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High-risk Long QT Syndrome Mutations in the Kv7.1 (KCNQ1) Pore Disrupt the Molecular Basis for Rapid K⁺ Permeation

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Abstract

Type 1 long QT syndrome (LQT1) syndrome is caused by loss-of-function mutations in the *KCNQ1*, which encodes the K⁺ channel (Kv7.1) that underlies the slowly activating delayed rectifier K⁺ current in the heart. Intragenic risk stratification suggests LQT1 mutations that disrupt conserved amino acid residues in the pore are an independent risk factor for LQT1-related cardiac events. The purpose of this study is to determine possible molecular mechanisms that underlie the loss-of-function for these high-risk mutations. Extensive genotype-phenotype analyses of LQT1 patients showed that T322M-, T322A-, or G325R-Kv7.1 confer a high risk for LQT1-related cardiac events. Heterologous expression of these mutations with KCNE1 revealed they generated non-functional channels and caused dominant negative suppression of WT-Kv7.1 current. Molecular dynamic simulations (MDS) of analogous mutations in KcsA (T85M-, T85A-, and G88R-KcsA) demonstrated that they disrupted the symmetrical distribution of the carbonyl oxygen atoms in the selectivity filter, which upset the balance between the strong attractive and K⁺-K⁺ repulsive forces required for rapid K⁺ permeation. We conclude high-risk LQT1 mutations in the pore likely disrupt the architectural and physical properties of the K⁺ channel selectivity filter.

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SUPPORTING INFORMATION AVAILABLE Supplemental Experimental Procedures, Supplemental Figure 1, Supplemental Movie 1, Supplemental Movie 2, Supplemental Movie 3, Supplemental Movie 4, Supplemental Table 1, and Supplemental Table 2 is available free of charge via the Internet at <http://pubs.acs.org> \

Keywords

Potassium channels; Electrophysiology; Genetic diseases; Molecular dynamics; Heart

Congenital Long QT syndrome (LQTS) is a monogenic cardiac arrhythmia syndrome that occurs in ~1:2,500 people and increases the risk for polymorphic ventricular tachycardia (torsades de pointes or torsades), syncope, and sudden death (1-3). Type 1 long QT syndrome (LQT1) accounts for ~35% of LQTS and is caused by loss-of-function mutations in the *KCNQ1* K⁺ channel (Kv7.1) (4-12). Kv7.1 combines with the β -subunit KCNE1 to mediate the slowly activating delayed rectifier K⁺ current in the heart (I_{Ks}). Intragenic risk stratification shows patients who have LQT1 missense mutations at conserved amino acids in the pore correlate with an increased risk for cardiac events (independent of QTc interval, gender, or β -blocker therapy) (13). The purpose of this study was to identify possible molecular mechanisms that underlie the loss-of-function for these high-risk LQT1 mutations.

The pore domain for all K⁺ channels is composed of two transmembrane segments joined together by a linker that contains the conserved amino acids of the selectivity filter. X-ray analysis of a K⁺ channel pore from *Streptomyces lividans* (KcsA) showed that the selectivity filter forms four contiguous K⁺ binding sites (S1-S4) (14). On the basis of this structure, and molecular dynamic simulations (MDS) that calculate the energetics of permeation, K⁺ conduction through the selectivity filter occurs by transitioning between two global states where two K⁺ ions occupy either S4-S2 or S3-S1 (15-21). The energetics for K⁺ ions moving between these binding sites is essentially barrierless, but short range K⁺-K⁺ repulsion between the ions is essential for promoting the exit of the K⁺ ion from S1 to a weak binding site outside the pore (S0) (15, 19).

The KcsA selectivity filter is nearly identical to Kv7.1 (as well as most other K⁺ selective channels). We made the simplifying assumption that the conserved amino acids in KcsA and Kv7.1 selectivity filters are structurally and functionally similar. This enabled us to study the impact of high-risk LQT1 pore mutations using the KcsA crystal structure. The advantage to using the KcsA structure is that it has been widely used to model K⁺ ion permeation (15-17, 19, 21, 22), and, similar to KCNE1 modified Kv7.1 channels, MDS show that the KcsA selectivity filter does not readily transit to non-conducting or inactivated states (15, 22).

