Mechanisms of Pharmacological Rescue of Trafficking-defective hERG Mutant Channels in Human Long QT Syndrome*

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Long QT syndrome type 2 is caused by mutations in the human ether-a-go-go-related gene (hERG). We previously reported that the N470D mutation is retained in the endoplasmic reticulum (ER) but can be rescued to the plasma membrane by hERG channel blocker E-4031. The mechanisms of ER retention and how E-4031 rescues the N470D mutant are poorly understood. In this study, we investigated the interaction of hERG channels with the ER chaperone protein calnexin. Using communoprecipitation, we showed that the immature forms of both wild type hERG and N470D associated with calnexin. The association required N-linked glycosylation of hERG channels. Pulse-chase analysis revealed that N470D had a prolonged association with calnexin compared with wild type hERG and E-4031 shortened the time course of calnexin association with N470D. To test whether the prolonged association of N470D with calnexin is due to defective folding of mutant channels, we studied hERG channel folding using the trypsin digestion method. We found that N470D and the immature form of wild type hERG were more sensitive to trypsin digestion than the mature form of wild type hERG. In the presence of E-4031, N470D became more resistant to trypsin even when its ER-to-Golgi transport was blocked by brefeldin A. These results suggest that defective folding of N470D contributes to its prolonged association with calnexin and ER retention and that E-4031 may restore proper folding of the N470D channel leading to its cell surface expression.

We have previously shown that the maturation of newly synthesized hERG protein in the ER is under stringent surveillance by the quality control system (16). The ER quality control system ensures that only properly folded and assembled proteins are exported from the ER to the Golgi. Misfolded, incompletely folded, and unassembled proteins are retained in the ER by the quality control system (17, 18). The ER quality control system involves molecular chaperones that transiently associate with newly synthesized proteins and promote their proper folding and assembly. However, proteins that are unable to fold and assemble correctly often exhibit prolonged association with these chaperones, which may contribute to their retention in the ER (17, 18). Although LQT2 mutant channels have been shown to display prolonged association with cytosolic chaperones Hsp70 and Hsp90, the interaction of hERG channels with ER molecular chaperones has not been reported (19).

Calnexin is an ER resident integral membrane chaperone protein that plays an important role in the biogenesis and quality control of glycoproteins (17, 18). Calnexin specifically interacts with glycan moieties of the glycoproteins and associates transiently with newly synthesized glycoproteins until they fold properly. If the proteins never fold correctly, the interaction between calnexin and the misfolded proteins is prolonged, leading to their retention in the ER. Because hERG undergoes N-linked glycosylation (20), we want to know whether calnexin interacts with hERG channels and whether calnexin plays a role in the ER retention of LQT2 mutant channels.

We previously showed that the LQT2 mutation N470D exhibits temperature-sensitive protein-trafficking defects (21). The N470D mutant is retained in the ER when expressed at 37 °C, whereas at 27 °C its trafficking to the plasma membrane is markedly improved. We also showed that the N470D mutant channel can be rescued by hERG channel-blocking drugs, including E-4031, astemizole, and cisapride (21, 22). The mechanisms of pharmacological rescue of trafficking-defective hERG mutant channels are poorly understood. It has been proposed that hERG channel-blocking agents may act as pharmacological chaperones to promote proper protein folding in a conformation that permits trafficking to the plasma membrane (10–12). However, the folding of LQT2 mutant proteins and effects of pharmacological chaperones on the folding have not been studied.

In the present work, we studied the role of calnexin in the quality control of mutant hERG channels. In addition, we analyzed protein folding of hERG channels using trypsin digestion and detergent extraction methods. We showed that both wild type and N470D mutant proteins associated with calnexin. However, the N470D mutant exhibited a prolonged association with calnexin. The prolonged association of N470D with calnexin may be due to defective folding of the mutant protein as evidenced by its increased sensitivity to trypsin digestion compared with wild type hERG. E-4031 improves the folding of the N470D mutant protein and shortens the time course of its association.
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with calnexin, leading to the cell surface expression of the mutant channel.

