Long QT Syndrome-associated Mutations in the Per-Arnt-Sim (PAS) Domain of HERG Potassium Channels Accelerate Channel Deactivation*

(Received for publication, January 14, 1999, and in revised form, February 5, 1999)

Jun Chen‡, Anrou Zou‡, Igor Splawski§, Mark T. Keating¶¶, Michael C. Sanguinetti∥∥∥

From the §Department of Medicine, Division of Cardiology, ¶Eccles Program in Human Molecular Biology and Genetics, ∥Howard Hughes Medical Institute, and ∥∥Department of Genetics, University of Utah, Salt Lake City, Utah 84112

Mutations in the human ether-a-go-go-related gene (HERG) cause long QT syndrome, an inherited disorder of cardiac repolarization that predisposes affected individuals to life-threatening arrhythmias. HERG encodes the cardiac rapid delayed rectifier potassium channel that mediates repolarization of ventricular action potentials. In this study, we used the oocyte expression system and voltage clamp techniques to determine the functional consequences of eight long QT syndrome-associated mutations located in the amino-terminal region of HERG (F29L, N33T, G53R, R56Q, C66G, H70R, A78P, and L86R). Mutant subunits formed functional channels with altered gating properties when expressed alone in oocytes. Deactivation was accelerated by all mutations. Some mutants shifted the voltage dependence of channel availability to more positive potentials. Voltage ramps indicated that fast deactivation of mutant channels would reduce outward current during the repolarization phase of the cardiac action potential and cause prolongation of the corrected QT interval, QTc. The amino-terminal region of HERG was recently crystallized and shown to possess a Per-Arnt-Sim (PAS) domain. The location of these mutations suggests they may disrupt the PAS domain and interfere with its interaction with the S4-S5 linker of the HERG channel.

Mutations in the human ether-a-go-go-related gene (HERG) cause LQT, an inherited disorder of cardiac repolarization (1–10). HERG encodes a potassium channel with properties nearly identical to IKr of cardiac myocytes (11, 12). Mutations in HERG can reduce IKr by loss of function, altered function, or a dominant negative effect (8, 13, 14). Reduction of IKr causes delayed myocardial repolarization (15) and an increased risk of life-threatening ventricular arrhythmia (16).

The combination of two properties, fast inactivation and slow deactivation, distinguish IKr from other cardiac currents (15). HERG channels inactivate much more rapidly than they activate. The net result is that most HERG channels are closed during the plateau phases of the cardiac action potential. Rapid recovery from inactivation during repolarization combined with a very slow subsequent transition to the closed state (deactivation) results in an increase of IKr during the terminal phase of cardiac repolarization. The structural basis of rapid inactivation and slow deactivation of HERG is partially understood. Inactivation can be completely removed by a double mutation in the pore region of the channel (17), indicating the importance of this region and its similarity to C-type inactivation (18, 19). This mechanism is in contrast to most rapidly inactivating K⁺ channels in which interaction of the amino terminus with the S4-S5 linker closes the channel by occlusion of the inner pore region (20). HERG channel deactivation is greatly accelerated by removal of the amino-terminal region or by mutation of specific residues in the S4-S5 linker (21, 22).

The crystal structure of the amino-terminal region of HERG has been determined and shown to possess a Per-Arnt-Sim (PAS) domain (23). This domain may interact with another region of HERG such as the S4-S5 linker to affect channel deactivation.

A negative shift in the voltage dependence of inactivation or acceleration in the rate of deactivation would reduce the contribution of IKr to repolarization and lengthen the duration of cardiac action potentials. Mutations in HERG that cause LQT and shift the voltage dependence of inactivation have been described (8). In this study, we describe several LQT-associated mutations in the amino-terminal region of HERG that accelerate the rate of deactivation. The location of these mutations in the recently described three-dimensional structure of the HERG amino-terminal region (23) suggests they may disrupt the function of the PAS domain.

EXPERIMENTAL PROCEDURES

Construction of HERG Mutations and in Vitro Transcription of cRNA—Mutations were introduced into wild type (WT) HERG by the megaprimer method (24). The mutant construct was then subcloned into the pSP64 (Promega, Madison, WI) plasmid expression vector. Before use in expression experiments, the constructs were characterized by restriction mapping and DNA sequence analyses.

cRNA for injection into oocytes were prepared with SP6 Capscribe (Boehringer Mannheim) following linearization with EcoRI.

