Hereditary long-QT syndrome (LQTS) is a disease characterized by a prolonged ventricular repolarization and a variable clinical course with arrhythmia-related syncope and sudden death. Mutations involving the human ether-a-go-go-related gene (HERG or KCNH2) account for LQTS subtype 2 (LQT2). The KCNH2 gene encodes the α-subunit of a voltage-gated potassium channel underlying LQT2. Functional KCNH2 channels are tetrameric proteins that result from coassembly of 4 individual KCNH2 subunits. Each subunit contains 6 transmembrane-spanning domains (S1 to S6). The region between S5 and S6 forms the ion-selective pore of the channel, whereas the N-terminus contains an eukaryotic Per-Arnt-Sim (PAS) domain.

The clinical course of the LQTS is influenced by the specific genotype of LQTS. Correlations between genotype and phenotype are useful for the clinician because they offer a tool for risk stratification and treatment of LQTS. In this regard, Moss et al recently reported a less favorable prognosis for patients with LQT2-causing mutations localized in the pore domain. In the present study, we have identified a novel nonpore missense mutation, K28E, in a large LQT2 family associated with severe clinical characteristics.

In vitro study of the K28E mutation indicated both defects in KCNH2 channel trafficking and gating. Trafficking-deficient mutations have become a focus of interest because curative therapy is feasible by restoration of intracellular trafficking. It remains to be established whether rescue of trafficking-deficient channels with aberrant gating characteristics is reasonable as therapy. This issue could be addressed by the study of the K28E mutation.

**Methods**

**Genetic Analysis**

Genomic DNA was extracted from peripheral blood lymphocytes with the use of standard techniques. Marker analysis was performed to include or exclude involvement of LQTS candidate genes (LQT1 to LQT3) as described previously. A denaturing high-performance liquid chromatography (D-HPLC) (Wave Transgenomics) protocol was used to analyze the entire coding region of KCNH2. Abnormal D-HPLC elution profiles were analyzed by direct sequencing analysis with the use of fluorescent dye chemistry (Big-Dye Terminator Ready Reaction Kit; Applied Biosystems) and by using the ABI-3100 genetic analyzer (Applied Biosystems). The study was performed in accordance with recommendations of the Ethics Committee of the University Hospital Leuven.

**Key Words:** long-QT syndrome ■ genetics ■ electrophysiology ■ ion channels
Site-Directed Mutagenesis and Transfection

Wild-type (WT) KCNH2 cloned in pCEP4 was a kind gift from Dr M. Sanguinetti (University of Utah, Salt Lake City). The K28E mutation was engineered by using the QuikChange-XL site-directed mutagenesis kit (Stratagene) and the following primers: 5'-GCCAGAGCCGTGAGTTACATCAAGGCACG-3' (sense) and 5'-CGTTGCGATGATGAACTCACGGCTCTGGC-3' (anti-sense). Underlined nucleotides indicate the altered sequence. WT and K28E mutant KCNH2 cDNA were subcloned upstream of the coding region of a green fluorescent protein (GFP) expression vector pEGFP-N1 (Invitrogen) by using restriction sites BamHI and HindIII. The K28E mutation and the in-frame fusion of GFP-KCNH2 protein were verified by automatic sequencing analysis and restriction enzyme analysis. HEK293 cells (ATCC-CRL-1573, Manassas) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% glutamine in a humidified 5% CO2 incubator at 37°C. HEK293 cells were transfected transiently with the use of Superfect reagents (Westburg), with 4 μg of either WT-KCNH2 or K28E-KCNH2 mutant plasmid, with 4 μg of WT and 4 μg of K28E mutant plasmid, or with 8 μg of WT plasmid. GFP plasmid (ratio GFP plasmid/KCNH2 plasmid 1/4) was cotransfected to allow identification of transfected cells. For imaging studies, 5 μg of either WT-GFP or K28E-GFP plasmid was used. After transient transfection, HEK293 cells were studied at 36 to 48 hours. The cells were seeded on coverslips coated with poly-l-lysine (Sigma-Aldrich) in a 12-well plate 12 to 24 hours before electrophysiological or imaging studies. WT- and K28E-expressing cells were cultured for 36 hours in E-4031 (Sigma-Aldrich) at different drug interactions. The cells were switched to E-4031–free culture medium for at least 1 hour to remove the drug before patch-clamp studies.