EXPERIMENTAL PROCEDURES

Clinical

We identified over 60 patients heterozygous for LQT1 missense mutations that disrupt a highly conserved threonine or glycine near the selectivity filter (T322M-, T322A-, or G325R-Kv7.1) (Table I; Supplemental Table 1) (23, 24). One of these families (O) was reported previously (25). Individual patient details are provided in Supplemental Table 1. The study was conducted according to the principles of the Helsinki Declaration. Blood samples were obtained after signed written informed consent for genetic analyses and after approval by the local institutional ethics committees. The respective protocols for research-based or commercially available genetic analyses of patients with LQTS were performed. Genomic DNA was isolated from blood leukocytes and mutational analyses were performed using standard methods. The index patients in the families are negative for additional LQTS-associated mutations in both *KCNQ1* and the major LQTS-susceptibility genes (except for the index patient in family A who has yet to be tested).

Mutagenesis, transfection, electrophysiology, and biotinylation

The appropriate nucleotide changes to generate the T322M-, T322A-, or G325R-Kv7.1 mutations were introduced into WT-Kv7.1 cDNA and used for electrophysiological studies in HEK293 as previously described (26). To recapitulate I_{Ks} -like currents, all transfections included equal amounts of Kv7.1 the auxiliary Kv7.1 β -subunit KCNE1 plasmid DNA (3 μ g each). For co-expression studies, equal amounts of WT-Kv7.1 (1.5 μ g) and mutant Kv7.1 (1.5 μ g) were used to maintain the equivalent Kv7.1 plasmid DNA concentration (3 μ g). The cells were cultured in MEM (with 10% Fetal Bovine Serum) at 37°C and functional analyses were done using standard whole-cell patch clamp technique 24-30 hrs after transfection. The external solution contained (in mM) 137 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4 with NaOH), and an internal pipette solution contained (in mM) 130 KCl, 1 MgCl₂, 5 EDTA, 5 MgATP, 10 HEPES (pH 7.2 with KOH). An Axopatch-200B patch clamp amplifier (Axon Instruments, Union City, CA) was used to measure membrane currents and cell capacitance. Only cells with stable membrane resistances > 1 G Ω were studied. pCLAMP 10.0 (Axon Instruments; Union City, CA) was used to generate the voltage clamp protocols, acquire current signals, and for data analyses. Origin 7.0 (Microcal; Northampton, MA) was used for performing Boltzmann fitting, generating current-voltage (I-V) relations, and plotting graphs. The Boltzmann equation used to describe the I-V relations was:

$$I = (I_{\text{MIN}} - I_{\text{MAX}} / 1 + e^{[(V - V_{1/2})/k]}) + I_{\text{MAX}}$$

I_{MIN} is the minimally activated current, I_{MAX} is the maximally activated current, $V_{1/2}$ is the mid-point potential for half maximal activation, and k is the slope factor (mV/ e -fold change). For all experiments the holding potential was -80 mV, and the dotted line in figures corresponds to the zero current baseline. Voltage clamp experiments were performed at 22-23°C within 1-2 hours of removing the cells from their culture conditions.

The biotinylation procedure was described previously (26). Western blots of the biotinylated proteins were analyzed using the Odyssey infrared imaging system (Li-Cor, Lincoln, NE). The membranes were incubated in anti-Kv7.1 (Alomone labs, Jerusalem, Israel) and anti-Na⁺/K⁺-ATPase (Abcam, Cambridge, MA) to normalize for the biotinylation reaction. The appropriate IRdye secondary antibodies (Li-Cor, Lincoln, NE) were used to detect anti-Kv7.1 and anti-Na⁺/K⁺-ATPase. To ensure that the cell surface biotinylation did not label intracellular proteins, the samples were probed with anti-calnexin (Abcam, Cambridge, MA). The Odyssey software (Li-Cor, Lincoln, NE) was used to quantify the density of the protein bands. The relative intensity of Kv7.1 to Na⁺/K⁺-ATPase intensity was calculated by normalizing the intensity of the Kv7.1 signal to the intensity of the Na⁺/K⁺-ATPase signal.

