Pharmacological Rescue of Human K⁺ Channel Long-QT2 Mutations

Human Ether-a-Go-Go–Related Gene Rescue Without Block

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Background—Defective protein trafficking is a consequence of gene mutations. Human long-QT (LQT) syndrome results from mutations in several genes, including the human ether-a-go-go–related gene (HERG), which encodes a delayed rectifier K⁺ current. Trafficking-defective mutant HERG protein is a mechanism for reduced delayed rectifier K⁺ current in LQT2, and high-affinity HERG channel–blocking drugs can result in pharmacological rescue.

Methods and Results—We postulated that drug molecules modified to remove high-affinity HERG block may still stabilize mutant proteins in a conformation required for rescue. We tested terfenadine carboxylate (fexofenadine) and terfenadine, structurally similar drugs with markedly different affinities for HERG block, for rescue of trafficking-defective LQT2 mutations. Terfenadine rescued the N470D mutation but blocked the channels. In contrast, fexofenadine rescued N470D with a half-maximal rescue concentration of 177 nmol/L, which is ~350-fold lower than the half-maximal channel block concentration. The G601S mutation was also rescued without channel block.

Conclusions—Pharmacological rescue can occur without channel block. This could represent a new antiarrhythmic paradigm in the treatment of some trafficking-defective LQT2 mutations. (Circulation. 2002;105:2830-2835.)

Key Words: genes □ long-QT syndrome □ proteins □ pharmacology □ fexofenadine
HERG channels are blocked by a remarkably diverse series of compounds, which act with a wide range of affinities. We postulated that small modifications to drug molecules might render a compound incapable of blocking the channel but still allow it to bind and stabilize the protein in a conformation important for trafficking. In the present report, we tested 2 drugs for their ability to rescue LQT2-linked trafficking-defective HERG channels. Terfenadine (Seldane) is a second-generation H₁-receptor antagonist that was withdrawn from the market because of high-affinity HERG channel block. Its principal metabolite, terfenadine carboxylate (fexofenadine or Allegra), retains H₁-receptor antagonist properties but only very weakly inhibits HERG channels. These drug molecules are structurally very similar, differing by only a single carboxyl group.

Methods

Site-Directed Mutagenesis and Transfection

The LQT2 mutations N470D (asparagine to aspartic acid), G601S (glycine to serine), and V822M (valine to methionine) have been shown previously to be trafficking-defective channel proteins. The HERG N470D, G601S, and V822M mutations were generated by site-directed mutagenesis of wild-type HERG cDNA with the use of the GeneEditor in vitro mutagenesis system (Promega). Transfections of human embryonic kidney (HEK293) cells with HERG wild-type, N470D, G601S, and V822M cDNA was carried out with Lipofectamine (Invitrogen). Stable cell lines were generated through G418 (Invitrogen) antibiotic selection, and successful transfection was confirmed by Western blot analysis. The cell lines were cultured in MEM, as previously described.

Patch-Clamp Recording Method

Whole-cell recordings were performed with the use of suction pipettes as described previously. Cells were superfused with Tyrode’s solution containing (mmol/L) NaCl 137, KCl 4, CaCl₂ 1.8, MgCl₂ 1, glucose 10, and HEPES 10 (pH 7.4 adjusted with NaOH). The bath solution was exchanged completely within 2 minutes. The internal pipette solution contained (mmol/L) KCl 130, MgCl₂ 1, EGTA 5, Mg-ATP 5, and HEPES 10 (pH 7.2 adjusted with KOH). An Axopatch 200B patch-clamp amplifier was used to record membrane current. Computer software (pCLAMP8.2, Axon Instruments) was used to generate voltage-clamp protocols, acquire data, and analyze current signals. Patch electrodes typically had resistances of 2 to 5 MΩ. Series resistance compensation was ~75%. Data were not leak-corrected. HEK293 cells during depolarizing steps display a small-amplitude endogenous current that is not present during the recording of tail current. All patch-clamp experiments were performed at 22°C to 23°C within 2 hours of removing cells from culture conditions.

Drugs and Experimental Protocols

Research-grade terfenadine (molecular weight 471.7) was obtained from Sigma Chemical Co. Research-grade fexofenadine HCl (molecular weight 538.1) was obtained from Hoechst Marion Roussel, Inc. E-4031 was obtained from Eisai, Ltd. Sodium phenylbutyrate (4PBA) was obtained from Triple Crown USA, Inc.

