid*_{Q3}/KCNQ2 heteromers, which showed a $\Delta V_{1/2} = -6.2$ mV, see Fig. 4D), and increased maximal I/I_{\max} values (Fig. 5B). In contrast, heteromeric KCNQ1-(L266W/V310L/V334L)-*sid*_{Q3}/KCNQ2-L299V channels were completely retigabine-

insensitive (Fig. 5C). From the reduced retigabine sensitivity as a result of the two KCNQ2 mutations, it can be concluded that the two leucines in the inner pore loop and in S6 are of general importance for the formation of the retigabine interaction site.

Discussion

The so-called M-current limits repetitive firing of numerous types of neurons, thereby controlling neuronal excitability (Brown and Adams, 1980). Its main molecular correlates are potassium channels of the Kv7 (KCNQ) family, especially Kv7.2 (KCNQ2) and Kv7.3 (KCNQ3) (Wang et al., 1998). The pharmacological activation of these channels by compounds such as retigabine or flupirtine provides therapeutic potential for disorders that are caused by neuronal hyperexcitability, including epilepsy, chronic pain, or migraine. Indeed, it has been shown that retigabine has a broad anticonvulsant activity in nearly every animal seizure model, and clinical trials are currently ongoing (Nicolson and Leach, 2001). Retigabine is known to activate exclusively the neuronally expressed Kv7.2–5 isoforms with different potencies but does not modulate Kv7.1 activity (Wickenden et al., 2000; Tatalian et al., 2001). This is of high pharmacological significance to avoid adverse effects on cardiac function. It has been shown recently that gene expression of Kv7.2–5 is not only restricted to neuronal cells (Ohya et al., 2003; Yeung et al., 2007; McCallum et al., 2008) but that they might play a role in controlling arterial tone (Yeung et al., 2007). Hence, it is important to describe the target site of retigabine in greater detail to understand the molecular mechanisms of Kv7.2–5 channel activation.

By using chimeric approaches, we and others have shown that a tryptophan residue within the S5 segment of KCNQ2 to KCNQ5 is necessary for retigabine interaction, and several other amino acids have been implicated in retigabine binding (Schenzer et al., 2005; Wuttke et al., 2005). However, upon introduction of the corresponding amino acids into the reti-

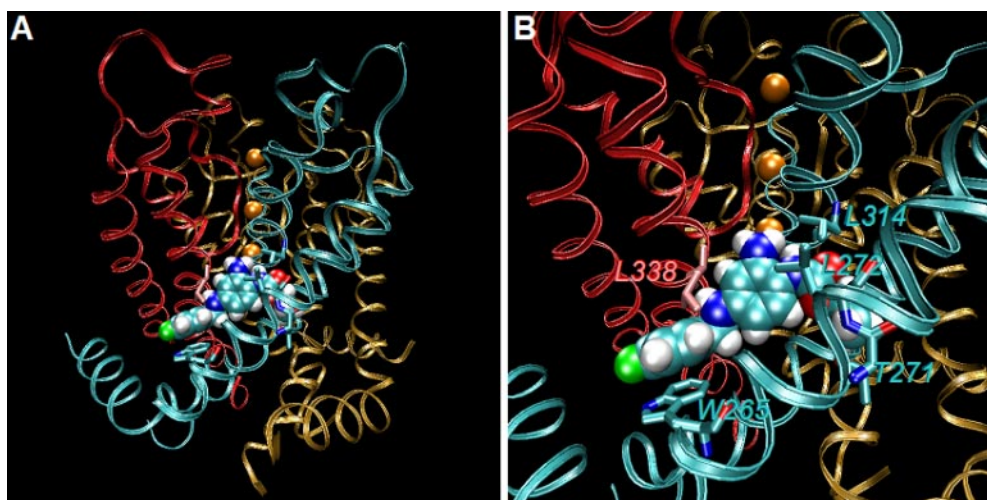
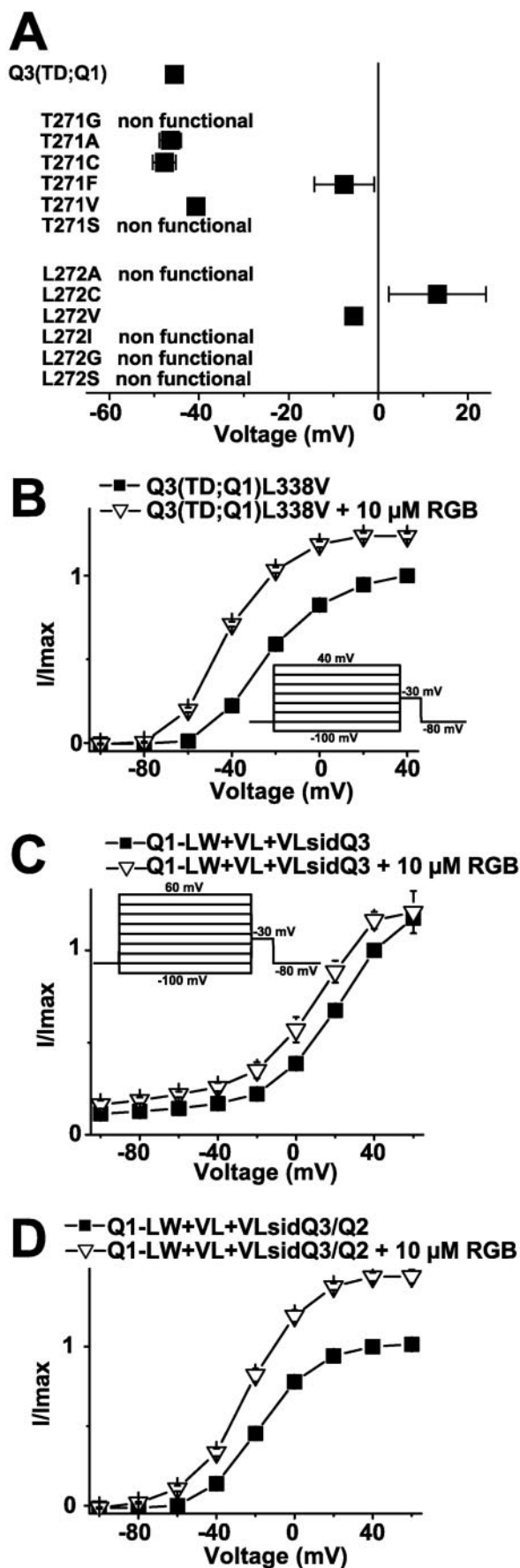