Molecular Dynamic Simulations

Details regarding the MDS simulation are provided in the Supplemental Methods. Briefly, the MDS systems for the KcsA channels (PDB:1k4c) (27) were constructed using the Visual Molecular Dynamics (VMD) (28) molecular graphics software, and KcsA simulations were performed using NAMD (Not just Another Molecular Dynamics program) (29). The KcsA structure was used to generate a channel embedded in a lipid bilayer consisting of 1-palmitoyl-2-palmitoleoyl-sn-glycero-3-phosphocholine (POPC) molecules, with explicit water, K⁺ ions, and Cl⁻ ions. Two K⁺ ions were placed in the selectivity filter and one was placed in the cavity. A series of short MDS simulations were performed to equilibrate the system as a whole. Production simulations were performed for up to 60 ns. A time-step of 2 fs was employed in all simulations and the atomic trajectory data was written at 1 ps intervals. The VMD program was used to analyze the atomic trajectories. The MDS showed

that the root mean square deviation (RMSD) values for α -carbon (C_α) backbone and amino acids quickly reached steady state after 5 ns. The relative RMSD values for the WT- and mutant simulations were similar to one another (data not shown), suggesting that the mutations did not grossly affect channel stability.

Statistical analysis

Data are reported as the mean $\pm$ standard error (SE) unless noted. A one-way ANOVA with a Bonferroni post-hoc test was used to determine if either of the mutant parameters differed from WT ($p < 0.05$ was considered significant).

RESULTS

The purpose of this study is to identify the possible molecular mechanisms for high-risk LQT1 mutations in the pore domain. Due to the wide range of LQT1 expressivity in patients, a large number of patients with the same mutation need to be studied to confirm that they confer a high risk for LQT1-related cardiac events. We identified over 60 patients from 26 families that are heterozygous LQT1 mutations T322M-, T322A-, or G325R-Kv7.1 (Figure 1; Table 1 and Supplemental Table 1) (11). Over 1/3rd of the patients have marked QT prolongation ($QT_c > 500$ ms) and about 85% of these families have histories of syncope, torsades, near drowning, or sudden cardiac death triggered primarily by exercise or swimming (30). Overall, the clinical data from this large cohort suggested that T322M-, T322A-, and G325R-Kv7.1 confer a high risk for LQT1-related events.

We studied the functional phenotypes for these mutations by voltage-clamping cells transiently expressing WT-, T322M-, T322A-, or G325R-Kv7.1 with KCNE1 (Figure 1A). Macroscopic currents were recorded by applying step-like pulses from -80 mV to 70 mV in 10 mV increments for 5 s, followed by a “tail” pulse for 5 s to -50 mV (Figure 1A). The peak current amplitude recorded at the start of the tail pulse was plotted as a function of the step pulse potential for cells expressing WT-, T322M-, T322A-, or G325R-Kv7.1 (Figure 1B). To mimic the patients’ heterozygous genotype, we also studied cells co-expressing WT- and T322M-, T322A-, or G325R-Kv7.1 (Figure 1A, 1C). Cells expressing WT-Kv7.1 generated large currents that resembled native I_{Ks} ; cells expressing T322M-, T322A-, or G325R-Kv7.1 did not generate current; and cells co-expressing WT- and T322M-Kv7.1, T322A-Kv7.1, or G325R-Kv7.1 conducted only small currents (Figure 1B). The I-V relations for the peak tail current were plotted as a function of the step voltage, and the data were described with a Boltzmann function to calculate changes in I_{MAX} , $V_{1/2}$, and k . Cells co-expressing WT- and T322M-, T322A-Kv7.1, or G325R-Kv7.1 reduced the I_{MAX} by $\sim 70\%$ with only small changes in activation gating (Supplemental Table 2). Importantly, the loss-of-function phenotype was not linked to co-expression of KCNE1, because cells expressing WT-, T322M-, T322A-, or G325R-Kv7.1 without KCNE1 showed that the mutations still did not generate any current (data not shown). These data demonstrate that T322M-, T322A-, and G325R-Kv7.1 caused a complete loss of Kv7.1 current and dominant negative suppression of WT-Kv7.1 current.

