Mechanisms of $I_{Ks}$ suppression in LQT1 mutants

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KvLQT1 mutations; cellular processing; cellular phenotype; clinical phenotype; slow delayed rectifier potassium current; long Q-T syndrome

SIX GENETIC LOCI HAVE BEEN LINKED to hereditary long Q-T syndrome (LQTS) (1, 6, 11, 35, 43–45). Five genetic loci encode for cardiac ion channels, two ($KCNQ1$ and $KCNH2$) being $K^+$ channels and two ($KCNE1$ and $KCNE2$) being accessory $K^+$ channel subunits. LQT1 is the most common hereditary LQTS and linked to $KCNQ1$ (voltage-gated $K^+$ channel subtype KvLQT1) on human chromosome 11 (44). LQT5, another type of LQTS, is rare and linked to $KCNQ1$. $I_{Ks}$ is major determinants of phase 3 repolarization of the cardiac action potential (4).

LQT1 has an autosomal dominant form (Romano-Ward syndrome; see Ref. 28), a severe recessive form expressing deafness (Jervell and Lange-Nielsen syndrome; see Ref. 14), and a mild recessive form without deafness (26). Numerous mutations have been identified in LQT1 families (2, 5, 9, 17, 22, 29–30, 36, 39, 41–42, 48), and the clinical manifestations have ranged from none to sudden cardiac death. It is possible that the heterogeneity among clinical phenotypes reflects differences in channel dysfunction (15, 31, 34, 50). A comparison among cellular and clinical phenotypes has never been attempted, and it is unknown whether the severity of channel dysfunction predicts the severity of the clinical disease. Some KvLQT1 mutations have been characterized electrophysiologically (5, 36, 48), but it is unknown whether nonfunctional subunits are transported to the cell surface. This possibility may be important because in LQT2 ($HERG$) mutants, it has become clear that, in many instances, trafficking is defective (12, 50).

In the present study, we have combined immunochamber and electrophysiological methods to determine the mechanism of dysfunction of one deletion-insertion and four missense LQT1 mutant KvLQT1 mutations (see Fig. 1) by expressing cognate KvLQT1 mutations in Xenopus oocytes. We have also examined whether there is a correlation among cellular and clinical phenotypes.

METHODS

Mutagenesis and cRNA preparation. Mutations were prepared by PCR using human wild-type (WT)-KvLQT1 cDNA...
as a template (kindly provided by Drs. M. C. Sanguinetti and M. T. Keating). The PCR products were subcloned into the plasmid pCR2.1 vector (Invitrogen) for amplification and sequencing. For cRNA synthesis, PCR fragments were exchanged with WT-KvLQT1 fragments by double digestion of plasmid pSP64 (Promega)-WT-KvLQT1 using EcoRI, and cRNA was prepared with the mMESSAGE mACHINE kit (Ambion) using SP6 RNA polymerase. cRNAs were dissolved in 0.1 M KCl, and their size and concentration were evaluated by comparison with markers of known concentration (Life Technologies). cRNA concentrations were evaluated by comparing cRNAs with markers of known concentration (Life Technologies). The PCR products were subcloned into the plasmid pCR2.1 vector (Invitrogen) for amplification and sequencing. For cRNA synthesis, PCR fragments were exchanged with WT-KvLQT1 fragments by double digestion of plasmid pSP64 (Promega)-WT-KvLQT1 using EcoRI, and cRNA was prepared with the mMESSAGE mACHINE kit (Ambion) using SP6 RNA polymerase. cRNAs were dissolved in 0.1 M KCl, and their size and concentration were evaluated by comparison with markers of known concentration (Life Technologies). cRNA concentrations were evaluated by comparison with markers of known concentration (Life Technologies). The PCR products were subcloned into the plasmid pCR2.1 vector (Invitrogen) for amplification and sequencing. For cRNA synthesis, PCR fragments were exchanged with WT-KvLQT1 fragments by double digestion of plasmid pSP64 (Promega)-WT-KvLQT1 using EcoRI, and cRNA was prepared with the mMESSAGE mACHINE kit (Ambion) using SP6 RNA polymerase. cRNAs were dissolved in 0.1 M KCl, and their size and concentration were evaluated by comparison with markers of known concentration (Life Technologies).

