The Binding Site for Channel Blockers That Rescue Misprocessed Human Long QT Syndrome Type 2 ether-a-gogo-related Gene (HERG) Mutations*

Eckhard Ficker‡, Carlos A. Obejero-Paz§, Shuxia Zhao, and Arthur M. Brown§

From the Rammelkamp Center for Education and Research, MetroHealth Campus, Case Western Reserve University, Cleveland, Ohio 44109, and the §Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio 44106

Mutations in the human ether-a-gogo-related gene (HERG) K+ channel gene cause chromosome 7-linked long QT syndrome type 2 (LQT2), which is characterized by a prolonged QT interval in the electrocardiogram and an increased susceptibility to life-threatening cardiac arrhythmias. LQT2 mutations produce loss-of-function phenotypes and reduce Ikr, currents either by the heteromeric assembly of non- or malfunctioning channel subunits with wild type subunits at the cell surface or by retention of misprocessed mutant HERG channels in the endoplasmic reticulum. Misprocessed mutations often encode for channel proteins that are functional upon incorporation into the plasma membrane. As a result the pharmacological correction of folding defects and restoration of protein function are of considerable interest. Here we report that the trafficking-deficient pore mutation HERG G601S was rescued by a series of HERG channel blockers that increased cell surface expression. Rescue by these pharmacological chaperones varied directly with their blocking potency. We used structure-activity relationships and site-directed mutagenesis to define the binding site of the pharmacological chaperones. We found that binding occurred in the inner cavity and correlated with hydrophobicity and cationic charge. Rescue was domain-restricted because the trafficking of two misprocessed mutations in the C terminus, HERG F805C and HERG R823W, was not restored by channel blockers. Our findings represent a first step toward the design of pharmacological chaperones that will rescue HERG K+ channels without block.

The cardiac potassium channel gene HERG1 (KCNH2) is mutated in the long QT syndrome type 2 (LQT2), a familial, autosomal dominant cardiac disease associated with prolongation of the QT interval and torsade de pointes (1, 2). LQT2 mutations are, with only one exception, loss-of-function mutations that reduce the repolarizing cardiac potassium current Ikr, thereby prolonging the cardiac action potential (3, 4). When expressed in heterologous cells loss of function is caused either by mal- or nonfunctioning tetrameric channels inserted into the plasma membrane or by trafficking-deficient mutant channels retained in the endoplasmic reticulum (ER) (5–8).

Mutations in hereditary channelopathies including cystic fibrosis, nephrogenic diabetes insipidus, and episodic ataxia (9–11) may produce misfolded proteins that are recognized and retained by quality control mechanisms in the ER. In cystic fibrosis the most common mutation, ΔF508, is trafficking-deficient and can be rescued by lowering the incubation temperature or by using chemical chaperones such as glycerol, dimethyl sulfoxide or trimethylamine N-oxide (12–14). These results have stimulated interest in the development of methods to correct folding defects and restore protein function. However, low incubation temperatures are of practical value only “in vitro,” and chemical chaperones work at high concentrations (10–1000 mM) that may restrict their use in patients. A more promising pharmacological strategy has been introduced for misfolded P-glycoprotein mutants using specific substrates and blockers to rescue the target protein (15). More recently, pharmaceutical strategies have been validated further with the rescue of misfolded vasopressin receptors mutants using small organic V2 receptor antagonists (16).

In LQT2, expression of two trafficking-deficient mutations, HERG N470D and HERG R752W, at low temperature resulted in incorporation of functional channels in the plasma membrane (6, 17). Incubation with chemical chaperones restored trafficking and rescued functional channels for HERG N470D, a mutation in the transmembrane domain, but was completely ineffective for HERG R752W, a mutation in the cyclic nucleotide binding domain. Similarly, pharmacological rescue of functional channel protein has been reported for HERG N470D when synthesized in the presence of the methanesulfonanilide E4031, the antihistamine astemizole, or the prokinetic drug cisapride, whereas E4031 was completely ineffective when incubated with HERG R752W (6, 17).

How blocking molecules interact with HERG channels to stabilize certain mutant conformational states for export to the plasma membrane but fail to stabilize others is unknown. Whether block and rescue rely on the same structural determinants of the channel protein and whether pharmacological rescue can be separated from block are also unknown. In the present work we used a series of channel blockers to probe the binding site for drug-induced rescue of protein trafficking in HERG G601S.
HERG G601S is a temperature-sensitive missense mutation in the pore region of the channel protein with most channel protein retained in the ER when expressed at physiological temperatures (18). In our experiments we were guided by more recent insights into the structure of the methanesulfonanilide binding site of HERG K_H11001 channels (19, 20). We used the known structure-activity relationship of the noncardiac drug astemizole to compare the relative efficacies of channel block and channel rescue. We extended our observations by comparing block produced by alkyl-TEA derivatives with their ability to rescue the mutant channels. We show that the inner vestibule of the HERG K_H11001 channel forms the receptor sites for compounds that produce both rescue and block and demonstrate that both sites share the properties of the internal quaternary ammonium receptor site present in most K_H11001 channels.