We tested whether T322M-, T322A-, and G325R-Kv7.1 caused a loss-of-function by simply reducing the amount of Kv7.1 expressed at the cell surface membrane. Biotinylation assays showed that cells expressing T322M-, T322A-, or G325R-Kv7.1 and KCNE1 reduced the cell surface membrane expression of Kv7.1 (Supplemental Figure 1A, 1C). However, the reduction was insufficient to explain a complete loss in current (Figure 1A, 1B). Additionally, although cells co-expressing WT-Kv7.1 and T322M-, T322A-, or G325R-Kv7.1 showed dominant negative functional effects (Figure 1A, 1C), they did not show a decrease in Kv7.1 at the cell surface membrane (Supplemental Figure 1B, 1C). Together,

these data suggest that a decrease in Kv7.1 expression at the cell surface membrane does not account for the mutations' complete loss-of-function or dominant negative phenotypes.

The mutated threonine and glycine are highly conserved pore residues in the K⁺ channel family (Figure 2A). Given their close proximity to the K⁺ channel selectivity filter (Figure 2B), we tested whether incorporating analogous mutations into the KcsA structure altered the architecture of the selectivity filter and permeation events. Figure 2C-2F shows representative MDS images for the WT-, T85M-, T85A-, or G88R-KcsA selectivity filter. The WT-KcsA selectivity filter formed four contiguous sites that could bind to K⁺ ions and water molecules in an alternating manner. For the most part, the oxygen atoms that lined the WT-KcsA selectivity filter faced the central pore axis; however, similar to what has been shown by others (17, 21, 22), the carbonyl oxygen atom between S2 and S3 (V76) on each α -subunit could briefly "flip" away from the central pore axis (Supplemental Movie 1). The representative selectivity filter images for the T85M-, T85A-, or G88R-KcsA simulations showed that these mutations essentially stabilized the flipped configuration (Figure 2D-2F; Supplemental Movies 2-4). T85M- and T85A-KcsA stabilized the flipped configuration for one of the α -subunits (Figure 2D 2E), and G88R-KcsA stabilized the flipped configuration for two adjacent α -subunits (Figure 2F). The transition of the flipped carbonyl oxygen away from the central pore axis reflects a change in the dihedral angles around the V76-G77 amide plane. This can be visualized in an overlay of Ramachandran plots calculated at each ps of the simulation for the four α -subunits. Figure 3A shows the overlay of the Ramachandran plots for the WT-KcsA α -subunits. The dihedral angles for V76 and G77 are the most dynamic, which reflects the carbonyl oxygen alternating between the central pore-axis and flipped configuration. The Ramachandran plots for each WT KcsA α -subunit is similar or symmetrical to one another. Figure 3B-3C show the Ramachandran plots for T85M-, T85A-, or G88R-KcsA, respectively. Notice the mutant α -subunits that stabilize the flipped configuration have very different Ramachandran plots when compared to the other α -subunits.

All the simulations were initiated with K⁺ ions at a weak binding site in the cavity (S5) and at S3-S1. The WT-KcsA simulation showed that the three K⁺ ions had moved from the initial (S5)-S3-S1 state to a S4-S2-(S0) state (Figure 2C). Based on the relative location of the K⁺ ions in the images from the mutant MDS (Figure 2D-2F), the α -subunit(s) in the flipped configuration delayed the K⁺ ions from moving into the S4-S2-(S0) state. To better appreciate the dynamic movement of the different K⁺ ions during the simulations, we plotted their z-axis coordinates over time (Figure 4). The WT-KcsA simulation showed that the K⁺ ion at S5 quickly approached S4, which was accompanied by the coordinated movement of the three K⁺ ions to the S4-S2-(S0) state (Figure 4A). This permeation step is qualitatively similar to the knock-on mechanism originally proposed by Hodgkin and Keynes (1955) (31) and described by in detail by Berneche and Roux (2001) for KcsA (15). The T85M- and T85A-KcsA simulations showed a similar permeation step but with a much longer delay (Figure 4B, 4C). Closer examination of the T85M- and T85A-KcsA simulations showed that the permeation could not occur as long as one of the α -subunits was in the flipped configuration. In contrast, the G88R-KcsA simulation showed dramatically different K⁺ ion trajectories (Figure 4D). The K⁺ ion at S5 approached S4 but then moved back into the cavity; the K⁺ ion at S3 then moved to S4; and the K⁺ ion at S1 stayed in place.