**Fig. 1.** Four LQT1 (the most common hereditary type of long Q-T syndrome (LQTS)) mutations. S1–S6 and H3 indicate transmembrane segments and the external pore, respectively. The tripeptide glycine-tyrosine-glycine (GYG) is predicted to form the selectivity filter. The accessory subunit of the voltage-gated K⁺ channel subtype KvLQT1 minK is also shown. Branches potential structures are potential glycyl-glycyl-tyrosine sites. E, extracellular; I, intracellular; N, NH₂-terminus; C, COOH-terminus; ABC motif, segment homologous to the ATP binding domain of the ATP-binding cassette (ABC) superfamily of transporters; underlined portion, the antibody epitope. LQTS-linked KvLQT1 mutations were previously reported in Refs. 19–20 and 25–26.

**Electrophysiological experiments** were performed 3–5 days after injection. Currents were recorded using a conventional two-microelectrode technique and an OC-725B amplifier (Warner Instrument). Pipettes were filled with 3 M KCl and had resistances of 1–2 MΩ. Oocytes were perfused with a bath solution containing (in mM) 120 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 HEPES; pH 7.4 with Tris·OH. For testing selectivity we used a solution containing (in mM) 100 NaCl, 5 KCl, 1.5 CaCl₂, 2 MgCl₂, and 10 HEPES; pH adjusted to 7.4 with NaOH.

We used the pCLAMP suite of programs for data acquisition and analysis. Currents were filtered at 0.2 kHz and subsequently digitized at 0.7 kHz.

**KvLQT1 antibody.** Anti-KvLQT1 antibodies were generated in rabbits. A glutathione S-transferase (GST) fusion protein corresponding to the COOH-terminal 116 amino acids of KvLQT1 was produced in *Escherichia coli* BL21 cells and then purified over a glutathione Sepharose 4B column using standard procedures (Pharmacia Biotech). The purified GST fusion protein was sent to Research Genetics for antisera production.

**Protein extraction and Western blotting.** All cell lines were cultured in minimal essential media (MEM; GIBCO-BRL) containing 10% heat-inactivated fetal bovine serum (PBS; GIBCO-BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin in 37°C in a humidified atmosphere containing 5% CO₂. Lipofectamine Plus (GIBCO-BRL) was used for transient transfections. Transfections were performed using the recommended DNA-to-lipid ratios, conditions, and times (GIBCO-BRL). Cells were washed three times with cold PBS and scraped into solubilization buffer (containing 150 mM NaCl, 50 mM Tris, 1 mM EDTA, and 1% Triton X-100; pH 7.5) plus a protease inhibitor mixture (Complete, Boehringer-Mannheim). Samples were collected in Eppendorf tubes, incubated on ice for 45 min, and then sonicated for 3 s. After an additional 45 min on ice, we spun the samples for 45 min at 4°C at 5,000 g. The pellet was discarded, whereas the supernatant was collected for separation with SDS-PAGE.

Three to five days after injection, 20–30 eggs were harvested, resuspended in 0.3 M sucrose plus 10 mM sodium phosphate (pH 7.4) containing the protease inhibitor mixture, and homogenized with 20 strokes in a glass homogenizer. Samples were spun at 3,000 g for 10 min at 4°C, the pellet was discarded, and the supernatant spun at 48,000 g for 1 h at 4°C. Pelleted membranes were resuspended in solubilization buffer plus protease inhibitor mixture and subjected to SDS-PAGE (47). All the samples were mixed with reducing SDS sample buffer (7% SDS) and heated at 90°C for 15 min before separation on 10 or 7.5% SDS-PAGE (16). Electrophoresed proteins were transferred onto Immobilon P membranes (Millipore). Membranes were blocked with 5% nonfat dry milk dissolved in Tris-buffered saline plus 0.1%...
Tweens 20 and probed with anti-KvLQT1 antibody (1:1,000). The ECL Plus system (Amersham) was used to detect the bound antibodies.

**Immunocytochemistry.** Staining of oocytes was performed as described previously (47). Briefly, 3–5 days after injection, oocytes were fixed at 4°C overnight with 4% paraformaldehyde. The next day, oocytes were washed four times at 5 min each in PBS, imbedded in low-melting-point agarose (3% in PBS), and cut in 50-μm-thick slices.

Slices were incubated overnight at 4°C in 0.2% BSA in PBS plus 0.1% Tweens 20 and subsequently incubated with anti-KvLQT1 antibody (1:100 in 1% BSA dissolved in PBS + 0.1% Tweens 20) for 1–2 h at room temperature.

