HERG Channel Dysfunction in Human Long QT Syndrome

INTRACELLULAR TRANSPORT AND FUNCTIONAL DEFECTS*

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Mutations in HERG are associated with human chromosome 7-linked congenital long QT (LQT-2) syndrome. We used electrophysiological, biochemical, and immunohistochemical methods to study the molecular mechanisms of HERG channel dysfunction caused by LQT-2 mutations. Wild type HERG and LQT-2 mutations were studied by stable and transient expression in HEK 293 cells. We found that some mutations (Y611H and V822M) caused defects in biosynthetic processing of HERG channels with the protein retained in the endoplasmic reticulum. Other mutations (I593R and G628S) were processed similarly to wild type HERG protein, but these mutations did not produce functional channels. In contrast, the T474I mutation expressed HERG current but with altered gating properties. These findings suggest that the loss of HERG channel function in LQT-2 mutations is caused by multiple mechanisms including abnormal channel processing, the generation of nonfunctional channels, and altered channel gating.

The congenital long QT syndrome is a disorder associated with delayed cardiac repolarization and prolonged electrocardiographic QT intervals and the development of ventricular arrhythmias (torsades de pointes) and sudden death (1). One cause of congenital long QT syndrome is mutation in the human ether-a-go-go-related gene (HERG) producing chromosome 7-linked congenital long QT syndrome (LQT-2) (2). HERG encodes a voltage-gated potassium channel (3). HERG channel current has been shown to have properties similar to the rapidly activating delayed rectifier K+ current (IKr), and it plays an important role in cardiac action potential repolarization in the mammalian heart (4–6). HERG channels are also an important target for block by many drugs, and suppression of HERG current causes action potential prolongation and cardiac arrhythmias (5–12). Therefore, HERG channels have emerged as an important cardiac ion channel.

More than 30 HERG mutations have been identified in LQT-2 patients (2, 13–18). The electrophysiological properties of a few LQT-2 mutations have been studied in Xenopus oocytes, where they have been shown to result in reduced or absent HERG current (19). Although the molecular basis for some congenital human diseases is known to involve multiple mechanisms including defective protein processing and abnormal protein function, the molecular basis for long QT syndrome causes has been studied. In this paper we present electrophysiological, biochemical, and immunohistochemical methods to study intracellular protein processing and functional properties of wild type and five LQT-2 mutant HERG channels. Our findings show that some mutant HERG proteins are not processed to the mature form of the channel. Other mutant HERG proteins undergo normal processing but do not form functional channels, or they gate abnormally. These findings provide new information about the molecular mechanisms for the failure of mutant LQT-2 channels to generate normal HERG current.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Transfection—The HERG LQT-2 mutations shown in Table I were generated by site-directed mutagenesis using the Altered Site II in vitro mutagenesis system (Promega, Madison, WI). Each mutation was verified by DNA sequencing using an automatic DNA sequencer. Wild type and mutant cDNAs were subcloned into pcDNA3 vector (Invitrogen, Carlsbad, CA), and HEK 293 cells were transfected transiently or stably with these constructs using a lipofectamine method as described previously (6). After transient transfection, HEK 293 cells were studied at 48 h. When transiently transfected cells were used for patch clamp experiments, green fluorescent protein cDNA (1 µg) was co-transfected with HERG cDNA (5 µg) to serve as an indicator. In our experiments, >90% of green fluorescent protein-positive cells express HERG channels. For LQT-2 mutants, we established four stable cell lines (T474I, I593R, Y611H, and G628S). LQT-2 mutant stably transfected cell lines were selected by their G418 resistance and identified by the presence of HERG protein on Western blot. A mock-transfected (pcDNA3 vector) cell line was selected by its G418 resistance.