EXPERIMENTAL PROCEDURES

Reagents and Cell Transfection—Anti-calnexin and anti-calreticulin antibodies were purchased from Stressgen (Victoria, BC, Canada). EXPRE35S protein labeling mix was purchased from PerkinElmer Life Sciences. Anti-hERG antibody was raised against the hERG C-terminal region as previously described (9). Stable transfection of HEK293 cells with wild type hERG, N470D, and N598Q has been previously described (5, 20, 21). The cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum.

Western Blot Analysis—Membrane protein preparation and Western blot were performed as previously described (5, 9). The membrane proteins were subjected to SDS-polyacrylamide gel electrophoresis and then electrophoretically transferred onto nitrocellulose membranes. The membranes were incubated with anti-hERG or anti-calnexin antibodies and visualized with the ECL detection kit.

Coimmunoprecipitation of Calnexin and hERG—HEK293 cells stably transfected with wild type hERG, N470D, and N598Q were lysed in 500 μl of immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 1 mM CaCl2, and 1% Triton X-100) with protease inhibitors. After centrifugation at 13,000 × g for 10 min at 4 °C, the cell lysates were precleared by incubation with protein A-agarose beads. The calnexin-hERG complexes were immunoprecipitated by incubating with 2 μg of antibody against calnexin at 4 °C overnight. The antigen-antibody complexes were isolated with protein A-agarose beads. The calnexin-hERG complexes were subjected to SDS-polyacrylamide gel electrophoresis and visualized with autoradiography.

Interaction of Calnexin with hERG Requires N-linked Glycosylation

FIGURE 1. Association of hERG channels with calnexin. Cell lysates of HEK293 cells stably expressing wild type hERG, N470D, N598Q, or untransfected HEK293 cells (control) were immunoprecipitated with anti-calnexin antibody. The precipitates were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-hERG antibody. The lower panels are inputs probed by anti-calnexin and anti-hERG antibodies.

Inhibitor mixture (50 mM Tris-HCl, pH 7.4, and 150 mM NaCl, 1 mM EDTA, and different concentrations of Triton X-100) and incubated at 4 °C for 30 min. Detergent-soluble and -insoluble proteins were separated by centrifugation at 100,000 × g for 20 min. The pellets were resuspended in the buffer containing 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl, 1 mM EDTA, 0.1% SDS, and protease inhibitor mixture. Equal fractions from supernatant (detergent soluble) and pellet (detergent insoluble) were analyzed by immunoblotting with anti-hERG antibody.

RESULTS

Interaction of Calnexin with Wild Type hERG and the N470D Mutant—To study the mechanism of defective trafficking of the N470D mutant, we examined the interaction of calnexin with wild type hERG and the N470D mutant. In these experiments, the physical association of hERG channels with calnexin was determined by immunoprecipitation with anti-calnexin antibody followed by Western blot with anti-hERG antibody. As shown in Fig. 1, hERG channels were coimmunoprecipitated with calnexin in wild type hERG and N470D-transfected cells. For both wild type hERG and the N470D mutant, calnexin associated with the core-glycosylated immature form of channel proteins but not with the fully glycosylated mature form. This is consistent with the function of calnexin as an ER chaperone. It is noted that the proportion of calnexin association with the hERG channel protein was greater for the N470D mutant than for wild type hERG, indicating that the mutant may have a prolonged association with calnexin. Similar analysis with anti-calreticulin antibody failed to detect any association of calreticulin with hERG channels (data not shown).

Interaction of Calnexin with hERG Requires N-linked Glycosylation—Calnexin is a lectin chaperone protein that interacts with glycan moieties of the glycoproteins. To study the role of N-linked glycosylation in the interaction of hERG channels and calnexin, we performed coimmunoprecipitation experiments in cells expressing the N598Q mutant. We have previously shown that the N598Q mutant disrupts N-linked glycosylation of hERG channels (20). Although the N598Q mutant was readily detected in Western blot analysis with anti-hERG antibody, it was not coimmunoprecipitated with calnexin (Fig. 1). This result suggests that N-linked glycosylation at Asn-598 is required for the interaction of calnexin with hERG channels.