Patch-Clamp Recordings

For electrophysiological studies, a coverslip was transferred to a small perfusion bath mounted on the stage of an inverted microscope equipped with epifluorescence optics. Cells were superfused with Tyrode’s solution containing (in mmol/L) 135 NaCl, 5.4 KCl, 1.8 CaCl2, 0.9 MgCl2, 10 glucose, 0.33 Na2HPO4, and 10 HEPES (pH 7.45 with NaOH). The internal pipette solution contained (in mmol/L) 155 KCl, 1 MgCl2, 5 Na2ATP, 1 EGTA, 0.1 Na2GTP, 5 HEPES (pH 7.25 with KOH). Membrane currents were recorded in whole-cell configuration with the use of pipettes with a tip resistance of 2 to 5 MΩ when filled with the internal solution. The electrodes were connected to an Axopatch 200B amplifier (Axon Instruments) and an analog-to-digital interface controlled by pClamp software. Signals were sampled at 1 kHz. Leak compensation was not used. All experiments were performed at room temperature. The membrane capacitance was 26±2.5 pF (n=31) for WT cells, 28±1.8 pF (n=39) for K28E mutant cells, and 28±4.8 pF (n=16) for cotransfected cells (P>0.05). Cells were held at −80 mV between test pulses. Specific voltage pulse protocols (see Results and figure legends) were used to study activation, inactivation, and deactivation of the KCNH2 current.

Figure 1. A, Family pedigree. Square indicates male; circle, female; filled symbol, carrier of K28E mutation; empty symbol with N, genotyped noncarrier; and open symbol, no data available. See text for clinical information. B, ECG of individual III:2. QTc is 458 ms. Arrow indicates notched T wave.
Western Blot Analysis

Membrane protein preparation and Western blot procedures were used as previously described. The proteins were subjected to a 7.5% SDS–polyacrylamide gel electrophoresis and subsequently transferred onto nitrocellulose membranes. The nitrocellulose membranes were initially incubated with the KCNH2 anti–C-terminus (Alomone Labs; 1:400 dilution) at room temperature during 3 hours followed by incubation with horseradish peroxidase–conjugated anti-rabbit antibody for another hour. Antibody detection was performed by using the ECL detection kit (Amersham Biosciences). Films were scanned, and densitometry of the bands was performed with NIH Image version 1.62 software.

Imaging Studies

Cells were transfected with either GFP-tagged WT or K28E mutant plasmids and prepared in a manner similar to that described for the electrophysiological experiments. To distinguish between membrane-bound and endoplasmic reticulum (ER)–retained KCNH2 proteins, plasma membranes were stained with the red fluorescent dye WGA-TR (Molecular Probes), and ER was labeled by cotransfection of red fluorescent plasmid pDsRed-ER (kindly donated by Dr D. Snyders, Antwerp, Belgium). Cells were photographed at ×630 magnification under a microscope (LSM510, Zeiss). Confocal imaging analysis was performed with an argon laser.

Figure 2. Less current density in K28E mutant cells. A, Examples of current recordings obtained during application of activation voltage-clamp protocol. B, Tail current amplitude (pA/pF) as a function of test potential. Repeated-measures ANOVA for potentials between −20 and +70 mV revealed statistically significant differences between WT and mutant \( (P=0.03) \) and between mutant alone and WT + mutant \( (P<0.001) \). Number between parentheses indicates n value. In these experiments, 4 μg of either WT-KCNH2 or K28E-KCNH2 mutant plasmid was used. For coexpression experiments, 4 μg of WT in combination with 4 μg of K28E mutant plasmid was used. C, Voltage dependence of activation was not different; \( V_{1/2} \) values of activation and slope factors were as follows: for WT, −14.2±1.9 mV and 7.5±0.3; for mutant, −15.5±1.7 and 6.8±0.6 mV; and for WT+mutant, −10.2±1.4 mV and 8.1±0.5.

Figure 3. No difference in time course of activation. A, Experimental protocol and original current tracings. Activation time course was measured by a single exponential fit of the envelope of tail currents. B, Time constants of activation were not statistically significant between WT and mutant channels. Number between parentheses indicates n value.
Statistical Analysis
Data are presented as mean±SEM. Student t test was used for statistical analysis. Repeated measures ANOVA was used for statistical analysis of data presented in Figure 2B. Data were considered significantly different at P<0.05.

Results
Clinical and Genetic Characteristics
The pedigree of the LQTS family is shown in Figure 1A. LQTS due to a defect in KCNH2 was suggested by the typical ECG, showing QT prolongation in combination with notched T waves (Figure 1B) in phenotypically affected family members. Marker analysis of 7 related and clinically affected family members showed an identical haplotype for the LQT2 locus (data not shown). Subsequently, a DNA sample from patient IV:7 was used for DNA amplification, and, by subsequent D-HPLC mutation detection analysis of the KCNH2 gene, an aberrant elution pattern was observed in exon 2. Sequencing analysis identified a single nucleotide transition (A82G), which changes lysine (K) into glutamic acid (E) at codon 28 within the N-terminus of the KCNH2 channel.