Pharmacological Rescue

Fexofenadine was dissolved in MEM to give stock solutions, and the final drug concentrations were made by further dilutions in MEM. Terfenadine and 4PBA were dissolved in absolute alcohol to give stock solutions, and the final drug concentrations were made by further dilutions in MEM (maximal alcohol concentration 0.01%). HEK293 cells expressing the N470D, G601S, or V822M mutations were cultured at 37°C in drug-containing MEM for 24 hours. Drugs were removed by culturing cells for 1 hour in drug-free MEM at 37°C before whole-cell recording.

Temperature-Dependent Rescue and Drug Block

Terfenadine or fexofenadine was dissolved in absolute alcohol to give stock solutions, and the final drug concentrations were obtained by diluting the stock solution with Tyrode’s solution (maximal alcohol concentration 0.01%). To examine drug block of N470D channels, HEK293 cells expressing the mutation were cultured at 27°C for 24 hours to induce temperature-dependent rescue of functional channels. E-4031 was dissolved as previously described.

Curve Fitting and Statistical Methods

Data are given as mean±SEM, where n is the number of cells. Voltage-dependence of activation was determined by fitting the peak tail current with a Boltzmann equation: \[ y = \frac{1}{1 + \exp \left( - \left( E - E_{0.5} \right) / k \right) } \] where \( E \) is membrane voltage, \( E_0 \) is the voltage at which 50% of channels are activated, and \( k \) is the slope factor. Concentration-dependent effects were fit to the Hill equation: \[ \frac{I_{\text{max}} - I_{\text{min}}}{I_{\text{max}} - I_{\text{min}}} = 1/[1 + (D/IC_{50})^n] \], where \( I \) is the drug concentration, \( X_{\text{on}} \) is the drug concentration for 50% pharmacological rescue (RC₅₀) or 50% block (IC₅₀), and \( n \) is the Hill coefficient. The Student t test was used to calculate statistical significance, and a value of \( P<0.05 \) was considered significant.

Results

Pharmacological Rescue of the N470D LQT2 Mutation and HERG Channel Block by Terfenadine

Pharmacological rescue of the N470D mutation by terfenadine is shown in Figure 1. The voltage-clamp protocol, which was applied at 15-second intervals, is shown in Figure 1A (upper trace). From a holding potential of ~80 mV, 4-second depolarizing steps were applied in 10-mV increments from -70 to 70 mV, followed by a 5.7-second step to -50 mV to record tail current. The control data show current recorded from a cell expressing the N470D mutation and cultured at 37°C. It displays a small-amplitude endogenous current during the depolarizing step and a very-small-amplitude HERG tail current after the repolarizing step to -50 mV (arrow). When the same cell line was cultured at 37°C in 1 μmol/L terfenadine for 24 hours, followed by drug-free culture conditions for 1 hour, HERG current was present (note large-amplitude tail current). The current-voltage (I-V) plots with activation curves fitted to the peak tail current amplitude are shown in Figure 1B. For control conditions, a very-small-amplitude HERG tail current was recorded (n=5). Culturing cells in 0.05 μmol/L terfenadine resulted in no pharmacological rescue (n=7). In contrast, culturing cells in 1 μmol/L terfenadine resulted in the pharmacological rescue of large-amplitude HERG current (n=5).

However, 1 μmol/L terfenadine blocks HERG channels. This is shown for wild-type HERG channels (Figure 1C, n=4) and for temperature-dependent rescued N470D channels (Figure 1D, n=4). From a holding potential of ~80 mV, cells were depolarized for 4 seconds to 20 mV, followed by a 5.7-second step to -50 mV to record tail current, and the protocol was repeated at 15-second intervals. In these experiments, the peak tail current amplitude in each cell was normalized to a control value recorded 1 minute before drug exposure. Application of 1 μmol/L terfenadine resulted in the complete block of HERG current in wild-type and temperature-dependent rescued N470D mutant cells (Figure 1C and 1D). These data agree with previous reports of
high-affinity HERG channel block by terfenadine (IC$_{50}$ 56 nmol/L), which contributed to its withdrawal from the market because of acquired LQT syndrome.