Isolation of Oocytes and Injection of RNA—Xenopus laevis frogs were anesthetized by immersion in 0.2% tricaine for 15–30 min. Ovarian lobes were digested with 2 mg/ml Type 1A collagenase (Sigma) in Ca²⁺-free ND96 solution for 1.5 h to remove follicle cells. Stage IV and V oocytes were injected with HERG cRNA (7.5 ng), then cultured in Barth’s solution supplemented with 50 μg/ml gentamycin and 1 μM pyruvate at 18 °C. Barth’s solution contained 88 mM NaCl, 1 mM KC1, 0.4 mM CaCl₂, 0.33 mM Ca(NO₃)₂, 1 mM MgSO₄, 2.4 mM NaHCO₃, 10 mM HEPES, pH 7.4.

Voltage Clamp of Oocytes—Oocytes were bathed in a modified ND96 solution containing 96 mM NaCl, 2 mM KC1, 2 mM MgCl₂, 0.1 mM CaCl₂, 5 mM HEPES, pH 7.6. Currents were recorded at room temperature (21–23 °C) using standard two-microelectrode voltage clamp techniques.
Mutations in PAS Domain of HERG

(25). Glass microelectrodes were filled with 3 M KCl, and their tips were broken to obtain tip resistances of 0.5–1.5 MΩ. Oocytes were voltage-clamped with a Dagan TEV-200 amplifier (Dagan Corp., Minneapolis, MN). Voltage commands were generated using pCLAMP software (Axon Instruments, Foster City, CA), a personal computer, and a TL-1 DA interface (Axon Instruments). Unless noted otherwise, the oocyte membrane potential was held at −80 mV between test pulses.

PCLAMP6.2 software was used to fit current traces to exponential functions. Exponential fits to current tracings were performed using the Chebyshev technique to determine the time constants (τ) and amplitudes (A) for exponential functions. The voltage dependence of HERG activation was determined from tail currents measured at −70 mV following 2- or 3-s test depolarizations. Normalized tail current amplitudes (Iτ) were plotted versus test potential (Vt) and fitted to a Boltzmann function using ORIGIN (Northampton, MA),

$$I_\tau = 1/(1 + \exp[(V_{1/2} - V_t)/k])$$  

(Eq. 1)

$V_{1/2}$ is the voltage at which the current is half-activated, and $k$ is the slope factor. The voltage dependence of HERG inactivation was determined using a three-pulse voltage pulse protocol as described previously (26). The peak of the inactivating current during the third pulse was normalized to the peak value, plotted as a function of the voltage of the second pulse, then fitted with a Boltzmann function. Data are expressed as the mean ± S.E. ($n$ = number of oocytes).

RESULTS

Identification of Long QT-associated Mutations in HERG—
The amino-terminal region is a highly conserved domain in all members of the eag potassium channel family that includes eag, erg, and elk. We and others (21, 22, 27) previously reported expression of HERG channels lacking this domain deactivate much faster than WT HERG channels, indicating the importance of this domain in normal gating. We hypothesized missense mutations within this domain that cause LQT would also accelerate deactivation and thereby reduce outward current through HERG channels during repolarization of the cardiac action potential. After solving the genomic structure of HERG (9), we used single strand conformation polymorphism and DNA sequence analyses to screen for mutations in individuals with LQT. Many mutations were found in the amino-terminal region of HERG.2 Eight missense mutations located in the amino-terminal region of HERG were chosen for electrophysiologic characterization (F29L, N33T, G53R, R56Q, C66G, H70R, A78P, and L86R). These mutations are located near the end of the amino terminus of HERG, which is about 400 amino acids in length.

Biophysical Characterization of Mutant HERG Channels—
Mutant channels were constructed using site-directed mutagenesis. cRNA for each construct was prepared and injected into Xenopus oocytes. Voltage clamp experiments were performed to assess the functional consequences of each mutation.

Unlike the majority of LQT-associated missense mutations in HERG (13, 14, 29), the 8-amino-terminal mutant HERG subunits formed functional channels when expressed alone in Xenopus oocytes. The I-V relationship for each oocyte was determined by pulsing to +40 mV for 1 s to allow currents to reach a steady-state level before repolarization for 3 s to a potential ranging from +40 to −120 mV (Fig. 1A). The currents at the end of the 3-s pulse were plotted as a function of voltage (Fig. 1B). The I-V relationships indicate that WT HERG and mutant HERG channels exhibit weak inward rectification. The peak of the I-V for some of the mutant HERG channels was shifted in the positive direction. As discussed below, this shift can be explained by an altered voltage dependence of channel inactivation.