HERG Antibody—The HERG protein antibody was generated using a fusion protein as antigen. The DNA fragment of HERG encoding 181 amino acid residues from the carboxyl terminus was subcloned into the pET-32a vector (Novagen, Madison, WI) to make a histidine-tagged thioredoxin-HERG fusion construct. This construct was expressed in Escherichia coli AD494(DE3)pLysS strain (Novagen). The histidine-tagged thioredoxin-HERG fusion protein was purified using the HisBind Buffer Kit (Novagen). The purified fusion protein was injected into rabbits to generate polyclonal antibody using a standard method (21). The specificity of the polyclonal anti-HERG antibody was tested by Western blot, immunohistochemical, and immunoprecipitation assays. Western blot showed that the HERG antibody recognized HERG protein bands only in HERG-transfected HEK 293 cells and not in mock-transfected and untransfected cells. Immunoprecipitation experiments also showed the absence of detectable HERG protein bands in mock-transfected cells.

Patch Clamp Recordings—Membrane currents were recorded in whole cell configuration using suction pipettes as described previously.

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(6, 22). Cells were superfused with HEPES-buffered Tyrode solution containing (in mM) 137 NaCl, 4 KCl, 1.8 CaCl2, 1 MgCl2, 10 glucose, and 10 HEPES (pH 7.4 with NaOH). The internal pipette solution contained (in mM) 137 NaCl, 20 KCl, 1.8 CaCl2, 1 MgCl2, 10 glucose, and 10 HEPES (pH 7.4 with KOH). All experiments were performed at 22–23 °C. Data are presented as mean ± S.E. Student's t test was used for statistical analysis.

**Western Blot Analysis—**Membrane protein preparation and Western blot procedures were previously described (6). The membrane proteins were subjected to SDS-polyacrylamide gel electrophoresis and then electroblotter transferred onto nitrocellulose membranes. The nitrocellulose membranes were incubated with the HERG antiserum (1:20,000 dilution) at room temperature overnight, and the antibody was detected with an ECL detection kit (6).

For proteinase K treatment of cells, wild type HERG- and LQT-2 mutant-transfected cells were washed with PBS and incubated with 2 μl of buffer containing 10 mM HEPES, 150 mM NaCl, and 2 mM CaCl2 (pH 7.4) with or without 200 μg/ml proteinase K (Sigma) at 37 °C for 30 min. The proteinase K activity was stopped by adding 1.3 ml of ice-cold buffer (pH 5.5) containing 15 mM KCl, 1 mM MgCl2, 5 mM EGTA, 5 mM MgATP, 10 mM HEPES (pH 7.4 with NaOH). All experiments were performed at 22–23 °C. Data are presented as mean ± S.E. Student's t test was used for statistical analysis.

**RESULTS**

**Electrophysiological Properties of Wild Type and Mutant Channels—** As shown in Table I, we performed site-directed mutagenesis of HERG to generate five reported LQT-2 mutations, T474I, I593R, G628S, and V822M (2, 13–15) and expressed these mutant HERG channels in HEK 293 cells. To study the functional expression of the HERG wild type and mutant channels, we performed patch clamp studies on transiently and stably transfected cells. Patch clamp recordings using transient transfection are shown in Fig. 1. Wild type HERG current showed voltage-dependent activation with inward rectification at more positive voltages as previously shown (6, 10). For mutants I593R, Y611H, G628S, and V822M, no HERG current was recorded; rather, only a small amplitude current endogenous to HEK 293 cells was present (6, 10). Similar results were obtained in stably transfected cells with the mutants I593R, Y611H, and G628S (data not shown). In contrast, the T474I mutation expressed functional channels but with altered gating properties. The T474I mutation activated at more negative voltages compared with wild type HERG channels. Similar results were obtained with stably transfected cells (data not shown). Fig. 1B shows the activation curves for wild type and T474I mutant currents. When fit as a Boltzmann function, the half-maximal activation voltages for wild type and the T474I mutation were −15.9 ± 1.1 mV (n = 17 cells) and −43.2 ± 1.2 mV (n = 16 cells), respectively (p < 0.05). The slope factors were 7.6 ± 0.3 and 6.8 ± 0.6, respectively (p > 0.05). Fig. 1C shows the I-V plots of wild type and T474I mutant current density measured at the end of the depolarization step. It shows that the maximal outward current in the T474I mutation was reached at −20 mV and that for wild type it was reached at 0 mV. The maximal outward current densities for wild type and T474I were 34.7 ± 4.0 and 26.6 ± 5.6 pA/pF, respectively (p > 0.05). Fig. 1C also shows marked inward rectification for both wild type and T474I currents at more positive voltages, and in the voltage range 0–60 mV the current amplitudes for the T474I mutation were decreased compared with wild type current amplitude (p < 0.05 at each voltage). Fig. 1B, B, and C, also shows that the threshold voltage for eliciting current was shifted negatively for the T474I mutation.