Fig. 3. Structural model of KCNQ3. (A) Three-dimensional model of the retigabine molecule docked to the pore domain of KCNQ3. Only the S5-P-S6 regions of the four subunits are shown, the voltage sensor parts encompassing transmembrane segments S1 to S4 were omitted for clarity. The figure shows a ribbon representation of four KCNQ3 subunits, which are energy-optimized homology models of the KCNQ3 pore domain derived from the crystal structure of Kv 1.2. Two subunits in the back are represented in yellow, whereas the two adjacent subunits in the front are shown in red and blue, respectively. (B) Close up of the binding site of retigabine, which is defined by the crucial Trp-265, the Thr-271, and the three leucines (Leu-272, Leu-314, Leu-338) (according to KCNQ3-numbering). In both panels, the retigabine molecule is depicted in Corey/Pauling/Koltun-colored spacefill mode, and the critical amino acids are portrayed in stick mode.



gabine-insensitive KCNQ1 isoform, a characteristic large shift in $V_{1/2}$ upon retigabine administration could not be obtained, indicating that the structural determinants involved in interaction with the drug were still incompletely recognized. Therefore, we extended our chimeric strategy and identified a leucine at position 314 in KCNQ3 within the inner pore vestibule as being important for retigabine binding.

The identification of the crystal structure of the mammalian voltage-gated K^+ channel Kv1.2 by Long et al. (2005a,b) provided a promising structural template for homology modeling of KCNQ structures based on the Kv1.2 coordinates. Using the knowledge about the involvement of these two amino acids, the Kv1.2-based KCNQ3 model revealed that Trp-265 and Leu-314 likely represent the outer limits of a well defined binding pocket for retigabine. In addition, the model indicated that Thr-271 and Leu-272 in the S5 helix should also delineate the retigabine pocket and, importantly, Leu-338 from the adjacent subunit. In the optimal docking position, the retigabine molecule apparently is in close contact with all the above-mentioned residues. It is noteworthy that retigabine could not be docked in a similar way in a KcsA-based closed state model, supporting the idea that retigabine stabilizes an open channel conducting conformation.

Two of the additional three amino acids, Thr-271 and Leu-272 in S5, which were predicted in molecular docking studies as candidates for retigabine binding, are apparently critical for channel function, because many mutations in these positions led to dysfunctional channels. Indeed, an interaction of KCNQ1 residue Leu-272 in S5 with Phe-340 in S6 and Val-310 has been proposed by us before (Seeböhm et al., 2005). This interaction is critical to macroscopic channel conduction and activation kinetics in KCNQ1 channels. It seems quite possible that retigabine modulates the concerted action of S5-S6 and the lower pore helix within one subunit to stabilize an open conformation resulting in larger macroscopic currents after retigabine application. The Thr-271 position seems to be tolerant for several conservative amino acid replacements, in agreement with the proposed interaction only with the protein backbone. However, when a bulky side chain such as phenylalanine is introduced, a marked loss of retigabine sensitivity is observed, indicating that the Thr-271 position is indirectly involved in interaction with the molecule. The lack of effect of smaller residues introduced at Thr-271 is in agreement with backbone interaction suggested by our model structure. Thr-271 is not conserved and exchange of this residue by a cysteine (as in KCNQ2) or an valine (as in KCNQ4 or KCNQ5) within the reference construct KCNQ3(TD;Q1) did not result in an altered retigabine sensitivity (Fig. 4A). These findings exclude that this amino acid directly acts as a retigabine sensitivity modifier. However, insertion of a bulky amino acid side chain in this posi-