Prolonged Association of the N470D Mutant with Calnexin—The ER retention of many mutant proteins has been linked to a prolonged association with calnexin (23–25). To determine whether the N470D mutant has a prolonged association with calnexin, we performed pulse-chase and sequential immunoprecipitation analysis (Fig. 2). In these experiments, HEK293 cells expressing either wild type hERG or the
N470D mutant were labeled with [35S]methionine/cysteine and then chased with unlabeled methionine/cysteine for various intervals up to 8 h. Following the chase, cell lysates were subjected to sequential immunoprecipitation with anti-calnexin antibody followed by anti-hERG antibody. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. The amount of hERG was quantified by phosphorimaging analysis and plotted as percentage of the value at time point 0 (D). The data points are means ± S.E. of the mean from three independent experiments.

E-4031 Shortens the Time Course of Association of the N470D Mutant with Calnexin—To study the effects of hERG channel blockers on chaperone interaction of hERG mutant channels, we carried out pulse-chase experiments in the presence of 5 μM E-4031 and performed sequential immunoprecipitation analysis with anti-calnexin antibody followed by anti-hERG antibody as described above. In the presence of E-4031, the time course of association of calnexin with wild type hERG and N470D was shortened (Fig. 2C and D) and the half-life of the calnexin interaction with wild type hERG was ~1 h, whereas the half-life of the calnexin interaction with N470D was ~6 h. Taken together, these data suggest that the N470D mutant protein was unable to undergo the forward folding reaction in the ER, which results in its prolonged association with calnexin. The prolonged association of the mutant channel with calnexin may contribute to its defective trafficking and ER retention.

FIGURE 2. Time course of the association of calnexin with wild type hERG and N470D. HEK293 cells stably transfected with wild type hERG (A) and N470D treated without (B) or with (C) 5 μM E-4031 were pulse-labeled for 30 min and chased for the indicated periods of time. The cell lysates were immunoprecipitated with anti-calnexin antibody followed by immunoprecipitation with anti-hERG antibody. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. The amount of hERG was quantified by phosphorimaging analysis and plotted as percentage of the value at time point 0 (D). The data points are means ± S.E. of the mean from three independent experiments.

Study of hERG Channel Folding by Trypsin Digestion and Detergent Extraction—To determine possible folding defects in hERG mutant channels, we performed trypsin digestion experiments. This method has been used to study protein folding of a variety of membrane proteins, including cystic fibrosis transmembrane conductance regulator, aquaporin-2, and P-glycoprotein (26–28). The rationale is that if there were differences in folding between wild type hERG and LQT2 mutants, exposure of trypsin-sensitive sites would be different, leading to variation in trypsin sensitivity. Crude membranes prepared from HEK293 cells stably expressing wild type hERG or the N470D mutant were treated with various concentrations of trypsin from 0.01 to 1000 μg/ml. The hERG channel proteins were then analyzed by Western blot using anti-hERG antibody. As shown in Fig. 3, wild type hERG expressed two protein bands: a lower band of 135 kDa and an upper band of 155 kDa, whereas the N470D mutant expressed primarily the 135-kDa band (5, 9). The 135-kDa band represents the core-glycosylated immature form of the channel protein located in the ER, and the 155-kDa band represents the complex-glycosylated mature form of the channel protein located in the plasma membrane (9, 20). The mature form of wild type hERG was quite resistant to trypsin, requiring >1000 μg/ml for complete digestion. In contrast, the immature forms of wild type hERG and the N470D mutant were much more sensitive to trypsin digestion. They were almost completely digested after treatment with 1–10 μg/ml of trypsin. These results suggest that the core-glycosylated immature forms of both wild type hERG and the N470D mutant are more loosely folded than the complex-glycosylated mature form of wild type hERG.

E-4031 Allows the Mutant Channel to Escape the Prolonged Association with Calnexin. By contrast, the N470D mutant had a prolonged association with calnexin. The estimated half-life of the calnexin interaction with wild type hERG was ~1 h, whereas the half-life of the calnexin interaction with N470D was ~6 h. Taken together, these data suggest that the N470D mutant protein was unable to undergo the forward folding reaction in the ER, which results in its prolonged association with calnexin. The prolonged association of the mutant channel with calnexin may contribute to its defective trafficking and ER retention.