Slices were washed three times for 5 min with PBS and incubated with fluorescein-conjugated sheep anti-rabbit antibody (1:50; Cappel, Organon Teknika) for 1 h at room temperature. After slices were washed three times for 5 min in PBS, they were mounted with VECTOREX medium (Vector) and photographed using an Olympus inverted microscope equipped with a Spot32 digital camera and software from Diagnostic Instruments. For detection of GFP-Δ544, oocytes were analyzed and photographed immediately after slicing. Images were analyzed and mounted with Adobe Photoshop 5.0 for Windows 95.

**RESULTS**

I<sub>Ks</sub> produced by coexpression of minK with WT and KvLQT1 mutations. Injection of minK cRNA alone produced a small I<sub>Ks</sub>, which was saturated at the concentrations of minK cRNAs used in the mutant KvLQT1 experiments. Injection of minK plus WT-KvLQT1 cRNAs produced a much larger I<sub>Ks</sub>, as previously reported (see Refs. 3, 32, and 49; Fig. 2A). None of the mutant KvLQT1s produced currents similar to WT. The amplitudes were decreased, and/or the voltage dependences were abnormal (Figs. 2–4). For the missense mutations Y281C and Y315C, the currents were similar to injection of minK alone. For the other two missense mutations, S225L and A300T, currents were expressed that were clearly distinguishable from minK alone, but the amplitudes were significantly reduced from coinjection of minK with WT-KvLQT1 (Fig. 2). The voltage dependence was altered; for S225L, it was shifted to more positive potentials, and for A300T, it was shifted to more negative potentials (Fig. 3). As a check on the shifts, we performed experiments using long (18 s) depolarizing pulses. The voltage dependence of all currents was affected by the longer duration, but the relative shifts persisted (Fig. 3, B and C). For A300T, voltage dependence was similar to minK coassembling with endogenous KvLQT1 (half-maximal voltage = 17.1 mV, slope factor = 17 mV). To check whether this voltage dependence was affected by contamination from background current, we compared A300T plus minK with WT plus minK currents at higher cRNA concentrations of A300T (Fig. 4). The currents now had amplitudes closer to WT yet still displayed the left shift of voltage dependence. Injection of higher A300T cRNA concentration (2.5 μg/μl) was accompanied by a higher minK cRNA concentration (0.5 μg/μl) to ensure a sufficient cofactor. We checked whether this concentration affected the amplitude of endogenous I<sub>Ks</sub> and, in the same batch of oocytes, we compared currents produced by A300T (0.5 μg/μl) plus minK at 0.1 and 0.5 μg/μl of minK cRNA. The currents were not significantly different, with amplitudes at +40 mV of 0.44 ± 0.05 μA (n = 9) and 0.45 ± 0.04 μA (n = 8), respectively, which were similar to the results reported by Splawski and co-workers (37).

Currents produced by S225L and A300T also had activation time constants that differed from WT I<sub>Ks</sub> (Fig. 4C). A300T had a faster activation at +40 and +60 mV, as did S225L. For S225L, activation was slower than that of WT, at +20 mV.

For a more severe mutation, we tested the insertion-deletion Δ544 mutation (19). We found that Δ544 expressed currents similar to minK alone.

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**Fig. 2.** I<sub>Ks</sub> of wild-type (WT) and LQT1 mutants coexpressed with minK in *Xenopus* oocytes. A: currents produced by minK (0.1 μg/μl) and minK + WT or mutant KvLQT1 (0.5 μg/μl), except for Y281C, where the cRNA concentrations were 0.05 and 0.25 μg/μl for minK and mutant, respectively. Currents were elicited by depolarizing steps from −30 to +70 mV from a holding potential (V<sub>H</sub>) of −80 mV in 20-mV increments. The return potential was −50 mV. B: comparison of I<sub>Ks</sub> current ratios (after correction for currents produced by minK alone) at +40 mV between mutant and WT-KvLQT1. Number of oocyte batches were the following: 4 for S225L + minK, 2 for Y281C + minK, 3 for A300T + minK, 4 for Y315C + minK, and 1 for Δ544 + minK, with 8 oocytes tested per sample per batch. Means ± SE were averaged from current ratios obtained in the different batches, except for Δ544. I<sub>max</sub>, maximum current produced.
Western blot analysis of mutant KvLQT1. We wondered whether the two inactive channels, Y281C and Y315C, were synthesized as full-length proteins and transported to the cell surface. Cellular trafficking of KvLQT1 or its mutations had not been reported at this time. The anti-KvLQT1 antibody recognized WT-KvLQT1 injected into oocytes or transiently transfected into Chinese hamster ovary, L, and human embryonic kidney-293 cells as a band at \( \sim 70 \) kDa (Fig. 5). Noninjected oocytes and nontransfected cells did not display a band with the same immunoreactivity. Membrane fractions from oocytes injected with Y281C and Y315C also contained the 70-kDa protein recognized by the KvLQT1 antibody (Fig. 5B).