Since T85M- or T85A-KcsA delayed (but did not prevent) the permeation step, we speculated that they alter the relative attractive/repulsive forces that underlie rapid K⁺ permeation. Accordingly, we calculated the change in the K⁺ ion restoring force at S3 and the interaction energy (mainly Columbic repulsion) between the different K⁺ ions (Figure 4E, 4F). When one of the α -subunits was in the flipped configuration, the K⁺ ion at S3

interacted more closely with the remaining carbonyl oxygen atoms and the net electrostatic repulsion with the K^+ ion at S1 was decreased. Together the data suggest that T85M- or T85A-KcsA upset the balance of forces needed to facilitate that rapid exit of the K^+ ion at S1.

To ensure the robustness of the MDS, we performed a negative control by testing a substitution in the KcsA selectivity filter that is not expected to negatively affect K^+ permeation. The V76 residue in the KcsA selectivity filter corresponds to an I313 in the Kv7.1 (Figure 2A). This is a very conservative substitution whose side chain differs by only one methylene group, and functional studies show that the engineered I313V-Kv7.1 substitution is not different when compared to WT-Kv7.1 (32). MDS of V76I-KcsA demonstrated it behaved similar to WT-KcsA (Figure 5A-5C).

DISCUSSION

A major challenge for clinicians is to determine which therapeutic strategy is the most appropriate for an individual LQT1 patient. The value of genetic testing in selecting the proper therapy for a given patient is limited because of its inability to either accurately predict functional phenotypes or forecast disease expressivity/severity. In an effort to move beyond genotype risk stratification simply based on the type of LQTS (i.e. LQT1, LQT2, LQT3, etc.), multiple studies are identifying intragenic approaches to correlate the risk for LQT-related cardiac events based on the type, location, and dysfunction of a mutation (9, 13, 33-35). These types of studies found that LQT1 mutations which disrupt conserved amino acids in the pore region are an independent risk factor for cardiac events (13). The purpose of this work was to provide molecular insight as to how these high-risk LQT1 mutations might cause channels to malfunction. We were able to generate a robust molecular model for high-risk LQT1 mutations by 1) identifying a number of different LQT1 patients with the same mutations in the pore, 2) studying their functional phenotypes *in vitro*, and 3) performing MDS of analogous mutations in KcsA. The clinical data confirmed that these mutations conferred a high-risk for LQT1-related events, and the *in vitro* data suggested that mutations generate non-functional Kv7.1 channels and dominant negative effects. The *in silico* data suggested a mechanism for the disruption of channel function, namely that a backbone conformational change disrupts permeation through the selectivity filter.

A major advantage for studying the LQT1 mutations using KcsA is that a model for K^+ permeation has been well developed for this structure (15). MDS studies that calculated the energetics of KcsA permeation suggest that the movement of K^+ ions between binding sites in the selectivity filter is essentially barrierless; however the K^+ ion at S1 is in a deep energy well and cannot exit towards S0 (15, 19). Berneche and Roux (2001) proposed that this is overcome by electrostatic repulsion between the other K^+ ions in the pore. As the K^+ ion at S5 approaches S4, the second K^+ ion at S3 initiates its transition to S2, electrostatic repulsion from the incoming K^+ ion at S4 and the K^+ ion transiting to S2 lift the bottom of the deep free energy well for the K^+ ion at S1. Ultimately the K^+ ion at S1 becomes unstable and exits toward S0. Without the repulsion from the other two incoming ions, the K^+ ion at S1 remains trapped in its deep energy well.

The K^+ trajectories for our WT-KcsA MDS are in good agreement with this energetic model of K^+ permeation (Figure 4A). We found that incorporating T85M- and T85A-KcsA primarily altered the S2-S3 binding site and decreased the net electrostatic repulsion from the incoming K^+ ion at S4 and the ion at S3 (Figure 4B). The simulations suggested that this would increase the trapping of the K^+ ion at S1 (Figures 3E, 3F) and decrease K^+

permeation. The effect that G88R-KcsA had on the S2-S3 binding site was much more severe and appeared to prevent the permeation step altogether.