**Protein Processing Studied by Western Blot Analysis—**We have shown previously that wild type HERG channel protein expressed in HEK 293 cells consists of two forms on Western blot, a 135-kDa (lower) band and a 155-kDa (upper) band, and that both species involve N-linked glycosylation (6). We proposed that the upper band was the complexly glycosylated, mature form of the HERG channel and that the lower band was a core-glycosylated, precursor form of the HERG channel. In order to study the mechanisms accounting for dysfunctional mutant channels, we analyzed HERG channel proteins by Western blot as shown in Fig. 2. The HERG antibody did not recognize protein in mock-transfected cells, whereas it recognized HERG proteins in wild type-transfected and all five mutant-transfected cells. Wild type HERG, as well as the T474I, I593R, and G628S mutations, expressed two protein bands, a lower band at 135 kDa and an upper broad band at 155 kDa.

**TABLE I**  
**LQT-2 mutations**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>HERG protein domain</th>
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<tbody>
<tr>
<td>T474I</td>
<td>S2–S3 intracellular loop or S2</td>
</tr>
<tr>
<td>I593R</td>
<td>SS pore region</td>
</tr>
<tr>
<td>Y611H</td>
<td>SS pore region</td>
</tr>
<tr>
<td>G628S</td>
<td>Pore region</td>
</tr>
<tr>
<td>V822M</td>
<td>Cyclic nucleotide binding domain</td>
</tr>
</tbody>
</table>
adjacent to the carboxylic group of aliphatic and aromatic amino acids (24). Under our experimental conditions, the cells remained intact after proteinase K treatment. Membrane proteins were then isolated and analyzed by Western blot. As shown in Fig. 3, the upper band of wild type HERG was sensitive to the proteinase K treatment with the complete disappearance of the band. This was associated with the appearance of lower molecular mass bands of 60–75 kDa that should represent degraded C terminus-containing fragments from the upper band. Thus, HERG channels are sensitive to digestion by extracellular proteases. In contrast, the 135-kDa band was resistant to proteinase K treatment. These results support the hypothesis that the upper band represents the mature form of the HERG channel located in the surface membrane and that the lower band is the precursor form located inside the cell.

Similar to wild type HERG, the upper band of the G628S mutant was also sensitive to proteinase K treatment, indicating that the G628S mutant protein traffics normally into the surface membrane. The lack of HERG current is probably due to the functional defects in the channel protein. For Y611H, the single lower band was resistant to proteinase K treatment. Similar findings with proteinase K were obtained with the V822M mutation. This suggests that these mutant channels are not transported to the cell surface membrane.

In Fig. 4, we used Endo H to study further the intracellular biochemical processing of HERG channel protein. Endo H digests high mannose oligosaccharides that are added during core glycosylation of newly synthesized proteins in the endoplasmic reticulum (ER), leaving a single GlcNAc residue attached to the protein. Once proteins reach the medial Golgi, they undergo complex oligosaccharide modification to become Endo H-resistant (25). As shown in the Western blot analyses in Fig. 4, the upper band of wild type HERG was resistant to Endo H digestion. In contrast, the lower band of wild type HERG was sensitive to Endo H treatment and was reduced in molecular mass from 135 to about 132 kDa. This suggests that Y611H mutant HERG protein undergoes core glycosylation in the ER but then fails to be transported to the medial Golgi to undergo complex glycosylation.
Mechanisms of HERG Channel Dysfunction in LQT-2