Analysis of the clinical history of the LQTS family revealed that carriers of the K28E mutation had either ECG abnormalities or cardiac events, although DNA samples were not available from all affected individuals. Four patients (II:4, III:7, IV:5, IV:6) died suddenly; patient II:4 at age 60 triggered by loud noise, patient III:7 at age 34 after exercise, and patients IV:5 and IV:6 during sleep at age 23 and 22 years, respectively. No autopsy was performed in any of these patients, nor were tissue or DNA samples available to identify their genotype. Because no other reasons for sudden death (SD) could be identified, they most likely died of ventricular arrhythmias, conceivably as carriers of the K28E mutation. Because of the autosomal-dominant pattern of inheritance of LQTS, individual I:1 or I:2 should be considered an obligate carrier of the K28E mutation. No clinical information was available for these individuals. Ultimately, 15 carriers of the K28E mutation were identified in this family, and 4 deceased patients, from whom no DNA was available, were highly suspected of carrying the K28E mutation. All carriers had either ECG abnormalities or cardiac events. The pathogenicity of the mutation was supported by exclusion of the K28E mutation in a control panel of 75 healthy unrelated individuals of Caucasian origin (150 alleles) and by the localization of the mutated amino acid in an evolutionary highly conserved domain of the potassium channel protein.

Electrophysiological Properties of K28E Mutant Ion Channels
Figure 2A shows representative current recordings of transfected cells expressing either WT or K28E mutant ion channels subjected to an activation protocol. Tail currents in cells expressing the K28E mutant were reduced to 53% of levels recorded in cells expressing the WT ion channel (P=0.03, analyzed with repeated-measures ANOVA for potentials between +20 and +70 mV; Figure 2B). Because the symptomatic carriers were all heterozygous for the K28E mutation, we also measured the current density in cells coexpressing both the WT and K28E mutant channels, using the same amount of each plasmid as used for the experiments, assessing the consequences of expressing the WT or mutant channel alone. As shown in Figure 2B, the current density in cotransfected cells equaled the sum of the currents in cells transfected with the same amount of each plasmid alone. These data indicate that the mutant ion channel did not have any dominant negative effect on the WT ion channel but that the overall current density was determined by the stoichiometric expression of each channel. Normalized currents show that the voltage dependence of activation was not altered (Figure 2C). There was no difference in time constants of activation between WT and mutant channels (Figure 2A and 3B). The decreased current of the mutant was further confirmed by measuring the I-V relation when channels were maximally activated (Figure 4).

As can be observed in Figure 2A and 3A, deactivation at −50 mV is much faster in K28E mutant than WT ion
channels when expressed alone. Time course of deactivation was obtained by fitting the difference of the tail currents in Figure 2A with the measured steady state current at −40 mV with a double exponential function. Compared with the WT, the relative amplitude of the fast component as well as the rate of the 2 components (τf and τs) was significantly increased in the mutant (Table). Interestingly, deactivation time constants for cotransfected cells were comparable to those for WT cells (Table).

Steady state channel availability was analyzed by applying a 3-pulse protocol (Figure 5). After maximal activation at +40 mV, the channels were assumed to reach steady state inactivation with a minimum of deactivation during the second pulse to different levels (50 ms for WT and 10 ms for K28E mutant ion channels) (Figure 5A). For each cell, tail currents, obtained at the second depolarizing pulse to +40 mV, were normalized to the maximum: V1/2 shifted toward more negative voltages in K28E mutant cells (Figure 5B).

### Table: Accelerated Deactivation in Mutant but Not in Cotransfected Cells and Restoration to Control Values in E-4031–Pretreated Cotransfected Cells

<table>
<thead>
<tr>
<th></th>
<th>WT (n=11)</th>
<th>K28E (n=12)</th>
<th>WT and K28E (n=16)</th>
<th>E-4031 K28E (n=5)</th>
<th>E-4031 WT and K28E (n=5)</th>
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</thead>
<tbody>
<tr>
<td>τf, ms</td>
<td>346±22</td>
<td>111±15</td>
<td>318±25</td>
<td>189±52</td>
<td>265±29</td>
</tr>
<tr>
<td>τs, s</td>
<td>3.23±0.2</td>
<td>1.01±0.2</td>
<td>2.94±0.3</td>
<td>1.41±0.4</td>
<td>3.0±0.3</td>
</tr>
<tr>
<td>A/(A0+A1)</td>
<td>0.34±0.02</td>
<td>0.60±0.03</td>
<td>0.39±0.02</td>
<td>0.55±0.03</td>
<td>0.33±0.01</td>
</tr>
</tbody>
</table>

τf and τs indicate slow and fast time constant, respectively; A, amplitude of the components.