The modeling raises several questions, including how do different mutations stabilize the flipped configuration? And why is this seen for only one or more of the α -subunits? The simulations showed that T85M-, T85A-, and G88R-KcsA similarly potentiated hydrogen bond formation between D80 and the R89 residue on the adjacent α -subunit (Figure 3B). We speculate the potentiation of this bond might increase the stability of the flipped configuration. The simulations also showed that when one of the T85M- or T85A-KcsA α -subunits entered the flipped configuration, the K^+ ion at S3 more closely associated with the remaining carbonyl oxygen atoms (Figure 4A). This likely prevented the remaining α -subunits from entering the flipped configuration. However, if the K^+ ion binding to S3 is lost, as it was in the G88R-KcsA simulation, then we suspect that all four of the α -subunits would eventually enter the flipped configuration. Indeed not only a second, but a third G88R-KcsA α -subunit entered the flipped configuration after 55 ns (data not shown).

There are several limitations to our approach. The functional data were obtained in a widely used heterologous expression system and may not completely recapitulate the native condition. Although the availability of a high quality structure for the closely related KcsA allowed us to investigate the molecular basis for the functional disruption by the different LQT1 mutations, not all of the KcsA pore residues are identical to Kv7.1. To circumvent this, we attempted to perform MDS using a published Kv7.1 homology model. However, this proved to be problematic because the selectivity filter was unstable and required the application of dihedral restraints in order to support permeation (data not shown) (36). A more recent Kv7.1 homology model also proved problematic because the selectivity filter is in a C-type inactivated configuration (37). A second limitation to using KcsA is that it does not include the obligatory I_{Ks} β -subunit KCNE1. KCNE1 profoundly affects Kv7.1 by eliminating C-type inactivation (38-41). Studies suggest that KCNE1 eliminates C-type inactivation by interacting with a phenylalanine (F340) in the second transmembrane segment of the pore domain, near the hinge region responsible for opening the channel. This phenylalanine is conserved in KcsA (F103) (42), and studies indicate that, as KcsA opens, F103 reorients to form a hydrogen bond network in the selectivity filter to facilitate C-type inactivation (43, 44). The interplay between KcsA opening and C-type inactivation is expected to occur in other K^+ channels, including Kv7.1 (42-44). Assuming KCNE1 prevents the reorientation of F340 in Kv7.1 to eliminate C-type inactivation, then the closed KcsA structure (which has F103 in an orientation that does not promote C-type inactivation) might represent an adequate surrogate to model the selectivity filter of a KCNE1 modified Kv7.1 channel. Lastly, MDS using KcsA does not provide mechanistic insight into how the mutations alter the cell surface membrane expression of Kv7.1 or its voltage-dependent gating. Based on the functional data, these changes do not likely account for the high-risk phenotypes in the patients. The cell surface expression of Kv7.1 was not decreased in conditions that mimicked heterozygosity (Supplemental Figure 1B) and there are only relatively small effects on gating (Supplemental Table 2).

In summary, we identified atomic-scale changes associated with a higher risk, dominant negative, pore-localizing LQT1-causative mutations. These data support the notion that MDS may assist in the pathogenic assessment of high-risk LQT1 mutations. We expect that subsequent advances in crystallography, homology modeling, and improvements in the force fields/computer hardware for MDS will further the applicability of this approach to non-conserved residues, other regions of Kv7.1, and other processes disrupted by LQT1 mutations (i.e. gating, co-assembly, phosphorylation, etc.)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

LQTS	long QT syndrome
LQT1	long QT syndrome type 1
MDS	molecular dynamic simulations
VMD	Visual Molecular Dynamics
NAMD	Not Just Another Molecular Dynamics program
POPC	palmitoyl-2-palmitoleoyl-sn-glycero-3-phosphocholine
RMSD	root mean squared deviation

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Figure 1. T322M-, T322A-, and G325R-Kv7.1 generate nonfunctional channels and cause dominant negative suppression of WT-Kv7.1 current