Immunostains of WT and mutant KvLQT1s. Immunofluorescence staining of WT and mutant KvLQT1 injections showed a diffuse staining in the cytoplasm, which was present in noninjected oocytes (data not shown). Clear fluorescence staining at the cell surface was present in all of the oocytes injected with WT-
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KvLQT1 (8 cells from 4 batches) as well as the KvLQT1 mutants (6–8 cells from 4 batches for each mutant) (Fig. 6). Neither noninjected (6 cells in 3 batches) nor HERG-injected (3 cells in 1 batch) oocytes displayed this staining at the periphery.

Therefore, the mutant subunits were transported to the cell surface regardless of the presence of the accessory subunit minK, which did not appear to dramatically influence the level of membrane associated fluorescence (Fig. 7A).

Fig. 5. Western blots of WT and mutant KvLQT1 channels. Proteins extracted from the indicated cell lines [L, Chinese hamster ovary (CHO), and human embryonic kidney (HEK-293 cell lines)] and from oocyte membrane fractions were separated on 10% (A) or 7.5% (B) SDS-PAGE. Proteins were transferred onto Immobilon P membranes, incubated with anti-KvLQT1 antibody (1:1,000), and subsequently with horseradish peroxidase-conjugated anti-rabbit (Amer sham). Thirty micrograms of total protein were loaded on each lane.

To Dominant negative effects of KvLQT1 mutants. To determine whether the mutants could exert a dominant negative effect on WT channels, we coexpressed them in equimolar amounts with WT-KvLQT1. Currents produced by ½ WT plus ½ minK were also slightly larger than 50%. Because the mutant alone did not produce any current, this suggests lack of coassembly with WT subunits. Analysis of the voltage dependence of currents produced by coexpression of minK with 50:50 mixtures of WT and mutated KvLQT1s revealed small shifts relative to control. Thus ½ WT plus ½ S225L or ½ Y315C activated at more depolarized potentials of −6 and 4 mV, respectively, and ½ WT plus ½ A300T and ½ Y281C or ½ WT plus ½ Δ544 activated at more hyperpolarized potentials of about −10 and −4 mV.

The tripeptide glycine-tyrosine-glycine (GYG; see Fig. 1) in H5 may be the selectivity filter of K+ channels (10). We examined the selectivity in Y315C because the dominant negative effect of this mutant indicates coassembly with WT channels, and we tested the other mutations as well. We compared the current reversal potentials in solutions containing 100 mM NaCl and 5 mM KCl and found that reversal potentials were similar to WT currents (in mV, WT = −70.9 ± 1.9, n = 10; ½ WT + ½ S225L = −70.4 ± 1.3, n = 8; ½ WT + ½ Y281C = −69.7 ± 1.4, n = 10; ½ WT + ½ A300T = −72.5 ± 0.7, n = 8; and ½ WT + ½ Y315C = −68.7 ± 0.5, n = 8).

Differences in channel dysfunction. Three of four missense mutations were severely dysfunctional. Y281C and Y315C were nonfunctional, and S225L produced very small currents that required increased depolarization for activation. Position 225 is in S4, the predicted voltage sensor, and a change in voltage sensitivity is not surprising (23). Y315C is in the selectivity filter (10) and produced nonfunctional subunits that coassembled with WT subunits yet preserved K+ selectivity, as reported by Chouabe and co-workers (5).

A300T is near H5 and, when coexpressed with minK, produced currents that were about 15% of WT. A300T was the only point mutation that did not suppress WT function, as reported by Chouabe and co-workers (5). A300T was near H5 and, when coexpressed with minK, produced currents that were about 15% of WT. A300T was the only point mutation that did not suppress WT function, as reported by Chouabe and co-workers (5).