Deactivation time constants and relative amplitude of the fast phase at −40 mV are shown. There is a statistically significant difference (P<0.0001) between WT and mutant. There is no statistically significant difference between WT and cotransfected cells, between WT and E-4031–pretreated (10 μmol/L) cotransfected cells, between untreated cotransfected cells and E-4031–pretreated (10 μmol/L) cotransfected cells, and between untreated mutant and E-4031–pretreated mutant cells.

Normalization of kinetics of mutant channels is dependent on the presence of WT but not on E-4031 pretreatment.

Time course of inactivation, measured by fitting the decay of the tail currents, were not different (data not shown). Time constants for recovery of inactivation, determined by fitting the first 10 to 20 ms after a 3-second activating pulse (protocol, Figure 4A), were faster for the mutant (Figure 5C).

### Protein Processing Studied by Western Blot Analysis and Confocal Imaging

In Western blot analyses of cells transfected with WT plasmid, 2 characteristic bands were detected: a 135-kDa (lower) band, representing the immature form of the KCNH2 channel retained in the endoplasmic reticulum, and a 155-kDa (upper) band, representing the complexly glycosylated mature form of the KCNH2 channel, expressed on the cell plasma membrane. In contrast, immunoblotting of cells expressing the K28E-KCNH2 protein revealed only minimal amounts of the 155-kDa band but abundant levels of the 135-kDa band (Figure 6A). This was confirmed by densito-

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**Figure 5.** Negative shift in voltage-dependent channel availability in K28E mutant cells. A, Experimental protocol and original tracings. Examples show currents for hyperpolarizing step to −110 mV (♦) and to −80 mV (●) and the subsequent test depolarization to +40 mV. Arrow indicates mutant ion current for 10-ms hyperpolarizing step to −110 mV. B, Negative shift in voltage-dependent channel availability for mutant current: V1/2 of −99.3±3.7 mV and k of 16.0±0.8 mV (n=10) for WT shifted in mutant cells to V1/2 of −54.8±6.2 mV and k of 20.7±1.7 mV (n=5), except for −90 mV and −100 mV, n=3; for −80 mV, n=2; for −70 mV, n=1; P=0.04 [V1/2] and 0.01 [k]). C, Time constants for recovery from inactivation were faster for the mutant.
Figure 6. Western blot analysis of WT and mutant proteins and confocal imaging of GFP-tagged WT and mutant channels. A, Lanes 1 and 2: 20 μg of protein loaded; lanes 3 and 4: 30 μg loaded. Top bands represent the glycosylated, membrane-bound mature form of KCNH2 protein (155 kDa); bottom band represents the immature KCNH2 protein retained in the endoplasmic reticulum (135 kDa). Top bands are fainter for mutant proteins. The ratio of densitometric analysis of top band and bottom band was 0.29, 1.18, 0.29, and 1.95, respectively. B, WT cells. Top row, This cell was cotransfected with WT-GFP, resulting in a strong green fluorescence signal in the cytosol and plasma membrane (left), and the ER-specific red fluorescent plasmid pDsRed-ER, resulting in red staining of the ER (middle). The yellow color after merging of the GFP and DsRed signal reveals that some of the WT channel is present in the ER (right). Bottom row, Another cell was transfected with WT-GFP (green signal in the cytosol and plasma membrane; left) and stained with a red fluorescent WGA-TR dye, which specifically labels the plasma membrane. With the use of these double-labeling techniques, the GFP-WT protein was found to be present in both the ER and plasma membrane (yellow signal in Figure 6B). When the subcellular localization of GFP-K28E was analyzed by similar methods, the mutant channels were detected predominantly in the ER, with only minimal expression in the plasma membrane (Figure 6C). These data thus confirm the trafficking defect of the mutant.

Pharmacological Rescue of Mutant Ion Channels With E-4031

Because it is important to know whether patients with a K28E mutation might benefit from a restoration of the reduced current density to normal levels, we assessed whether the trafficking defect could be rescued by E-4031, a high-affinity KCNH2 channel–blocking drug previously shown to rescue trafficking-defective LQT2 mutations. As shown in Figure 7A, when cells were pretreated with E-4031 (10 μmol/L), the current density of the mutant ion channels was restored to WT levels. Current density of cells coexpressing WT and mutant channels and pretreated with E-4031 was similar to that of untreated cells, expressing double the amount of WT channels (Figure 7B). Western blot analysis confirmed that E-4031 increased the 155-kDa mature form of the K28E mutant protein. Most importantly, deactivation time constants and the ratio of fast to slow phases in cotransfected cells pretreated with E-4031 (10 μmol/L) were not significantly different from data in WT cells (Table).