Protein Processing Studied Using Metabolic Labeling—To study the biosynthesis of wild type HERG and transport-deficient LQT-2 mutant proteins, we performed pulse-chase experiments using metabolic labeling. As shown in Fig. 5, the wild type HERG protein was initially synthesized as a precursor form of 135 kDa, which was gradually converted to a larger form of 155 kDa. These findings provide direct evidence for the precursor and product relationship of the 135- and 155-kDa bands of HERG channel protein found in Western blot experiments. The pulse-chase data also show that the rate of conversion of the precursor form to the mature form is relatively slow when compared with other membrane channel proteins expressed in mammalian cells (26, 27). For the Y611H mutant, the protein was initially synthesized as a 135-kDa form; however, it was not converted to a larger molecular mass form for chase times up to 24 h. Rather, the mutant protein underwent progressive degradation with the appearance of smaller molecular mass bands and with the nearly complete disappearance of the 135-kDa band by 24 h. These findings show that the Y611H mutation fails to generate the mature form of the channel protein and that the immature form of the channel protein is rapidly degraded.

Immunolocalization of Wild Type and Mutant HERG Channels—We studied the subcellular localization of HERG protein in wild type and the five LQT-2 mutants by immunostaining permeabilized transfected HEK 293 cells. As shown in Fig. 6, the mock-transfected HEK 293 cells showed no detectable immunofluorescence staining. In cells transfected with wild type HERG, the immunofluorescence staining pattern is visible throughout the cells and their processes, showing the widespread distribution of HERG protein. Similar immunofluorescence patterns were obtained with the T474I, I593R, and G628S mutations. In contrast, cells transfected with the Y611H and V822M mutations display an immunofluorescence staining pattern that is more restricted to a perinuclear region. This restricted intracellular distribution pattern is consistent with the results of the Western blot experiments and shows that the mutant proteins are retained intracellularly.

To study whether transport-deficient HERG mutant protein is trapped in the ER, we performed double immunofluorescence staining of HERG proteins and the ER-resident chaperone BiP (28–30). Fig. 7 shows results of cells transfected by the Y611H mutation (A–D) and by wild type HERG (E–H). For the Y611H mutant, the green HERG and red BiP immunoreactivities are restricted to the same perinuclear distribution. This is confirmed in the superimposed image, which shows a uniform yellow color, indicating that the mutant HERG and BiP are colocalized. In contrast, for the wild type-transfected cell, HERG protein is widely distributed, whereas BiP shows a more restricted intracellular localization. These findings suggest that some LQT-2 mutations result in intracellular protein processing defects with retention of mutant HERG channel protein in the ER.

DISCUSSION

Our results provide new data about the biosynthesis patterns of a human, voltage-gated ion channel protein studied in a heterologous mammalian expression system. Wild type HERG channel protein is initially synthesized in the ER as the core-glycosylated precursor form with a molecular mass of 135 kDa. It is then modified in the Golgi apparatus, where it becomes the mature form of the channel with a molecular mass of about 155 kDa. As we noted previously, much of the increased protein size results from the addition of complex oligosaccharides by N-linked glycosylation, although other post-translational modification may occur (6). The 155-kDa protein is then transported into the plasma membrane. The fact that only 155-kDa protein is sensitive to externally applied proteinase K suggests that 155-kDa proteins form functional HERG channels in the cell surface membrane. Therefore, the 155-kDa band observed on Western blot serves as a useful marker to assess HERG protein maturation.