EXPERIMENTAL PROCEDURES

Molecular Biology—HERG mutations were generated by overlap extension PCR, verified by sequencing, and subcloned into full-length HERG-pcDNA3 as described previously (6).

HERG Antibody—The polyclonal HERG antibody used in Western blots and immunostaining was generated in rabbits against a glutathione S-transferase fusion protein containing the last 112 amino acids of HERG (residues 1048–1159). HERG antiserum was purified on an affinity column consisting either of a short C-terminal peptide corresponding to HERG residues 1102–1121 (TLTLDSLSQVSQFMACEELP) or the entire fusion protein.

Western Blot Analysis—HEK293 cells maintained in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum plus penicillin/streptomycin at 37 °C, 5% CO_2 were transiently transfected with 2 μg HERG wild type (WT) or HERG mutant cDNA in 60-mm culture dishes using LipofectAMINE/Plus as recommended by manufacturer (Invitrogen). Some experiments were performed using COS-7 cells. After transfection, cells were incubated for 48 h at either 26 or 37 °C. For Western blot experiments, HERG channel blockers were diluted in prewarmed culture medium and added for 12–16 h to cells 36 h post-transfection. Trafficking of HERG WT channels was studied using a stable cell line established in HEK293 cells.

Cells were solubilized for 1 h at 4 °C in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100), containing protease inhibitor mix (Complete, Roche Biochemicals). Protein concentrations were determined by the BCA method (Pierce). N-Glycosidase F

FIG. 1. Restoration of HERG G601S trafficking by incubation with cisapride, E4031, and quinidine. Panel A, schematic representation of the inner vestibule of HERG K⁺ channels with residues crucial for binding of high affinity pore blockers in the S6 transmembrane domain (Phe-656, Tyr-652, Gly-648) and the pore helix (Thr-623, Val-625). Location of mutation in HERG G601S is depicted in the extracellular part of pore region. Panel B1, at 37 °C HERG G601S is synthesized only as a core-glycosylated protein of 135 kDa (con). Protein processing is shown in the presence of increasing amounts of cisapride with individual drug concentrations in the micromolar concentration range as indicated above the immunoblot. With increasing cisapride concentrations an additional 155-kDa form of the membrane protein can be detected on Western blots. Blotted are equal amounts (20 μg) of total protein from cells expressing HERG G601S. Panel B2, left lane, 135- and 155-kDa forms of HERG G601S channel protein expressed in cells treated with 5 μM cisapride at 37 °C and blotted prior to treatment with N-glycosidase F (PGNase F, +). Right lane, after treatment with N-glycosidase F (PGNase F, –) all carbohydrate residues were removed, and the protein band at 155 kDa collapsed into a single protein band of about 130 kDa. Blotted is 15 μg of total protein. Panel C, processing of HERG G601S is shown in the presence of increasing amounts of quinidine applied in the micromolar concentration range. Panel D, processing of HERG G601S in the presence of increasing amounts of E4031 in the micromolar concentration range. Panel E, concentration-dependent rescue of HERG G601S by cisapride, E4031, and quinidine. Half-maximal rescue concentrations RC₅₀ were 0.6 ± 0.1 μM for cisapride, 1.1 ± 0.2 μM for E4031, and 1.7 ± 0.4 μM for quinidine (n = 3 or 4). On all Western blots, markers indicate core- and fully glycosylated protein bands at 135 and 155 kDa, respectively.
treatments (NEB P0704S) were performed as recommended by the manufacturer. For Western blotting, proteins were separated on 7.5% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked overnight with 5% nonfat dry milk in phosphate-buffered saline plus 0.1% Tween and immunoblotted with rabbit polyclonal HERG antibody (1:100 dilution; 1 h at room temperature) followed by horseradish peroxidase-conjugated secondary antibody (1:3,000; 1 h at room temperature; Amersham Biosciences, Inc.). ECL Plus (Amersham Biosciences, Inc.) was used for blot development.

Immunoblot images were captured directly on a Storm PhosphorImager and analyzed using ImageQuant (Molecular Dynamics). Image densities were normalized to densities measured in untreated control cells (five independent experiments). Differences between treatment groups are not statistically significant as determined by one way analysis of variance on ranks (p = 0.197).