A) Representative families of currents recorded from cells expressing WT-, T322M-, T322A-, or G325R-Kv7.1 using the voltage protocol shown. Also shown are representative families of currents from cells co-expressing WT- and T322M-, T322A-, or G325R-Kv7.1. **B)** The graph shows the corresponding mean I-V relations for the peak tail current plotted as a function of the test voltage for WT-Kv7.1 (black solid squares), T322M- (grey solid circles), T322A-Kv7.1 (grey upward triangles) or G325R-Kv7.1 (grey downward triangles). **C)** The graph shows the corresponding mean I-V relations for the peak tail current plotted as a function of the test voltage for WT-Kv7.1 (black solid squares), WT- & T322M-Kv7.1 (grey solid circles), WT- & T322A-Kv7.1 (grey upward triangles) or WT- & G325R-Kv7.1 (grey downward triangles). The mean peak I-V relations were described using a Boltzmann equation (grey line). All of these studies included KCNE1.

Figure 2. Sequence alignment of K⁺ channel pore domain and the position of the conserved threonine and glycine with respect to the K⁺ channel selectivity filter

A) Sequence alignments of the selectivity filter for different K⁺ channels: Kv7.1 (NP_000209); KcsA (NP_631700.1); GluR0 = ionotropic glutamate receptor from *Nostoc punctiforme* (Swiss-Prot ID: P73797); c. ele = voltage-dependent K⁺ channel from *Caenorhabditis elegans* (GenBank ID: AAB95119.1); KvAP = voltage-dependent K⁺ channel from *Aeropyrum pernix* (Swiss-Prot ID: Q9YDF8.1); Kv1.1 (GenBank ID: NP_000208); Kv2.1 (GenBank ID: AAB88808.1); Kv4.1 (GenBank ID: NP_004970.3); Kv8.1 (GenBank ID: NP_055194.1); Kv9.1 (GenBank ID: NP_002242.2). Only amino acid residues that correspond to the pore helix, the selectivity filter (SF), and the linker between the selectivity filter and the second transmembrane segment (TM2) in the pore domain are shown. The asterisks denote the conserved threonine and glycine disrupted by T322M-, T322A-, or G325R-Kv7.1 mutations. Red residues = conserved in Kv7.1 and KcsA; blue residues = conserved in Kv7.1 but not KcsA; green residues = conserved in KcsA but not Kv7.1; black residues = not conserved in Kv7.1 or KcsA. **B)** Shown is a ribbon structure of two adjacent KcsA α-subunits (I, II) (purple) with the space filled van der Waal spheres for the conserved threonine (T85) and glycine (G88) (C = light blue; O = red; N = dark blue; H

= grey; the glycine atoms are all colored yellow for clarity). Also shown are the van der Waal spheres for the conserved aspartate in the K^+ channel selectivity filter (D80), conserved arginine/lysine (R89), the carbonyl oxygen atoms that line the selectivity filter from all four α -subunits, and the K^+ ions (green) in the selectivity filter. The threonine and glycine are in close proximity to the aspartate in the selectivity filter of the adjacent subunit. **C)** A representative atomic-scale image of the selectivity filter for WT, **D)** T85M-, **E)** T85A-, or **F)** G88R-KcsA MDS. Shown are the main chain backbone for selectivity filter residues TVGYG (C = light blue; O = red; N = dark blue) for all four α -subunits, the bound K^+ ions (green), and several adjacent water molecules (O = red; H = grey). The oxygen atoms that line the filter and the K^+ ions are shown as large spheres and water molecules are shown as small spheres for emphasis. S0-S4 approximates the different binding sites. The arrows highlight the α -subunits in the flipped configuration.

Figure 3. T85M-, T85A-, G88R-KcsA α -subunits in the flipped configuration disrupt the bond angle symmetry for the amino acid residues in the selectivity filter

An overlay of Ramachandran plots calculated at each ps from the trajectory file for the different selectivity filter amino acid residues (T74 = black, T75 = brown, V76 = red, G77 = orange, Y78 = yellow, G79 = green, D80 = blue, L81 = violet, Y82 = purple, and P83 = grey) for **A**) WT-, **B**) T85M-, **C**) T85A-, or **D**) G88R-KcsA α -subunits (I-IV). The arrows highlight the Ramachandran plots for the mutant α -subunits that have a stable flipped configuration.