An important finding of the present study is that some LQT-2 disease-causing mutations result in protein processing defects that lead to failure of the channel protein to undergo normal transport to the cell surface. Rather, mutant channels are retained in the ER, where they are rapidly degraded. The Y611H and V822M mutations are examples of this defect. These mutations are most easily identified on Western blot analysis, where they generate only the single lower molecular mass precursor band. Several lines of evidence show that these mutant channels are located intracellularly. They are not sensitive to externally applied proteinase K. The Y611H mutation is not converted to the mature form, as shown in the pulse-chase experiments. The immunolocalization experiments confirm a restricted intracellular distribution for both mutations and suggest that the mutant channels are retained in the ER. The mechanism of the retention of the mutant HERG channel protein in the ER is not identified in these experiments. It is well recognized that export of newly synthesized proteins from the ER to the Golgi is regulated by a “quality control” mechanism (31, 32). This mechanism ensures that only properly folded and assembled proteins leave the ER. Misfolded, unassembled, and incompletely assembled subunit proteins are retained in the ER and undergo degradation without reaching the Golgi complex (33, 34). Thus, the LQT-2 mutations Y611H and V822M may cause structural abnormalities with protein misfolding or improper assembly, and they are retained and degraded in the ER. Whether these channel mutations are capable of forming functional channels is unknown.
Defective protein processing has been recognized as an important mechanism in some congenital human diseases. For cystic fibrosis transmembrane conductance regulator chloride channels, the most common mutation is the deletion of phenylalanine at position 508 (ΔF508), which causes 70% of cystic fibrosis cases. This mutation leads to retention of the channel protein within the cell and failure of channel trafficking to the plasma membrane (26). Protein processing defects have also been shown to be important in the low density lipoprotein receptor in familial hypercholesterolemia (35), in the Na+/glucose symporter in glucose-galactose malabsorption (36), and in several other congenital human diseases (37). Our results with LQT-2 mutations are the first to show this mechanism for a human, voltage-gated K+ channel.

In addition to transport-deficient mutants, other mechanisms for HERG channel dysfunction in LQT-2 are shown by our findings. The I593R and G628S mutations generate channel proteins that are processed similarly to wild type HERG protein, yet these proteins fail to form functional ion channels. The experiments showing sensitivity of the G628S mutant protein to digestion by proteinase K confirm its cell surface location. The immunolocalization experiments also show immunofluorescence staining patterns throughout the cell and its processes. These mutations are close to the pore region of HERG, which may result in defective channel gating or ion permeation; hence, we conclude that these channels cannot open or conduct ions normally. In a brief report, Nie et al. (38) have suggested that the I593R mutation also is able to insert into the plasma membrane, since epitope-tagged mutant protein can be detected on the cell surface. It is also interesting in our experiments that the I593R mutation causes a weakly stained upper band on Western blot. This could suggest that
intracellular protein transport may not be completely normal and that some LQT-2 mutations could have both protein processing and functional defects.

The T474I mutation represents another mechanism of channel dysfunction. This mutation generates HERG current with altered gating properties. The channels activate at more negative voltages (activation $V_1/2$ shifted negatively by 27.3 mV). Although maximum current amplitude was similar to that of wild type current, the peak of the $I-V$ plot was shifted negatively by about 20 mV as shown in Fig. 1C. At more positive voltages, inward rectification was present for both the wild type and T474I mutation, and there was a reduction in outward current for the mutation compared with wild type current. This decrease in current at more positive voltages may account for its QT-prolonging phenotype. This mutation has been proposed to be in the S2-S3 intracellular loop (3) or in the S2 intramembrane domain (39) of the putative channel protein structure. Interestingly, another LQT-2 mutation in the S2 region (N470D) also results in functional channels that gate at more negative voltages (19). These results suggest that the S2 region or adjacent S2-S3 loop contribute to the voltage dependence of HERG channel activation. A role of the S2 region in channel gating also has been reported in other voltage-gated K+ channels (40, 41).

LQT-2 is an autosomal dominant inherited disease with both normal and mutant genes present in patients. The present results were obtained by expressing LQT-2 mutations and wild-type HERG protein as homomultimeric channels. Since HERG proteins are thought to form tetrameric channels, the co-expression of some LQT-2 mutations with wild type HERG proteins are thought to form tetrameric channels, the results were obtained by expressing LQT-2 mutations and HERG mutants in LQT-2: 1) some mutations result in intra-

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REFERENCES