Image densities measured in vehicle-treated (ethanol or dimethyl sulfoxide) controls were fit to Hill equations of the form shown in Equation 1,

\[
I_{\text{drug/control}} = 1/(1 + (D/IC_{50})^n)
\]

(Eq. 1)

where \( I \) indicates current, \( D \) is drug concentration, \( n \) is the Hill coefficient, and \( IC_{50} \) is the concentration necessary for half-maximal block. Patch pipettes were filled with (in mS): 100 potassium aspartate, 20 KCl, 2 MgCl₂, 1 CaCl₂, 10 EGTA, 10 HEPES (pH 7.2). The extracellular solution had the following composition (in mS): 140 NaCl, 5 KCl, 1 MgCl₂, 1.8 CaCl₂, 10 HEPES, 10 glucose (pH 7.4). No leak subtraction was applied. All current recordings were performed at room temperature (20–22 °C). To analyze changes in current densities membrane capacitances were measured using the analog compensation circuit of an Axon 200B patch clamp amplifier. Pclamp software (Axon Instruments) was used for generation of voltage clamp protocols and data acquisition. Whenever possible data are presented as mean ± S.E. of \( n \) experiments. Drugs—Astemizole and norastemizole were kindly provided by Janssen (Beerse, Belgium). E4031 was a gift from Eisai (Tokyo, Japan). Cisapride was obtained from Research Diagnostics (Flanders, NJ); quinidine was purchased from Sigma Chemical. C8-TMA was bought as a commercial salt from Fluka and TCI America. Asymmetrical tetraethyl ammonium compounds (C-TEA) were synthesized as described previously (21). Briefly, one part of triethylamine was added to one part alkylbromide in the presence of absolute ethanol and heated under gentle refluxing for 8–10 h. Recrystallized alkyl-TEA compounds were tested for identity by NMR and elementary analysis.

**RESULTS**

Restoration of HERG G601S Trafficking by Incubation with Cisapride, E4031, and Quinidine—HERG G601S is located in the S5-pore helix linker and was described as a trafficking-deficient, hypomorphic LQT2 mutant expressing greatly reduced, kinetically unaltered currents when expressed in mammalian cells at physiological temperature (18). Much larger currents were recorded on expression in Xenopus oocytes, indicating restoration of protein trafficking at lower incubation temperatures. The trafficking defect was reflected in Western blots in which the channel protein was detected only as a core-glycosylated immature protein of 135 kDa when isolated from transiently transfected cells incubated at 37 °C (Fig. 1B1). On incubation at 26 °C an additional band at 155 kDa appeared representing the fully mature channel protein (see Fig. 5B). These results showed that HERG G601S is a temperature-sensitive trafficking mutant with few channels evading quality control at 37 °C.

We tested three HERG channel blockers for restoration of G601S trafficking at 37 °C. These blockers appear to interact with different portions of the extended binding site for methanesulfonanilides in the inner vestibule of HERG (19). We used E4031, a methanesulfonanilide drug in which the methanesulfonyl group is thought to bind to a pocket formed between the pore helix and the S6 helix and its aromatic piperidine ring to aromatic amino acid residues at Tyr-652 and Phe-656. We also
tested the prokinetic drug cisapride, which is thought to interact with Tyr-652 and Phe-656 but not with the S6/pore helix pocket. Finally we tested the antiarrhythmic drug quinidine, which is thought to interact mainly with Phe-656 but not with the inactivated state of the HERG channel (20).

In electrophysiological experiments HERG currents were blocked in mammalian cells by E4031 with an IC₅₀ of 7–10 nM (22, 23). Cisapride blocked HERG currents with an IC₅₀ of 6–7 nM (24, 25), and quinidine blocked with an IC₅₀ of about 1 μM (26, 27). We tested whether all of these drugs restored HERG G601S trafficking in a concentration-dependent manner and whether the difference in interaction with the methanesulfonamide binding site was reflected in their ability to rescue HERG G601S.

HEK293 cells were transiently transfected with HERG G601S cDNA, incubated for 36 h at 37 °C, and then exposed overnight to different concentrations of cisapride (Fig. 1 B1), quinidine (Fig. 1 B2), and E4031 (Fig. 1 B3) before immunoblotting. In vehicle-treated control cells only a core-glycosylated protein band of 135 kDa could be detected (Fig. 1 B, D). With increasing drug concentrations an additional 155 kDa band appeared which could be reduced to a smaller form of about 130 kDa by treatment with N-glycosidase F, an enzyme used to remove all carbohydrate residues from glycoproteins (Fig. 1 B2). The fully glycosylated 155-kDa form of the channel protein which could be detected after exposure of G601S cells to the different channel blockers was quantified using a PhosphorImager, normalized, and plotted as a function of drug concentration (Fig. 1 E). All three drugs restored trafficking of HERG G601S, with half-maximal rescue concentrations (RC₅₀) of 0.6 ± 0.1 μM for cisapride, 1.1 ± 0.2 μM for E4031, and 1.7 ± 0.4 μM for quinidine (n = 3 or 4). For WT protein both the 135- and the 155-kDa forms are synthesized at 37 °C (Fig. 2 A, con), and