**Pharmacological Rescue of the N470D LQT2 Mutation by Fexofenadine**

Pharmacological rescue of the N470D mutation by fexofenadine is shown in Figure 2. Current was elicited with the voltage-clamp protocol shown in Figure 2A (same protocol as in Figure 1A). The control record shows current recorded from a cell expressing the N470D mutation cultured at 37°C. A small-amplitude endogenous current is present during depolarization along with a very-small-amplitude HERG current (note diminutive tail currents, arrow). Culturing the same cell line at 37°C for 24 hours resulted in the emergence of HERG current (note large-amplitude tail current), which was not increased further by culturing cells for 48 hours in drug. In contrast to terfenadine, fexofenadine-rescued HERG current could be recorded without culturing cells in drug-free MEM before whole-cell recording. To confirm that the pharmacologically rescued current was carried by HERG channels, in some experiments the cells were exposed to E-4031 (100 to 300 nmol/L), which completely blocked the fexofenadine-rescued current (n = 10, 0.05 μmol/L terfenadine (V$_{1/2}$ = 20.8 ± 9.1 mV, k = 4.1) and 1 μmol/L terfenadine (V$_{1/2}$ = 27.2 ± 4.4 mV, k = 7.2). C and D, Normalized peak tail current amplitude vs time and block of current by application of 1 μmol/L terfenadine (arrows).

![Figure 1. Effect of terfenadine on HERG wild-type (WT) and N470D mutant channels. A, Voltage-clamp protocol and representative current records for control conditions and terfenadine treatment. B, Activation curves for peak tail current amplitude fit with Boltzmann equation for control conditions (V$_{1/2}$ = 30.6 ± 2.3 mV, k = 6.4) and after 24 hours of treatment with 0.05 μmol/L terfenadine (V$_{1/2}$ = 27.2 ± 1.2 mV, k = 6.3). C and D, Normalized peak tail current amplitude vs time and block of current by application of 1 μmol/L terfenadine (arrows).](image)

![Figure 2. Effect of fexofenadine on HERG WT and N470D mutant channels. A, Voltage-clamp protocol and representative current records for control conditions and after fexofenadine treatment. B, Activation curves for peak tail current amplitude fit with Boltzmann equation for control conditions (V$_{1/2}$ = 30.6 ± 2.3 mV, k = 6.4) and after 24 hours of treatment with 0.05 μmol/L fexofenadine (V$_{1/2}$ = 33.6 ± 4.8 mV, k = 8.4), 1.0 μmol/L fexofenadine (V$_{1/2}$ = 30.0 ± 1.2, k = 6.3), or 5 μmol/L fexofenadine (V$_{1/2}$ = 27.5 ± 2.3, k = 8.5). C, Concentration-dependent relation for pharmacological rescue by fexofenadine. Hill equation intersects y-axis at control current value. Number of experiments for each data point is given in parentheses. D, Concentration-dependent relations for fexofenadine block of WT (●) and N470D (○) channels obtained by fitting data points with Hill equation. Number of experiments for each data point is given in parentheses.](image)
amplitude HERG current was present for control conditions, and fexofenadine resulted in concentration-dependent pharmacological rescue. Quantitative analysis of the concentration dependence of pharmacological rescue was performed by plotting peak tail current amplitude recorded at -50 mV after full activation at 20 mV as a function of the rescuing fexofenadine concentration. The resulting plot when fit with a Hill equation gave an RC50 value of 177 ± 6 nmol/L and Hill coefficient of 1.01, consistent with high-affinity rescue through a single drug-binding site (Figure 2C).

Fexofenadine is reported to block HERG wild-type channels only weakly and, therefore, may rescue trafficking-defective HERG protein without blocking channel function. Consequently, we studied the ability of fexofenadine to block HERG wild-type and temperature-dependent rescued N470D channels (Figure 2D). Tail currents were recorded by the same protocol as used in Figure 1C and 1D. Peak tail current amplitude at steady-state drug block in each cell was normalized to a control value recorded 1 minute before drug exposure. Averaged normalized peak tail current amplitude is plotted versus the fexofenadine concentration (3, 30, 100, and 300 μmol/L), and these data were fit with the Hill equation. The IC90 value for HERG wild-type channels was 65.1 ± 8.3 μmol/L with a Hill coefficient of 1.07; for N470D channels, the IC90 value was 57.7 ± 2.8 μmol/L with a Hill coefficient of 0.96. The IC50 values were not statistically different (P > 0.05). The IC90 values for fexofenadine block of HERG wild-type and N470D channels exceed the RC50 value for pharmacological rescue of the N470D channel by 368- and 326-fold, respectively.