Fig. 4. Retigabine sensitivities of KCNQ3(TD;Q1)/T271X, KCNQ3(TD;Q1)/L272X, KCNQ3(TD;Q1)-L338V, KCNQ1-(L266W/V310L/V334L)-sid_{Q3}, and KCNQ1-(L266W/V310L/V334L)-sid_{Q3}/KCNQ2. Summary of the $V_{1/2}$ values of KCNQ3(TD;Q1)/T271X (T271A, $n = 16$; T271C, $n = 10$; T271F, $n = 10$; T271V, $n = 10$) and KCNQ3(TD;Q1)/L272X (L272C, $n = 9$; L272V, $n = 9$) mutants (A). Voltage-dependent I/I_{max} curves of KCNQ3(TD;Q1)-L338V ($n = 22$; the voltage protocol is shown as an inset) (B), KCNQ1-(L266W/V310L/V334L)-sid_{Q3} ($n = 7$; the voltage protocol is shown as an inset) (C), and KCNQ1-(L266W/V310L/V334L)-sid_{Q3}/KCNQ2 ($n = 12$) (D) as obtained from tail current analyses. See Table 1 for $V_{1/2}$ values and $V_{1/2}$ shifts.

tion might result in displacement of the protein backbone, thereby indirectly distorting the binding pocket, as can be inferred from the reduced retigabine sensitivity of the T271F mutant.

Leu-272 is of more general functional importance because most mutations at this position lead to dysfunctional channels. However, all Leu-272 mutants that could be functionally expressed were essentially retigabine-insensitive, implicating that this amino acid is critically involved too. Because Leu-272 is conserved in the analogous positions in all KCNQ subunits, the involvement of this amino acid could only be inferred from structure predictions. The results of the mutagenesis studies are in good agreement with the side-chain interaction of Leu-272 with retigabine, as predicted by the docking prediction.

It is noteworthy that the model also predicted that Leu-338 within S6 of the adjacent subunit is involved in delineating the retigabine binding site. Indeed, when this hypothesis was tested with the KCNQ3(TD;Q1)-L334V mutant, a largely reduced $V_{1/2}$ shift was observed upon retigabine addition. Furthermore, testing a KCNQ1-based triple mutant in a heteromeric expression scheme with KCNQ2 revealed both a $V_{1/2}$ shift and a pronounced augmentation of the maximal I/I_{\max} values.

The contribution of Leu-314 in the pore loop and Leu-338 in S6 to the retigabine-binding site could also be shown with heteromeric KCNQ subunit assemblies. Therefore, constructs resulting from mutation of the homologous residues in KCNQ2 (KCNQ2-L275V and -L299V) were coexpressed with the triple mutant subunit KCNQ1-(L266W/V310L/V334L)-*sid*_{Q3} to test for the mutual involvement of residues from different subunits to a single binding site. Channels assembled from KCNQ1-(L266W/V310L/V334L)-*sid*_{Q3}/KCNQ2-L275V subunits displayed a reduced $V_{1/2}$ shift compared with

the KCNQ1-(L266W/V310L/V334L)-*sid*_{Q3}/KCNQ2 heteromers, and increased maximal I/I_{\max} values were observed. Even more so, heteromeric KCNQ1-(L266W/V310L/V334L)-*sid*_{Q3}/KCNQ2-L299V channels were completely retigabine-insensitive. Thus, the reduced retigabine sensitivity resulting from the two KCNQ2 mutations indicates, that the two leucines in the inner loop and in S6 are of general importance for the formation of the retigabine-binding pocket.