**FIG. 2. Rescue of HERG G601S currents by high affinity HERG channel blockers.** Panel A, representative whole cell current recordings from HEK293 cell expressing HERG G601S (upper part, control) and from an HEK293 cell incubated for 48 h with 5 μM cisapride (lower part of panel). Incubation temperature was 37 °C. Currents were elicited with 650-ms depolarizing voltage commands from −60 mV to +80 mV, return potential −100 mV, and holding potential −80 mV. Panel B, G601S, current densities measured in HEK cells transfected with HERG G601S and treated post-transfection with vehicle (control) or for 48 h with 5 μM cisapride, 5 μM E4031, 100 μM quinidine, or 5 μM astemizole. Note that current densities are increased significantly in all experiments with drug incubation (*p < 0.001, **p < 0.006). WT, to evaluate wash-out of drugs prior to patch clamp recordings we incubated cells expressing WT currents for 48 h with 5 μM astemizole and compared current densities after wash-out with densities in untreated WT controls. More than 80% of WT current levels could be recovered, indicating that the partial rescue observed in HERG G601S was not entirely the result of residual drug block. Panel C, normalized current inhibition measured after steady-state block with 20 nM astemizole in HERG WT and HERG G601S, HERG F656C, HERG G601S/F656C mutant channels. The number of cells studied is shown in parentheses.

**FIG. 3.** Rescue of HERG G601S currents by high affinity HERG channel blockers. Panel A, representative whole cell current recordings from HEK293 cell expressing HERG G601S (upper part, control) and from an HEK293 cell incubated for 48 h with 5 μM cisapride (lower part of panel). Incubation temperature was 37 °C. Currents were elicited with 650-ms depolarizing voltage commands from −60 mV to +80 mV, return potential −100 mV, and holding potential −80 mV. Panel B, G601S, current densities measured in HEK cells transfected with HERG G601S and treated post-transfection with vehicle (control) or for 48 h with 5 μM cisapride, 5 μM E4031, 100 μM quinidine, or 5 μM astemizole. Note that current densities are increased significantly in all experiments with drug incubation (*p < 0.001, **p < 0.006). WT, to evaluate wash-out of drugs prior to patch clamp recordings we incubated cells expressing WT currents for 48 h with 5 μM astemizole and compared current densities after wash-out with densities in untreated WT controls. More than 80% of WT current levels could be recovered, indicating that the partial rescue observed in HERG G601S was not entirely the result of residual drug block. Panel C, normalized current inhibition measured after steady-state block with 20 nM astemizole in HERG WT and HERG G601S, HERG F656C, HERG G601S/F656C mutant channels. The number of cells studied is shown in parentheses.

**AST**

**NOR-AST**

0.6 ± 0.1 μM for cisapride, 1.1 ± 0.2 μM for E4031, and 1.7 ± 0.4 μM for quinidine (n = 3 or 4). For WT protein both the 135- and the 155-kDa forms are synthesized at 37 °C (Fig. 2 A, con), and

**FIG. 4. Chemical structures of astemizole (AST) and norastemizole (NOR-AST).**
expression of the mature fully glycosylated channel protein at 155 kDa is not modified significantly when cells expressing HERG WT are exposed to channel blockers at concentrations that maximally restore trafficking in HERG G601S (Fig. 2B).

To test whether the increased production of fully glycosylated mature G601S protein by HERG blockers was accompanied by increased expression of functional channels we measured G601S currents in vehicle-treated control cells and in cells exposed 48 h to saturating concentrations of cisapride (5 μM), E4031 (5 μM) and quinidine (100 μM). In these experiments the different drugs were washed out 2 h before the start of patch clamp recordings. Fig. 3A illustrates current recordings from a control cell expressing G601S at 37 °C and from a cell treated for 12 h with 5 μM cisapride. Rescued G601S channels showed no alteration in kinetic properties compared with untreated mutant channels (data not shown). However, current densities increased in the presence of saturating concentrations of cisapride, E4031, and quinidine from 54 ± 7 pA/pF (control) to 116 ± 17, 147 ± 39 and 96 ± 8 pA/pF, respectively (Fig. 3B).

Despite a significant increase, current densities remained smaller in G601S than in cells transiently transfected with equal amounts of WT cDNA (382 ± 50 pA/pF). This difference was not the result of incomplete washout of HERG blockers because in control experiments about 80% of WT current density was recovered after treatment with 5 μM astemizole (309 ± 32 pA/pF, Fig. 3B).