The most notable change caused by the mutations was an accelerated rate of current deactivation. Fig. 2A shows tail currents for WT HERG and three mutants elicited by a similar voltage pulse protocol illustrated in Fig. 1. The rate of current deactivation was determined by fitting tail currents to a two-exponential function at potentials ranging from −70 mV to −120 mV for WT HERG and from −50 mV to −120 mV for the mutant channels. Deactivation was too slow to measure during the 3-s period at −50 mV and −60 mV for WT HERG channels. All 8 mutant HERG channels deactivated more rapidly than WT HERG channels at potentials positive to −90 mV. The rate of deactivation varied for each mutant and was fastest for R56Q HERG and slowest for H70R HERG. For example, at −70 mV, WT HERG channels deactivated with time constants of 0.42 s and 1.90 s. By contrast, the mutant channels had time constants that ranged from 0.067 to 0.22 s for the fast component and 0.18 to 1.20 s for the slow component (Fig. 2B and C). The rate of HERG channel activation between −40 mV and 0 mV was best described with a two-exponential function. The rates of activation were similar for WT HERG and most of the mutant HERG channels. The exceptions were R56Q and N33T, which activated more slowly than WT HERG (Fig. 3).

The voltage dependence of steady-state activation was determined for several mutants (N33T, R56Q, H70R, L86R) by measuring the relative amplitude of tail currents measured at −70 mV after a 30-s-activating pulse to potentials ranging from −70 to 0 mV (Fig. 4A). These mutants were chosen because they caused the greatest changes in kinetics or shifts in the voltage dependence of the I-V relationship. The $V_{1/2}$ and slope factor for WT HERG was −52.1 ± 0.6 mV and 6.7 ± 0.3 mV ($n = 7$). The $V_{1/2}$ for H70R and L86R HERG channels were similar to WT, but the $V_{1/2}$ was shifted by +7.6 mV for N33T HERG and +11.3 mV for R56Q HERG (Fig. 4B and D).

The voltage dependence of channel availability was determined using a three-pulse protocol described previously (17, 26). A 1-s pulse to +35 mV to inactivate most channels was followed by a 20-ms pulse to a variable potential (−145 to +35
mV) to allow a variable recovery of channels from inactivation but little or no deactivation. The third pulse was to +35 mV to assess the relative number of channels that recovered from inactivation during the second pulse (Fig. 5A). The peak current elicited by the third pulse was estimated by extrapolation of a single exponential fit of the current trace to the beginning of the pulse. These data were plotted as a function of the voltage for pulse two and then fit with a Boltzmann function to determine the peak current to be used for normalization. The normalized data was averaged and fit with a Boltzmann function to determine the $V_{1/2}$ and slope factor of the relationship (Fig. 5B). For these experiments, we characterized the four mutants that caused the greatest positive shift in the peak of the steady-state I-V relationship. The $V_{1/2}$ for the voltage dependence of steady-state channel availability was $-89.3 \text{ mV}$ for WT HERG channels. All four mutants (N33T, R56Q, H70R, L86R) caused a positive shift in this relationship. The $V_{1/2}$ was shifted by $+19.6 \text{ mV}$ for N33T, +9.5 mV for H70R, +9.0 mV for L86R (Fig. 5B), and +35.8 mV for R56Q HERG (Fig. 6C). The slope factor of the relationship was not significantly altered by any of the mutant channels. The shift in the voltage dependence of channel availability explains the shift in the peak of the steady-state I-V relationship shown in Fig. 1.

The mutations of HERG studied here are inherited in a dominant manner. Thus, affected individuals have one normal allele and one mutant allele. To mimic the disease condition, the currents induced by coexpression of WT HERG subunits and one of the mutant subunits (R56Q) were compared with

**Fig. 2. Mutations in the amino-terminal domain of HERG accelerate deactivation.** A, pulse protocol and currents recorded from oocytes expressing WT HERG (top panel) and three of the mutant HERG channels (lower 3 panels). Tail currents were fitted with two exponential function. B and C, voltage-dependent time constants for fast (B) and slow (C) components of deactivation ($\alpha = 8-16$). •, WT; ▽, F29L; □, R56Q; ●, G53R; ○, N33T; △, L86R; △, H70R; ▽, A78P; ●, C66G HERG.