Discussion

In the present study we report a novel mutation, K28E, in the PAS domain of the KCNH2 channel that is associated with a severe clinical outcome in a single large LQT2 family. In a recent study by Moss et al, 15% of 35 patients with a pore mutation in KCNH2 experienced SD or aborted SD compared with only 6% of 166 patients with nonpore mutations. In the present study 6 of 19 suspected carriers of the K28E-KCNH2 gene defect experienced SD or aborted SD, a potential fatality rate of 32%. Although we cannot rule out an adverse effect of an additional unknown genetic or environmental susceptibility risk factor in this family, our data indicate that a PAS domain mutation may result in a malignant clinical outcome. A previously reported mutation in the PAS domain (T65P) was also associated with SD, with a fatality rate of 37.5% in a total of 6 carriers and 2 suspected carriers. Interestingly, both mutations have a serious impact on current density. Eight of the 14 N-terminus mutations reported in the study by Moss et al have been shown to accelerate the rate of deactivation in vitro but without affecting the current density.
We have used the Luo-Rudy dynamic model\cite{17} to study the contribution of fast deactivation kinetics and reduced current density to the prolongation of the action potential (not shown). This analysis reveals that even a 5-fold decrease in time constants of deactivation prolonged the action potential much less than a 2-fold reduction in current density. It is thus conceivable that the reduction in current density caused by the K28E and T65P mutations is responsible for the more malignant clinical phenotype compared with other N-terminus mutations with documented faster deactivation kinetics but without reduced current density.

The rarity of each individual LQTS mutation in the population makes it difficult to perform accurate statistical analysis in predicting prognosis when one causative mutation is compared with another. Grouping of mutations according to the functional site may be a clinically relevant strategy that might help in prognostic assessment. Our data suggest that, besides mutations in the pore, those in the PAS domain may also have a deleterious impact. Thus, pore mutations should still be considered to be associated with more severe phenotypical manifestations, but caution is warranted that there may be serious exceptions to this “rule,” as exemplified by this report.

Recent work identified the highly conserved KCNH2 initial domain of the N-terminus as a PAS domain.\cite{4} Proteins with PAS domains are frequently involved in signal transduction.\cite{18} To our knowledge, the K28E mutation reported in this study is hitherto the most proximally located KCNH2 PAS domain mutation that has been characterized in vitro.

The K28E mutation accelerates deactivation by shortening the fast and slow time constants and increasing the amplitude ratio of the fast over slow component. From a structure-function point of view, this implies an important role of the PAS domain in KCNH2 channel gating. Several other PAS domain mutations affect deactivation kinetics and have been implicated in causing a LQT2 phenotype.\cite{19} It is important to note, however, that the deactivation kinetics of the heteromultimeric channels (ie, consisting of both WT and mutant subunits) were normal and comparable to those of WT channels, which is an unprecedented finding to the best of our knowledge.

The reduction in current density in conjunction with the decreased sarcolemmal expression, as indicated by Western blot and confocal microscopy imaging of the GFP-tagged ion channels, suggests a possible underlying deficient trafficking mechanism. Defective trafficking is a well-known pathophysiological mechanism responsible for a LQT2 phenotype.\cite{13} Restoration of normal intracellular transport with low-affinity channel antagonists is an attractive strategy for treating patients carrying these mutations.\cite{20} Our data indicate that E-4031–rescued K28E mutants show a normal current density and have normalized kinetics under condition of cotransfection with WT channels.

In conclusion, we have identified a novel KCNH2 mutation in a large LQT2 family with severe clinical characteristics. Our data indicate that a PAS domain mutation, which is no constituent of the channel pore region, may show overt malignant features. Therefore, at this stage, we would propose caution with regard to making an unambiguous clinical risk assessment based only on the localization of the mutation within the channel protein, nonpore versus pore mutation. The K28E mutation causes a challenging and unique biophysical phenotype as it alters trafficking and gating of KCNH2 channels. It indicates an important regulatory role for the PAS domain in KCNH2 channel function. By analyzing the K28E mutation in vitro, we were able to evaluate the possible efficacy of a specific therapy. The use of modified KCNH2 channel–blocking drugs is promising as mutation-specific therapy in this family but clearly requires detailed clinical evaluation.

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References


Novel Mutation in the Per-Arnt-Sim Domain of KCNH2 Causes a Malignant Form of Long-QT Syndrome
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