Structure-Function Studies of Pharmacological Rescue Using Astemizole and Its Metabolite Norastemizole—Astemizole is a second generation antihistamine that blocked HERG with an IC₅₀ of 0.9 nM (28). In humans it undergoes oxidative N-alkylation generating norastemizole (Fig. 4) reported to have a reduced IC₅₀ of 27.7 nM with about 50% of HERG current resistant to block by norastemizole (28). This observation suggests that hydrophobic side chains attached to tertiary amines in noncardiac HERG-blocking antihistamines are important for block (29). We asked whether these side chains were also important for rescue of HERG G601S. In immunoblotting experiments the RC₅₀ for G601S at 37 °C and from a control cell treated for 12 h with 5 μM cisapride. Rescued G601S channels showed no alteration in kinetic properties compared with untreated mutant channels (data not shown). However, current densities increased in the presence of saturating concentrations of cisapride, E4031, and quinidine from 54 ± 7 pA/pF (control) to 116 ± 17, 147 ± 39 and 96 ± 8 pA/pF, respectively (Fig. 3B).
reported that high affinity drug binding in HERG is dominated by a phenylalanine in position 656 (19, 20). We mutated the Phe-656 to Cys-656. This mutation did not modify HERG currents (data not shown) but dramatically reduced the affinity for astemizole (Fig. 3C). HERG F656C was not trafficking-deficient but was retained in the ER when combined with the trafficking mutation 601S (HERG G601S/F656C) with trafficking being restored by incubation at 26°C (Fig. 5B). In contrast to G601S, however, the affinity of the double mutant for astemizole block was dramatically reduced (Figs. 3C and 5B). In line with our hypothesis we found that the double mutated channel was not rescued by astemizole (Fig. 5, C and D). Even at high concentrations only about 15% of fully glycosylated protein was recovered compared with HERG G601S. The suppression of pharmacological rescue by the F656C mutation provides further evidence that just as for block, rescue involves interactions with the inner vestibule of the HERG channel.

**Relationship between Quaternary Ammonium Block and Rescue**—For antihistamines and class III antiarrhythmic blockers of HERG a general structure has been proposed consisting of aromatic benzene rings, which are connected via a short chain to a basic nitrogen atom with the basic nitrogen carrying additional hydrophobic substituents (29). In this model the tertiary amine at physiological pH is mainly in the protonated quaternary form and resembles quaternary ammonium ions such as TEA, which are known to be potent potassium channel blockers.

We used alkyl-TEA derivatives to probe the molecular characteristics of the rescue site in HERG G601S. In squid axon it was shown that block by TEA compounds increased with increasing alkyl chain length and that at a given chain length block decreased with changes in the size of the remaining N-substituents (30, 31). Transfected HEK cells were incubated for 12 h with increasing concentrations of TEA derivatives comprised of a constant TEA headgroup that was combined with alkyl tails consisting of six (C6-TEA), eight (C8-TEA), and ten (C10-TEA) carbon atoms. In immunoblots we found that rescue of fully mature HERG G601S protein by these TEA derivatives was concentration-dependent (Fig. 6, A–C). The image density of the emerging 155 kDa band was quantified, normalized, and used to determine RC50 values. We found that RC50 values increased with the addition of methylene groups from 57.5 ± 11.5 μM for C6-TEA to 8.7 ± 3.1 μM for C8-TEA and 4.1 ± 1.4 μM for C10-TEA (Fig. 6D). To correlate the rescue efficacy of alkyl-TEA compounds with their potency as channel blockers we performed electrophysiological experiments using a stable HEK cell line expressing WT channels. A stable WT cell line was selected because it expressed much higher current densities than cells transfected with G601S and thus allowed for a more reliable determination of IC50 values. Moreover, we could demonstrate in experiments with astemizole that drug block was similar between HERG WT and G601S channels (Fig. 3C). Patch clamp experiments were performed in the whole cell mode with extracellular application of alkyl-TEA derivatives to mimic the experimental situation in rescue experiments. Steady state was reached within a few min after start of perfusion. To ensure that the observed drug effects were not the result of the interaction of alkyl-TEA derivatives with an external TEA receptor we monitored the inactivation time course of C-type inactivation. We did not detect slowing of C-type inactivation with micromolar drug concentrations. This result was not unexpected because the external TEA receptor was blocked half-maximally by TEA in the 10 mM range (32). In 5 mM external [K+]ex we found IC50 values for C6, C8, and C10 TEA of 183 ± 38, 8.4 ± 1.4 and 3.6 ± 1.2 μM, respectively (n = 3–11, Fig. 7). These IC50 values were similar to the RC50 values determined in immunoblotting experiments even though in electrophysiological experiments [K+]ex was 5 mM, whereas in rescue experiments HERG channels were most likely exposed to symmetrical 140 mM K+. A weak dependence of IC50 values on [K+]ex has been reported for organic HERG channel blockers (33).