Mutant KvLQT1 subunits transported to cell surface: cellular consequences. Our immunological studies showed that nonfunctional and dysfunctional KvLQT1 subunits were transported to the cell surface, regardless of the presence of the accessory subunit minK. MinK did not appear to modify the amount of membrane-associated fluorescence (Fig. 7), but its effects were not quantified. It appeared that nonfunctional channels were synthesized in amounts similar to WT, but again the two effects were not quantified. These results rule out one possible mechanism of Iκs suppression; namely, intracellular retention of mutant sub-
units. Similar results have been reported for two HERG missense mutations, but for three other missense mutations, the protein was retained in the endoplasmic reticulum (12, 50). For LQT2/HERG mutants, misprocessing may be more common than dominant negative suppression of trafficking-competent heteromultimers. At this point, misprocessing has not been reported for LQT1/KvLQT1 mutants.

The surface immunostaining suggested the possibility of coassembly of mutant and WT subunits into heteromultimeric channels. The subsequent demonstration of dominant negative suppression of $I_{Ks}$ by the nonfunctional mutations Y281C and Y315C and the severely dysfunctional mutation S225L confirmed this hypothesis. This interpretation assumes that trafficking of minK-KvLQT1 or minK-KvLQT1 mutants in Xenopus oocytes is similar to human ventricular myocytes. For ion channels, this is generally the case; the exceptions being the electrophorus Na$^+$ channel (40) and the skeletal muscle Ca$^{2+}$ channel (24). We also assume that immunostaining at the cell surface corresponds to the presence of exogenous KvLQT1 subunits in the plasmalemma. The assumption seems reasonable for defolliculated oocytes because the only other structure present is the vitelline membrane, which was not stained in our control experiments. Furthermore, the anti-KvLQT1 antibody seems unable to detect endogenous KvLQT1 because un.injected and HERG-injected oocytes do not display any membrane-associated fluorescence. This failure could be due to either the very low level of endogenous KvLQT1 protein or to divergence of the Xenopus laevis COOH-terminal sequence from the human sequence. Because only a partial frog KvLQT1 clone is available, we cannot discriminate between these two possibilities. Because the endogenous KvLQT1 was not detectable immunochemically, it should not interfere with our immunostains or Western blots.

$\Delta 544$ is a severe mutation resulting in the change of a sequence of 107 amino acids at the COOH-terminus of the protein with a premature stop at codon 651. This mutant did not produce any current when expressed with minK but, like A300T, did not interfere with WT function. The lack of dominant negative effects of $\Delta 544$ on WT were not due to misprocessing, because the mutant subunit was de-
ected at the cell surface, suggesting an inability of the mutant subunits to form heteromultimers with WT (19). This mutation may produce its effects as a result of haploinsufficiency.

Lack of correlation with clinical phenotypes. The severe cellular phenotypes displayed by S225L, Y281C, and Y315C are in striking contrast with the mild clinical phenotypes of the carriers. A300T was the only mutation in which there was a correlation between mild cellular phenotype and mild clinical phenotype. The clinical phenotypes were extensively studied and have been reported (20, 25, 26). In brief, Y315C was identified in an elderly woman with no cardiac history and a borderline Q-T interval who developed a markedly prolonged Q-T and torsade des pointes during treatment with the antigastroesphogeal reflux drug cisapride, which is known to block HERG (27). The mutation is also present in her two asymptomatic sons, both of whom have normal Q-T intervals (20). For Y281C, eight of nine family members carrying this mutation had no clinical manifestations. One youth, for whom an electrocardiogram was not available, died suddenly. The three individual carriers of the S225L mutations were asymptomatic and never showed clinical manifestations of the disease (25). For A300T, heterozygotes had normal Q-T intervals and an absence of symptoms. It was only in the homozygote that Q-T prolongation occurred. The proband is the first homozygote for KvLQT1 without the auditory changes associated with recessive Jervell and Lange-Nielsen syndrome (26). The lack of agreement between cellular and clinical phenotypes for S225L, Y281C, and Y315C suggests that factors other than the primary genetic
abnormality may play a major role in defining the clinical phenotype.

We thank W.-Q. Dong, C.-D. Zuo, R. Bialecki, and R. Bryskin for assistance, and we thank Dra. A. L. George, D. M. Miller III, and J. Barnett at Vanderbilt University, Nashville, for the use of some of the laboratory equipment.

This study was supported by National Institutes of Health Grants NS-23877, HL-36930, and HL-55404 (to A. M. Brown), by American Heart Association Grant 9804566 (to L. Bianchi), and by Italian Telethon Foundation Grants 748 and 1058 (to S. G. Priori, C. Napo- politano, and P. J. Schwartz). Present address of L. Bianchi: Dept. of Pharmacology, Vanderbilt Univ., Nashville, TN 37232.

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