**Fig. 3. Voltage dependence of HERG activation rate.** A, activating currents for WT HERG and N33T HERG were fitted with two exponential functions, and the fitted traces were superimposed over current traces. B and C, voltage-dependent time constants for fast (B) and slow (C) components of activation ($\alpha = 8-10$). N33T and R56Q HERG channels activated slower than WT HERG channels. •, WT; ▽, F29L; □, R56Q; ●, G53R; ○, N33T; △, L86R; △, H70R; ▽, A78P; ●, C66G HERG.
currents induced by expression of WT HERG or R56Q HERG subunits alone. Coexpression of R56Q HERG and WT HERG subunits resulted in a current that was similar or larger in magnitude compared with WT HERG alone but had intermediate kinetics of deactivation and voltage dependence of activation (Fig. 6A). For example, at –70 mV, the time constants for deactivation were 0.42 s and 1.90 s for WT HERG, 0.067 s and 0.18 s for R56Q HERG, and 0.11 s and 0.58 s for cells expressing WT + R56Q HERG channel subunits. The shift in the \( V_{1/2} \) for steady-state activation induced by coexpression of WT and R56Q HERG channel subunits was +7.4 mV, about half that caused by R56Q HERG alone (Fig. 6B). As shown in Fig. 6C, the shift in the voltage dependence of channel availability induced by R56Q HERG (+35.8 mV) was about twice that measured for oocytes expressing WT + R56Q HERG subunits (+18.9 mV). These data indicate that R56Q HERG does not have a dominant negative effect when coexpressed with WT HERG but does alter the kinetics and voltage dependence of heteromultimeric channels.

To simulate the voltage transition that occurs during a cardiac action potential, we applied a voltage step from –80 mV to +40 mV followed by a slow (1.7 s) voltage ramp back to –80 mV. Although this voltage protocol does not accurately simulate a ventricular action potential, it is useful to demonstrate the gradual increase then decrease in current magnitude during membrane repolarization. The amplitude of current during the declining voltage ramp is determined by (1) the number of channels that recover from inactivation, which increases current magnitude, (2) the declining driving force for outward flux of \( K^+ \), and (3) the rate of channel deactivation, which decreases current magnitude. The voltage dependence of channel availability (Fig. 6C) is the only important determinant of the number of channels that recover from inactivation because the rate of recovery from inactivation is extremely fast (11). Because R56Q HERG caused the greatest shift in channel availability (Fig. 6C) and rate of deactivation (Fig. 2), we compared the current induced by voltage ramps for this mutant with WT HERG. As shown in Fig. 6D, R56Q HERG current is initially larger than WT HERG current. However, later in the voltage ramp, corresponding to voltages negative to –48 mV, WT HERG current is larger than R56Q HERG current. The ramp current recorded in oocytes that were coinjected with cRNA for WT and R56Q HERG subunits was intermediate in magnitude to oocytes expressing either WT or R56Q HERG alone. These data indicate that although the R56Q HERG current activated during step depolarizations can be larger than WT HERG because of the positive shift in the voltage dependence of channel availability, the rapid deactivation caused by this mutation results in a smaller current when the membrane is slowly repolarized.
FIG. 6. Currents induced by coexpression of WT HERG and R56Q HERG subunits have properties intermediate to that induced by either subunit alone. A, examples of currents during depolarizations from –70 to 0 mV for oocytes expressing WT, R56Q, and WT + R56Q HERG channels. B, voltage-dependent activation of WT (same as Fig. 4), R56Q (Δ), and WT + R56Q HERG (○, V1/2 = −44.7 ± 0.9 mV, k = 7.3 ± 0.3 mV, n = 7). C, voltage dependence of channel availability for WT (same as Fig. 5), R56Q (V1/2 = −53.5 ± 1.3 mV, k = 25.3 ± 1.0 mV, n = 8), and R56Q + WT HERG (V1/2 = −70.4 ± 2.4 mV, k = 27.6 ± 1.0 mV, n = 10). D, fast deactivation of R56Q HERG channels reduces amplitude of current during repolarization phase of a voltage ramp. Currents were normalized to amplitude of instantaneous tail current at −120 mV to account for variable levels of channel expression between individual oocytes. Instantaneous tail current was measured by extrapolation of the deactivating phase to the time of initial repolarization from a prepulse to +40 mV. The traces represent the mean current for 5 oocytes + or −1 S.E. (n = 5 for each trace).

DISCUSSION

Mutations in HERG that cause LQT can reduce the amplitude of Im, by several different mechanisms. The most common mechanism is a loss of channel function and a dominant negative effect when mutant subunits are coexpressed with WT HERG subunits. Loss of function can result from an alteration of a critical structural component, exemplified by mutations in the pore region of HERG (13, 14), or abnormal channel processing (14). Frameshift or deletion mutations that cause truncation of the encoded protein can cause haplo-insufficiency without a dominant-negative effect (13). Only a few LQT-associated HERG mutants encode proteins that can form functional channels when expressed alone in a heterologous expression system (8, 13, 30). We have found that eight LQT-associated missense mutations in the amino terminus of HERG channels form functional channels with altered properties when expressed alone in oocytes. These mutations cause an accelerated rate of channel deactivation. This alteration in gating reduces outward current through HERG channels during repolarization from the plateau phase of the cardiac action potential, prolongs the QTc interval, and is the likely cause of the increased risk of torsades de pointes arrhythmia in affected individuals (16).