**Structure-Function Studies Using Quaternary Ammonium Derivatives with Variable Headgroup Size**—To evaluate the contribution of smaller hydrophobic side chain substituents to the rescue of HERG G601S we used C8-TMA. C8-TMA was less potent than C8-TEA, and trafficking was restored only to about 80% of C8-TEA levels. The RC50 decreased from about 8.7 ± 3.1...
sensitive mutations localized to the transmembrane domain: HERG G601S and HERG N470D (17). We may ask whether pharmacological rescue by pore blockers is restricted to temperature-sensitive mutants in the transmembrane domain or whether blocking molecules might be able to induce more global conformational changes and rescue mutants localized to other domains of the HERG protein. Recently, we demonstrated that a temperature-sensitive trafficking mutation located in the cyclic nucleotide domain, HERG R752W, was not rescued by incubation with E4031 at 37 °C (6). In the present experiments we analyzed two additional LQT2 mutations in the cyclic nucleotide domain region, HERG F805C and HERG R823W. Both mutants were rescued at lower incubation temperatures as shown in Fig. 9B. After incubation at 26 °C trafficking of HERG F805C was restored. A strong protein band was detected at 155 kDa in addition to the protein band at 135 kDa present in cells cultured at 37 °C. In electrophysiological recordings of HERG 805C incubated at 26 °C current densities reached WT levels, whereas current expression was negligible at 37 °C (Fig. (9, A and C)). The mutant phenotype of HERG R823W was more severe in that only a weak protein band at 155 kDa was detected after incubation at 26 °C (Fig. 9B). Similarly, current densities were much smaller than those measured for WT or F805C after incubation at 26 °C (Fig. 9C). To achieve pharmacological rescue both temperature-sensitive cyclic nucleotide domain mutants were expressed at 37 °C and cultured overnight in the presence of either 5 μM astemizole or 100 and 300 μM C8-TEA (Fig. 9D). Although both treatments restored the maturation of HERG N470D, trafficking was not restored for mutations in the cyclic nucleotide binding domain.

**DISCUSSION**

We tested astemizole, cisapride, and E4031, three different high affinity blockers of HERG, and the nonspecific antiarrhythmic drug quinidine for their ability to stabilize the trafficking-deficient LQT2 mutant HERG G601S in a conformation for release from the ER at physiological temperature. We showed that all four blockers were effective as pharmacological chaperones and significantly increased the expression of functional channels at the cell surface.

The HERG channel blockers we have used block the open state (23, 24, 28) indicating that pharmacological rescue of HERG G601S was mediated by the binding of these blockers to a receptor site in the hydrophobic inner vestibule of HERG soon after assembly of channel tetramers. This interpretation was supported further by our observation that rescue was reduced strongly when the pore residue HERG Phe-656 was mutated into a more hydrophilic cysteine and by our experiments with astemizole/norastemizole in which a hydrophobic substituent was replaced by a hydrophilic group and the binding to the inactivated state and competitive interactions with luminal K+ ions and blocking molecules (31, 33). How-
Nevertheless, channel inactivation and luminal [K⁺] should affect most open channel blockers used in the present study in a similar manner, which was clearly not the case (see Table I).

The large differences in efficacy between drug block and rescue were not simply the result of permanent, mutation-induced structural changes in the methanesulfonanilide binding site of HERG G601S because after expression at the cell surface G601S currents were blocked to the same extent by astemizole as WT currents. We propose that low affinity RC₅₀ values may be the result of small structural aberrations in the drug binding site formed early during assembly of HERG G601S channels. These aberrations are corrected subsequently when folding progresses toward the native state, and the protein is finally exported to the cell membrane.

To characterize further the mechanism by which blocking molecules stabilize HERG G601S for surface expression we used alkyl-TEA derivatives thought of as generic pore blockers of potassium channels (30, 39). We found that the interaction of alkyl-TEA derivatives with HERG mirrored observations made in Shaker K⁺ channels (30, 31, 39). Block of HERG increased in parallel with increasing lipophilicity of alkyl-TEA derivatives (39). Moreover, as described first for potassium channels in squid axons block of HERG K⁺ channels depended strongly on the size of the terminal headgroup and was substantially reduced when the trimethyl headgroup of C8-TEA was replaced with the smaller trimethyl headgroup of C8-TMA (31). Pharmacological rescue of HERG G601S followed the same rules outlined for quaternary ammonium ions as blocking molecules.

The rescue capacity of alkyl-TEA increased with longer, more lipophilic hydrocarbon side chains, and rescue was reduced strongly when the pore residue HERG Phe-656 was mutated, as if Phe-656 was both part of the methanesulfonanilide and the quaternary ammonium binding site of HERG. We speculate that C8-TEA and C10-TEA rescue and block HERG channels with about the same potency most likely because of their hydrocarbon chains sensing the overall hydrophobicity of the inner vestibule rather than specific amino acid side chains that project into the inner vestibule to form the methanesulfonanilide receptor (19, 30).

We found two chaperones, C6-TEA and C8-TMA, that were less efficient as channel blockers than as pharmacological chaperones. This result indicates that rescue and block may be separated when the inner cavity of HERG is probed with molecules of various sizes. In analogy to observations made in Shaker K⁺ channels we attribute this to a reduction in either the size of the terminal headgroup or the length of the hydrocarbon chain, both of which may restrict the interaction of C6-TEA/C8-TMA molecules with residues in the pore helix region known to be important for internal TEA binding in Shaker channels (40).