Figure 4. T85M-, T85A-, and G88R-KcsA mutations delay or disrupt the K^+ permeation step, and T85M- and T85A-KcsA increase the K^+ restoring force at S3 and decrease K^+ K^+ repulsion at S1

A) The graphs show the movement of the three K^+ ions for WT-, B) T85M-, C) T85A-, or D) G88R-KcsA simulations along the z-axis over time. The origin for the z-coordinate is the center of the membrane (0 Å) and S0-S4 approximates the different binding sites from the WT-KcsA simulation. E) The graph shows the mean restoring force calculated at S1 and S3 for WT- (black), T85M- (light grey), or T85A-KcsA (dark grey) ($n = 3$ simulations each) (* $p < 0.05$ compared to control). F) The graph shows the mean interaction energies between the different K^+ at S1 (K^+_{S1}), S3 (K^+_{S3}), or K^+ ion moving into S4 (K^+_{S4-S5}) for WT- (black), T85M- (light grey), or T85A-KcsA (dark grey) ($n = 3$ simulations each) (* $p < 0.05$ compared to control).

Figure 5. The V76I-KcsA substitution does not disrupt the binding sites in the selectivity filter or K⁺ permeation

A) A representative atomic image of the selectivity filter for V76I-KcsA. Shown are the TIGYG amino acid residues (C = light blue; O = red; N = dark blue) for all four α -subunits, the bound K⁺ ions (green), and several adjacent water molecules (O = red; H = grey). The oxygen atoms that line the filter and the K⁺ ions are shown as large spheres and water molecules are shown as small spheres for emphasis. S0-S4 approximates the different binding sites. **B)** The graphs show the movement of the three K⁺ ions along the z-axis over time. The origin for the z-coordinate axis is the center of the membrane and S0-S4 approximates the different binding sites from the WT-KcsA simulation. V76I-KcsA does not delay or disrupt the permeation step. **C)** Cumulative Ramachandran plots for the different selectivity filter amino acid residues (T74 = black, T75 = brown, I76 = red, G77 = orange, Y78 = yellow, G79 = green, D80 = blue, L81 = violet, Y = purple, and P83 = grey) calculated for the four (I-IV) V76I-KcsA α -subunits are shown. The Ramachandran plots for each V76I-KcsA α -subunit is similar to one another and to the WT-KcsA α -subunits (Figure 3A).

Table 1

Clinical characteristics for genotype positive patients with T322M-, T322A-, or G325R-Kv7.1. Symptomatic is defined as experiencing syncope, torsades, or near drowning. See Supplemental Table 1 for individual patient details.

Genotype positive families, n	26
family history of symptoms, n	22
T322M, n	13
family history of symptoms, n	9
T322A, n	2
family history of symptoms, n	2
G325R, n	11
family history of symptoms, n	11
Genotype positive subjects, n (female, male)	63 (35, 28)
symptomatic, n	29 (19, 10)
mean QTc $\pm$ SD ms	483 $\pm$ 44 (490 $\pm$ 32, 473 $\pm$ 55)
average age $\pm$ SD years	29 $\pm$ 18 (31 $\pm$ 20, 26 $\pm$ 17)
T322M, n	29 (16, 13)
symptomatic, n	12 (7, 5)
mean QTc $\pm$ SD ms	497 $\pm$ 43 (505 $\pm$ 32, 488 $\pm$ 54)
average age $\pm$ SD years	29 $\pm$ 18 (29 $\pm$ 20, 26 $\pm$ 16)
T322A, n	12 (7, 5)
symptomatic, n	2 (2, 0)
mean QTc $\pm$ SD ms	463 $\pm$ 27 (473 $\pm$ 22, 447 $\pm$ 27)
average age $\pm$ SD years	20 $\pm$ 16 (24 $\pm$ 18, 15 $\pm$ 12)
G325R, n	22 (12, 10)
symptomatic, n	15 (10, 5)
mean QTc $\pm$ SD, ms	474 $\pm$ 47 (479 $\pm$ 31, 468 $\pm$ 63)
average age $\pm$ SD, years	35 $\pm$ 19 (39 $\pm$ 20, 30 $\pm$ 18)