Pharmacological Rescue of the G601S LQT2 Mutation by Fexofenadine

To test whether fexofenadine might rescue multiple HERG mutations at similar drug concentrations, we studied another trafficking-defective LQT2 mutation, G601S.10,11 Fexofenadine-mediated pharmacological rescue of the G601S mutation is shown in Figure 3. The voltage-clamp protocol and representative current records are shown in Figure 3A (same protocol as used in Figures 1A, 1B, 2A, and 2B). The control record shows current recorded from HEK293 cells stably expressing the G601S mutation cultured at 37°C. The G601S-expressing cells have a small-amplitude HERG current (arrow), indicating that some mutant channels reach the plasma membrane.10,11 Culturing the same cell line in 1 μmol/L fexofenadine at 37°C for 24 hours resulted in the appearance of large-amplitude HERG current. Similar to the N470D mutation, the HERG current could be recorded without culturing cells in drug-free MEM solution before whole-cell recording, and the pharmacologically rescued current was blocked by E-4031 (100 to 300 nmol/L, n = 3; data not shown). The I-V relations for peak tail current amplitude for the G601S mutation are shown in Figure 3B (same protocol as used in Figures 1B and 2B). For these experiments, cells were cultured for 24 hours in control (no drug, n = 4) conditions or in 0.05 (n = 3) or 1.0 μmol/L (n = 6) fexofenadine. The results show a small-amplitude HERG current for control conditions and its concentration-dependent pharmacological rescue with fexofenadine.

Lack of Pharmacological Rescue of the V822M LQT2 Mutation by Fexofenadine

We studied a third LQT2 mutation, V822M, located in the C-terminus of the HERG channel in the nucleotide binding domain that we have previously confirmed to express the immature form of the protein that is trafficking defective.4 The voltage-clamp protocol and representative current records are shown in Figure 4 (same protocol as used in Figures 1A, 1B, 2A, 2B, and 3A). The control record shows current recorded from HEK293 cells stably expressing the V822M mutation cultured at 37°C. A small-amplitude endogenous current is present with the depolarizing steps, but unlike the N470D- and G601S-transfected cells, there is no HERG current (arrow, n = 4). Culturing in 1 μmol/L fexofenadine at 37°C for 24 hours resulted in no pharmacological rescue of HERG current (n = 4), and this is shown in the I-V plots in Figure 4B.

Discussion

These data are the first to show pharmacological rescue of human LQT2 mutations at drug concentrations that do not block HERG channels. Although fexofenadine and terfenadine rescued mutated HERG channels, only fexofenadine did so at drug concentrations that did not cause channel block. Our data show that the RC50 value for rescue of the N470D mutant channel protein is ~350-fold less than the IC90 values for block of HERG wild-type or N470D mutant channels. The concentration-response curve for pharmacological rescue (Figure 2C) is below the concentration-response curves for channel block (Figure 2D), and there is no overlap, except for
minimal block at the highest pharmacological rescue concentration.

Our data show that the half-maximal activation voltage ($V_{1/2}$) values for the N470D mutation are shifted negatively compared with the values obtained for wild-type or G601S mutant channels (Figures 1B, 2B, and 3B). This is in agreement with previously published data and confirms that the pharmacologically rescued channels retain electrophysiological properties similar to those of the channels expressed in oocytes and mammalian cells at reduced temperature.9,11,14 Thus, fexofenadine does not alter these biophysical properties of the pharmacologically rescued channels. Weak block by fexofenadine of HERG wild-type channels has been reported previously.13 Our data extend this observation to show that HERG wild-type and temperature-dependent rescued N470D mutant channels have similar low affinities for block by 4PBA or glycerol, have been shown to improve trafficking of mutant proteins in disease models, such as cystic fibrosis and nephrogenic diabetes insipidus,8,18,19 and are thought to act as protein-stabilizing agents (chemical chaperones). These compounds frequently require very high concentrations (millimolar to molar). We obtained similar findings with glycerol (~1 mol/L) in the N470D mutation,9 whereas culture for 24 hours in 2.5 to 5.0 mmol/L 4PBA, the concentration range required for rescue of the cystic fibrosis ΔF508 mutation, did not rescue N470D current (n=3, data not shown).