Effect of E-4031 on the Folding of the N470D Mutant—To study whether hERG channel blocker E-4031 can promote proper folding of the N470D mutant, we treated the cells with 5 μM E-4031 for 24 h. As shown in Fig. 6, E-4031 treatment rescued the N470D mutant as evidenced by the appearance of the 155-kDa complex-glycosylated mature form. We showed previously that the mature form of N470D is expressed on the cell surface and forms functional hERG channels (21).
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**DISCUSSION**

The present experiments demonstrate that both wild type hERG and the N470D mutant associated with calnexin. The association of hERG channels with calnexin requires \( N \)-linked glycosylation. Recently, it has been reported that more than one glycan is needed for efficient trimming of glucose by glucosidase II and, as a consequence, many glycoproteins with a single glycan fail to associate with calnexin (30). We have previously shown that although hERG contains two consensus \( N \)-linked glycosylation sites, Asn-598 and Asn-629, only Asn-598 is used for glycosylation (20). The fact that newly synthesized hERG proteins associate with calnexin suggests that a single glycan is sufficient to mediate the interaction with calnexin. Several other glycoproteins with a single glycan, especially membrane proteins such as erythrocyte anion exchanger AE1, V2 vasopressin receptor, and Kv1.2 potassium channel, have been reported to associate with calnexin (25, 31–33). One possibility is that many of these membrane proteins are known to form dimers or tetramers and activation of glucosidase II may occur during oligomerization (14, 25, 30, 33, 34).

The observation that the immature, but not the mature, form of hERG associates with calnexin is consistent with the function of calnexin as an ER chaperone. Calnexin is a lectin chaperone protein that interacts with glycan moieties of the glycoproteins. Calnexin plays an important role in the quality control of glycoproteins in a process termed calnexin cycle. In this process, calnexin associates with glycoproteins that are monoglucosylated intermediates of the \( N \)-linked core glycan. The association of calnexin with monoglucosylated glycoproteins is regulated by a cycle of deglucosylation, by glucosidase II, and reglucosylation, by UDP-glucose:glycoprotein glucosyltransferase. Because UDP-glucose:glycoprotein glucosyltransferase preferentially acts on unfolded proteins, only the incompletely folded proteins reenter the cycle, whereas the properly folded proteins leave the ER and move farther along the secretory pathway. Thus, our results suggest that the wild type hERG channel transiently associates with calnexin during the early stages of biogenesis and dissociates from calnexin when it folds properly. In contrast, the N470D mutant has a prolonged association with calnexin, suggesting that N470D fails to fold properly, is recognized by UDP-glucose:glycoprotein glucosyltransferase, and reenters the calnexin cycle.

The interaction of calnexin with voltage-gated potassium channels has been reported in Shaker and Kv1.2 channels (33, 35). For Shaker channels, calnexin is not involved in the quality control and ER retention of mutant Shaker channels (35). However, transient calnexin interaction confers long-term stability of folded Shaker channel proteins in the ER and promotes surface expression of correctly assembled Shaker channels (36, 37). Similarly, calnexin facilitates cell surface expression of Kv1.2 potassium channels (33).

To study the folding of hERG channels, we performed trypsin digestion experiments. The results show that the immature and mature forms of wild type hERG were different in their sensitivity to digestion by trypsin. The core-glycosylated hERG was ~100-fold more sensitive to trypsin digestion than the complex-glycosylated mature form. This suggests that the trypsin-sensitive sites are more readily accessible in the core-glycosylated immature form but are probably hidden in the mature form as a result of proper folding. Our results also show that in the presence of BFA, a fraction of the 135-kDa form of wild type hERG is in a folding conformation that is comparable with the 155-kDa mature form even though its trafficking to the Golgi is blocked by BFA. This fraction may represent the properly folded hERG protein that would have trafficked to the cell surface in the absence of BFA. This result

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**FIGURE 4. Effect of brefeldin A treatment on protease sensitivity of wild type hERG and the N470D mutant.** A, HEK293 cells stably transfected with wild type hERG were treated with 5 \( \mu \)g/mL brefeldin A for the indicated times, followed by immunoblot analysis with anti-hERG antibody. B, HEK293 cells expressing wild type hERG and the N470D mutant were treated with brefeldin A for 24 h prior to the isolation of total cell membranes. The membranes were treated with trypsin, followed by immunoblotting with anti-hERG antibody.