It is noteworthy that the Leu-275 residue of KCNQ2, which is homologous to Leu-314 of KCNQ3, has also been implicated in interaction with zinc-pyrithione (ZnPy), a different KCNQ-activating substance (Xiong et al., 2007, 2008). On KCNQ2, ZnPy induces a more than 5-fold augmentation of maximal conductance and a -25.5 -mV shift in $V_{1/2}$ (Xiong et al., 2007). However, the effects of both substances were complex for the KCNQ2-L275A mutant, because ZnPy induced an even augmented increase in conductance, and retigabine caused a significant increase in currents, whereas the shift in $V_{1/2}$ was reportedly reduced for both substances. Only in the L249A/L275A double mutant was the ZnPy-induced $V_{1/2}$ shift completely absent. Because of the overlapping action pattern, a possible allosteric effect mediated by both drugs is difficult to test by analysis of macroscopic currents. Different techniques, such as photoaffinity labeling, may help to clarify whether there is an overlap between the interaction sites. It is likely that the determinants of ZnPy interaction with KCNQ channels need further refinement, because the KCNQ2 residues Leu-249, Leu-275, and Ala-306 found to be important for interaction with ZnPy are conserved in KCNQ2, -3, -4, and -5, but KCNQ3 is not functionally modified by the compound, whereas a significant effect on KCNQ1 currents was observed. Thus, further experiments will be needed to completely delineate the ZnPy interaction site on KCNQ channels.

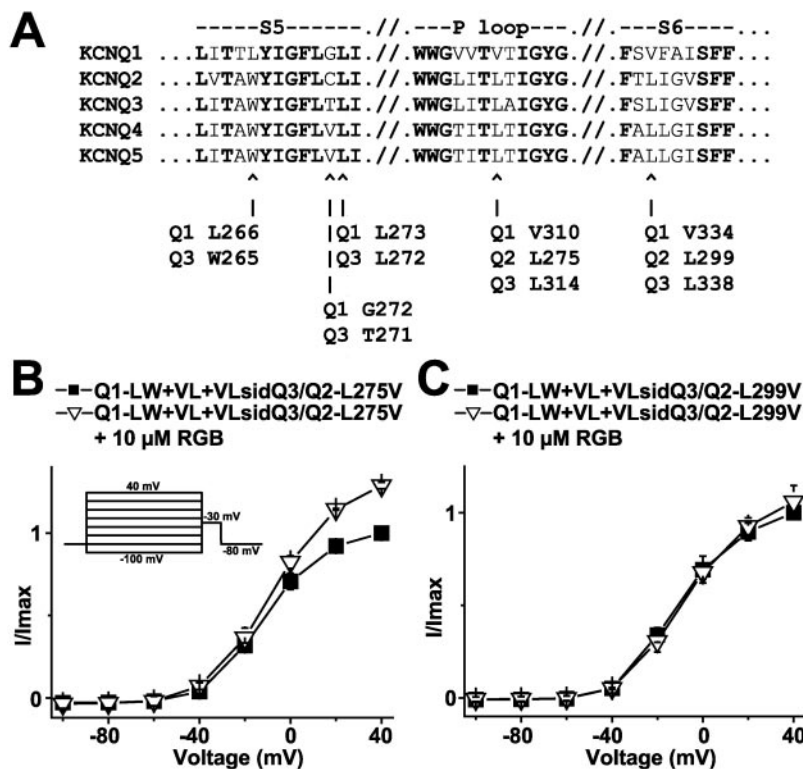


Fig. 5. Retigabine sensitivities of KCNQ1-(L266W/V310L/V334L)-*sid*_{Q3}/KCNQ2-L275V and KCNQ1-(L266W/V310L/V334L)-*sid*_{Q3}/KCNQ2-L299V. Sequence alignment of human KCNQ channels in the pore region encompassing large parts of the α -helices S5 and S6 and the pore loop. Residues belonging to the different portions of the pore loop are labeled, respectively. For clarity, carets (^) indicate the amino acids at the respective position in the different KCNQ α -subunits that have been identified and mutated in this study. Voltage-dependent I/I_{\max} curves of KCNQ1-(L266W/V310L/V334L)-*sid*_{Q3}/KCNQ2-L275V ($n = 11$; the voltage protocol is shown as an inset) (B), and KCNQ1-(L266W/V310L/V334L)-*sid*_{Q3}/KCNQ2-L299V ($n = 11$) as obtained from tail current analyses.

In conclusion, our results strongly suggest that, according to the KCNQ3 numbering scheme, the following four amino acids are critically involved in formation of the complete retigabine binding site: Trp-265 as well as Leu-272 in S5, Leu-314 within the inner pore loop, and Leu-338 in S6 of the neighboring subunit. Thus, by binding to a pocket at the interface between two adjacent channel subunits the retigabine molecule may act in stabilizing the open channel conformation. The four critical amino acids are found in all retigabine-sensitive KCNQ channels but are not present at the equivalent positions in the retigabine-insensitive KCNQ1, apart from the conserved Leu-272, which apparently is of more general importance for channel function. The aforementioned residues most likely contribute in general to the retigabine binding site on KCNQ channels; however, subtle differences in their spatial arrangement within the 3D structure might account for the different retigabine sensitivities of the KCNQ channel isoforms.

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