Taken together our results highlight the significance of hydrophobic interactions for both rescue and block. The hydrophobic central cavity of HERG K⁺ channels appears to form a common receptor site that stabilizes the binding of both high affinity HERG blockers and lipophilic quaternary ammonium blockers to the channel protein. At the same time, ligand binding stabilizes the native state of the HERG G601S channel protein. Our conclusions are in line with the localization of the methanesulfonanilide binding site to the inner cavity of HERG (19) and with more recent crystallographic data demonstrating a binding site for tetrabutylammonium (TBA), a quaternary ammonium ion very similar to TEA derivatives used in the present study, in the central cavity of the potassium channel KcsA (34, 35). The structural data in KcsA show that TBA draws the inner (S6) helices closer...
toward the center of the inner vestibule. Based on this observation we propose that pharmacological chaperones first bind to a slightly distorted inner cavity and then reorient and stabilize the S6 helices of HERG G601S sufficiently to facilitate folding into the native, exportable state. More recently, it has been suggested that native cardiac IKr channels may be heteromultimeric proteins assembled from HERG/H9251 and MiRP1/H9252 subunits. HERG/H18528 MiRP1 complexes showed alterations in kinetics and increased sensitivity to block by E4031 upon expression in heterologous systems (22). Neither Mink nor MiRP1 proteins restored trafficking of HERG G601S when coexpressed (data not shown); however, based on our observation that rescue by pharmacological chaperones varies directly with their blocking potency it is possible that heteromultimeric HERG G601S-MiRP1 mutant channels might be rescued by E4031 more effectively than HERG G601S alone.

For the development of pharmacological chaperones in the treatment of LQT2 we should consider that all of the rescue molecules tested to this point appear to stabilize the inner vestibule of HERG. A direct implication is that rescue with channel blockers can occur only for properly assembled channel tetramers. Impaired assembly might explain best why temperature-sensitive HERG proteins such as HERG R752W, F805C, or R823W with mutations in the cyclic nucleotide binding domain cannot be rescued using channel blockers. Indeed two of these mutations, HERG R752W and R823W do not exert dominant-negative effects when coexpressed with WT channels, suggesting improper assembly (6, 41). The observation that lower incubation temperatures rescue both C-terminal and pore mutants of HERG whereas pore blockers seem to rescue only mutants in the pore region is explained best by a cotranslational folding model in which the different functional domains of large proteins fold sequentially and independently during translation (42). In such a model lower incubation temperatures could stabilize the folding of multiple domains, whereas the effects of ligand or blocker binding would be mostly restricted to protein domains harboring the particular binding site.

Up until now successful pharmacological refolding in LQT2 has only been reported for two mutations in the transmembrane domain, HERG N470D and G601S, both of which repre-
sent a mild phenotype expressing small currents at 37°C (17). Many other trafficking-deficient mutations in the transmembrane or the cyclic nucleotide binding domain do not respond to channel-blocking pharmacological chaperones (6–8). To overcome the specific problems posed by diverse LQT2 mutations multiple rescue strategies should be developed which target channel assembly as well as properly assembling mutants such as A561V, which are insensitive to known pharmacological chaperones (7). Most important, however, is the need to identify target sites on the HERG channel protein other than the inner vestibule so that rescue can be achieved without the unwanted side effect of channel block.

Acknowledgment—we thank Dr. M. Sanguinetti for the generous gift of HERG F805C and R823W vector constructs.