It was recently reported by Ficker et al10 that pharmacological rescue of the G601S mutation by high-affinity HERG blocking drugs failed to rescue 2 other C-terminus mutations. Our data also show that the N470D and G601S mutations, but not the V822M mutation. Thus, although fexofenadine rescued multiple LQT2 mutations, it is not capable of rescuing all trafficking-defective LQT2 mutations.

The N470D mutation is located within the S2 transmembrane-spanning domain, and the G601S mutation is located within the S5-pore extracellular linker of the HERG channel protein. Fexofenadine rescued both mutations, demonstrating that a single pharmacological rescue mechanism may exist for pharmacological rescue of LQT2 mutations.

These observations led them to propose that pharmacological rescue of the G601S mutation involved drug binding to a distorted inner vestibule in the pore-S6 region, which stabilized the protein in a configuration that improved trafficking to the plasma membrane. In preliminary experiments, we have also obtained similar results.20 Our present findings provide new insight. Fexofenadine-mediated pharmacological rescue had an $RC_{50}$ value of 177 nmol/L, with a Hill coefficient of 1.01, suggesting a single high-affinity drug-binding site for rescue. Furthermore, high-affinity HERG channel block is not a requirement for pharmacological rescue because this can occur without HERG channel block; thus, rescue and block are not inextricably linked, and these processes can be uncoupled. Taken together, these data suggest that the mechanism for fexofenadine-mediated rescue may not involve drug binding within the pore-S6 drug-binding domain that is postulated to mediate HERG channel block, or fexofenadine might bind to the pore-S6 drug-binding domain but does so without impeding ion flow through the channel at the drug concentrations required for pharmacological rescue. A possibility is that multiple mechanisms may exist for pharmacological rescue of LQT2 mutations.

Figure 4. Lack of pharmacological rescue of HERG V822M mutation. A, Voltage-clamp protocol and representative current records for control conditions and fexofenadine treatment. B, I-V plots for control conditions and after 1 μmol/L fexofenadine treatment, with current measured at beginning of the 5.7-second step to −50 mV.

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The N470D mutation is located within the S2 transmembrane-spanning domain, and the G601S mutation is located within the S5-pore extracellular linker of the HERG channel protein. Fexofenadine rescued both mutations, demonstrating that a single pharmacological agent is capable of rescuing mutations in different regions of the channel. However, fexofenadine did not rescue the V822M mutation. Thus, although fexofenadine rescued multiple LQT2 mutations, it is not capable of rescuing all trafficking-defective LQT2 mutations, and this observation agrees with the recent report by Ficker et al10 that high-affinity HERG channel–blocking drugs failed to rescue 2 other C-terminus mutations. Our data also show that the N470D and G601S mutations, but not the V822M mutation, express very-small-amplitude HERG currents when cultured under control conditions, suggesting that small numbers of channels escape the quality-control mechanism to insert into the plasma membrane. A possibility is that the presence of small-amplitude HERG current recorded under control conditions could serve as a "signature" for LQT2 mutant channel proteins that might undergo successful
pharmacological rescue. Our observations add importance not only to identifying gene mutations but also to elucidating their biological consequences and potential for rescue through functional expression.

These findings have the potential for therapeutic application. Fexofenadine is a Food and Drug Administration–approved drug that is widely available by prescription as an antihistamine agent. It is thought to not affect the QT interval on the ECG even when administered in doses that exceed 10-fold recommendations. After the oral administration of 60 or 180 mg fexofenadine to healthy volunteers, the mean maximum plasma concentration was 141 and 494 ng/mL, respectively, or 262 and 918 nmol/L, respectively, and protein binding of fexofenadine is reported to be 60% to 70%. Thus, serum concentrations achieved in patients are within the concentration range required for the pharmacological rescue of mutant LQT2 channels found in our experiments. Consequently, our results have the potential for therapeutic application and could represent a new paradigm for antiarrhythmic drug therapy in some trafficking-defective LQT2 mutations. This approach is likely to be mutation specific (eg, trafficking-defective channel proteins), and it is unknown whether it might be applicable to other ion channel diseases. Whether this therapeutic approach can be translated to an effective therapy for human disease will require further research and carefully performed controlled clinical trials.

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References
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