Nakajima et al. (8) recently reported on the functional consequences of two LQT-associated missense mutations in HERG (V630L, A614V) that cause loss of function. These mutants suppressed current by a dominant negative effect and by altering properties of inactivation when coexpressed with WT HERG subunits. These mutant residues are located in the outer mouth of the channel pore, a region known to be an important determinant of fast C-type inactivation of HERG channels (17). Both mutant subunits enhanced inactivation by causing a negative shift in the voltage dependence of channel availability when coexpressed with WT HERG subunits. This effect would increase channel rectification and reduce repolarizing current during an action potential and prolong action potential duration and the QTc interval. Surprisingly, we found that several missense mutations in the amino-terminal domain of HERG shifted the voltage dependence of channel availability in the positive direction. In the absence of any other effect, this shift would cause an increase in outward current during repolarization and, inferentially, a decrease in QTc interval. However, these mutations also accelerated the rate of channel deactivation, an effect that dominates the shift in channel availability and would cause a net reduction in outward current during slow repolarization typical of a cardiac action potential. This effect is exemplified by R56Q, the mutation that caused the greatest shift in the voltage dependence of channel availability (+36 mV) and the most pronounced increase (7- to 10-fold at −70 mV) in the rate of deactivation (Fig. 2).

The altered channel deactivation properties caused by mutations in the amino-terminal region of HERG indicate the important role of this domain in gating. The crystal structure of this domain was recently solved and shown to be a PAS domain (23). PAS domains are basic helix-loop-helix structures that have been shown to be the site of protein-protein interactions for factors that function in sensing and signal transduction. Examples include PAS-mediated dimerization of the transcription factors tracheless (Trh) with single-minded (Sim) (31), the transcription factors PER with TIM (32), and the dioxin receptor with the basic helix-loop-helix PAS factor Arnt (33, 34). Sensing proteins with a PAS domain include phototact yellow protein that senses light in bacteria (35). Although the interactive regions in these PAS domains are understood, it is unclear how the PAS domain of HERG modulates channel gating. Physiologic studies suggest that this domain may interact with the S4-S5 linker of the same channel. Removal of the amino-terminal region of HERG channels accelerates deactivation (21, 22). Alternatively spliced variants of erg channels that lack a portion of the amino-terminal domain also exhibit fast deactivation (36, 37). Missense mutations or chemical modification of the S4-S5 linker of HERG mimic the effects of amino-terminal deletion on channel deactivation (28, 38). Similar interactions have been noted in the eag channel, which also contains the same PAS domain in the amino terminus (27). Deletion of amino acids 2–12 from HERG speeds deactivation but has no effect on inactivation gating (38). Removal of a larger portion of the amino-terminal region (residues 2–354) also shifts the voltage dependence of inactivation to more positive potentials (21) and slows the rate of C-type inactivation.
(38). Our studies indicate that mutation of a single residue (i.e., R56Q) can alter the gating of both deactivation and inactivation. Thus, it appears that the amino-terminal region of HERG slows deactivation by binding to the S4-S5 linker. The mechanism by which this interaction affects inactivation is unknown. F29L, N33T, G53R, R56Q, C66G, and L86R are conserved in all known erg, eag, and elk channels, emphasizing their critical role for normal channel function. The functional effect of a mutation in this region has been studied previously. Morais Cabral et al. (23) reported that the mutation F29A HERG sped the rate of deactivation the most of any missense mutation by a factor of about two. Deletion of residues 2–9 had a similar effect, and further deletion (32–26) was as effective as complete truncation of the amino terminus. In general, all the mutations studied here are likely to disrupt the structural integrity of the PAS domain and alter its required interaction with the rest of the channel to modulate deactivation.

In summary, LQT-associated mutations in HERG accelerate the rate of channel deactivation. It is likely that this effect is caused by disruption of the interaction of the amino-terminal domain with the S4-S5 linker of HERG subunits, which normally slows the gating associated with channel deactivation. The increased rate of channel deactivation decreases the contribution of HERG current to cardiac repolarization, resulting in prolongation of ventricular action potentials and predisposes affected individuals to life-threatening arrhythmias.

Acknowledgments—The technical support of M. Lin and M. Martines is gratefully acknowledged.

REFERENCES