REFERENCES
1. Curran, M. E., Splawski, I., Timothy, K. W., Vincent, G. M., Green, E. D., and Keating, M. T. (1995) Cell 80, 795–803
2. Sanguinetti, M. C., Jiang, C., Curran, M. E., and Keating, M. T. (1995) Cell 81, 299–307
3. Sanguinetti, M. C., Curran, M. E., Spector, P. S., and Keating, M. T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2208–2212
4. Less-Miller, J. P., Duan, Y., Teng, G. Q., Thorstad, K., and Duff, H. J. (2000) Circ. Res. 86, 507–513
5. Zhou, Z., Gong, Q., Epstein, M. L., and January, C. T. (1999) J. Biol. Chem. 274, 21061–21066
6. Ficker, E., Thomas, D., Viswanathan, P. C., Dennis, A. T., Priori, S. G., Napolitano, C., Memmi, M., Wible, B. A., Kaufman, E. S., Iyengar, S., Schwartz, P. J., Rudy, Y., and Brown, A. M. (2000) Am. J. Physiol. 278, H1748–H1756
7. Ficker, E., Dennis, A. T., Obejero-Paz, C. A., Castaldo, P., Tagilatela, M., and Brown, A. M. (2000) J. Mol. Cardiol. 32, 2327–2337
8. Kagan, A., Yu, Z., Fishman, G. I., and McDonald, T. V. (2000) J. Biol. Chem. 275, 11241–11248
9. Kopito, R. R. (1999) Physiol. Rev. 79, S167–S173
10. Tamarappoon, B. K., and Verkman, A. S. (1998) J. Clin. Invest. 101, 2257–2267
11. Zerr, P., Adelman, J. P., and Maylie, J. (1998) J. Neurosci. 18, 2842–2848
12. Denning, G. M., Anderson, M. P., Amara, J. F., Marshall, J., Smith, A. E., and Welch, M. J. (1992) Nature 358, 761–764
13. Sato, S., Ward, C. L., Krouse, M. E., Wine, J. J., and Kopito, R. R. (1996) J. Biol. Chem. 271, 635–639
14. Brown, C. R., Hong-Brown, Q., Biwersi, J., Verkman, A. S., and Welch, W. J. (1996) Cell Stress Chaperones 1, 117–125
15. Loo, T. W., and Clarke, D. M. (1997) J. Biol. Chem. 272, 709–712
16. Morello, J.-P., Salaphour, A., Laperriere, A., Bernier, V., Arthus, M.-F., Lonergan, M., Petaeae-Repo, U., Angers, S., Morin, D., Bichet, D. G., and Schwartz, P. J., Rudy, Y., and Brown, A. M. (1992) J. Gen. Physiol. 100, 453–463
17. Zhou, Z., Gong, Q., and January, C. T. (1999) J. Biol. Chem. 274, 31123–31126
18. Furutani, M., Trudeau, M. C., Hagiwara, N., Seki, A., Gong, Q., Zhou, Z., Imamura, S., Nagashima, H., Kasanuki, H., Takao, A., Momma, K., January, C. T., Robertson, G. A., and Matsuoka, R. (1999) Circulation 99, 2290–2294
19. Mitcheff, J. S., Chen, J., Lin, M., Cullerson, C., and Sanguinetti, M. C. (2000) Proc. Natl. Acad. U. S. A. 97, 12329–12333
20. Less-Miller, J. P., Duan, Y., Teng, G. Q., and Duff, H. J. (2000) Mol. Pharmacol. 57, 367–374
21. Jarolimek, W., Soman, K. V., Alam, M., and Brown, A. M. (1995) Pfizers Arch. 430, 672–681
22. Abbott, G. W., Sesti, F., Splawski, I., Buck, M. E., Lehmann, M. H., Timothy, K. W., Keating, M. T., and Goldstein, S. A. (1999) Cell 97, 175–187
23. Zhou, Z., Gong, Q., Ye, B., Fan, Z., Makielski, J. C., Robertson, G. A., and January, C. T. (1998) Biophys. J. 74, 230–241
24. Rampe, D., Roy, M., Dennis, A., and Brown, A. M. (1997) FEBS Lett. 417, 28–32
25. Mohammad, S., Zhou, Z., Gong, Q., and January, C. T. (1997) Am. J. Physiol. 273, H2534–H2538
26. Poo, S. S., Wang, D. W., Yang, I. C., Johnson, J. P., Nie, L., and Bennett, P. B. (1999) J. Cardiovasc. Pharmacol. 33, 181–185
27. Yang, T., and Boden, D. M. (1996) Circulation 93, 407–411
28. Zhou, Z., Vorperian, V. R., Gong, Q., Zhang, S., and January, C. T. (1999) J. Cardiovasc. Electrophysiol. 10, 836–843
29. Zhang, M. Q. (1997) Circ. Res. 81, 171–184
30. Armstroong, C. M. (1971) J. Gen. Physiol. 58, 413–437
31. Sweeney, R. P. (1981) J. Gen. Physiol. 77, 255–271
32. Smith, P. L., Baukrowitz, T., and Yellen, G. (1996) Nature 379, 833–836
33. Kiehn, J., Lacerda, A. E., Wible, B. A., and Brown, A. M. (1996) Circulation 94, 2572–2579
34. Doyle, D. C., Morais Cabral, J., Pfuetzner, R. A., Kao, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) Science 280, 69–77
35. Zhou, M., Morais Cabral, J. H., Mann, S., and MacKinnon, R. (2001) Nature 411, 657–661
36. Hrnejz, B. J., Song, J. C., Prasad, M., Mayol, J. M., and Matthews, J. B. (1999) Am. J. Physiol. 277, G521–G531
37. Nomurauchi, H., Mullins, F. M., Johnson, J. P., Johns, D. C., Po, S. S., Yang, I. C., Tomaselli, G. F., and Balser, J. R. (2000) Circ. Res. 87, 1012–1018
38. Wang, S., Morales, M. J., Liu, S., Strauss, H. C., and Rasmusson, R. L. (1999) FEBS Lett. 417, 43–47
39. Choi, K. L., Mossman, C., Aube, J., and Yellen, G. (1993) Neuron 10, 533–541
40. Yellen, G., Jurman, M. E., Abramson, T., and MacKinnon, R. (1991) Science 252, 97–113