**FIGURE 5. Detergent extractability of wild type hERG and the N470D mutant.** HEK293 cells stably transfected with wild type hERG or the N470D mutant were solubilized in lysis buffer containing different concentrations of Triton X-100. Detergent-soluble and -insoluble proteins were separated by centrifugation. Equal fractions from supernatants (S) and pellets (P) were analyzed by immunoblotting with anti-hERG antibody.

**FIGURE 6. Effect of E-4031 on protease sensitivity of the N470D mutant.** HEK293 cells stably transfected with the N470D mutant were cultured in the presence of 5 \( \mu \)g/mL E-4031 (N470D + E-4031) or 5 \( \mu \)g/mL E-4031 plus 5 \( \mu \)g/mL BFA (N470D + E-4031 + BFA) for 24 h. Total cell membranes from the cells were treated with trypsin, followed by immunoblotting with anti-hERG antibody.

Similar to wild type hERG, the mature form of N470D was quite resistant to trypsin, requiring 1000 \( \mu \)g/mL trypsin for complete digestion. The effect of E-4031 on trypsin sensitivity of N470D was independent of subcellular localization, because in the presence of BFA, the core-glycosylated form of N470D had similar trypsin sensitivity to the complex-glycosylated mature form. These results indicate that E-4031 improves folding of N470D even when its trafficking from the ER to the Golgi was blocked by BFA.
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suggests that the conformation change from the loosely folded form to the proper folded form occurs in the ER.

Defective protein trafficking has been recognized as an important mechanism for an increasing number of inherited human diseases (38). In many cases, trafficking-defective mutant proteins are functional if they can be rescued to their final destinations. Recently, the use of specific ligands as pharmacological chaperones has emerged as a strategy for rescue of trafficking-defective proteins (39, 40). It has been hypothesized that binding of specific ligands to the unfolded or misfolded proteins promotes correct folding. We and other investigators found that the trafficking-defective LQT2 mutations K28E, T65P, N470D, and G601S can be rescued by hERG channel blockers (21, 41–43). It has been shown that pharmacological rescue requires binding of the drugs to the inner vestibule of the pore region of the hERG channel (41).

Our present results show that in the presence of E-4031, the N470D mutant is able to escape the calnexin cycle and exit the ER. Therefore, E-4031 binding may improve proper folding of the mutant channel so that it is no longer a substrate for UDP-glucose:glycoprotein glucosyltransferase. The poteasome sensitivity experiments show that the complex-glycosylated mature form of the N470D mutant protein rescued by E-4031 becomes more resistant to trypsin digestion, suggesting that it has a compact conformation that is similar to the mature form of wild type hERG. In addition, the results demonstrate that pharmacological rescue of the N470D mutant by E-4031 is associated with its increased resistance to trypsin even when its trafficking from the ER to the Golgi is blocked by BFA. This indicates that the E-4031-induced conformational changes take place in the ER.

LQT2 mutations that can be rescued by hERG channel-blocking agents appear to express small amplitude currents under control conditions (21, 41–43). This observation indicated that the immature form of wild type hERG is in an incompletely folded intermediate state, which can become the properly folded mature form and traffic to the plasma membrane. However, the presence of the mutation in N470D may result in the formation of a hemodynamic hurdle that inhibits maturation of the mutant channel and consequently causes its ER retention. Taken together, the results from the protease digestion and detergent extraction experiments and previous pulse-chase experiments suggest that the N470D mutant does not cause significant aggregation of the hERG channel protein.

Our previous pulse-chase experiments showed that the immature form of wild type hERG is more resistant to trypsin digestion, suggesting that it is no longer a substrate for UDP-glucose:glycoprotein glucosyltransferase. The poteasome sensitivity experiments show that the complex-glycosylated mature form of the N470D mutant protein rescued by E-4031 becomes more resistant to trypsin digestion, suggesting that it has a compact conformation that is similar to the mature form of wild type hERG. In addition, the results demonstrate that pharmacological rescue of the N470D mutant by E-4031 is associated with its increased resistance to trypsin even when its trafficking from the ER to the Golgi is blocked by BFA. This indicates that the E-4031-induced conformational changes take place in the ER.

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