Novel Mechanism of HERG Current Suppression in LQT2
Shift in Voltage Dependence of HERG Inactivation

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Abstract—In a *Xenopus* oocyte heterologous expression system, we characterized the electrophysiology of 3 novel missense mutations of HERG identified in Japanese LQT2 families: T474I (within the S2-S3 linker), A614V, and V630L (in the outer mouth of pore-forming region). For each of the 3 mutations, injection of mutant cRNA alone did not express detectable currents. Coinjection of wild-type (WT) along with each mutant cRNA (T474I/WT, A614V/WT, and V630L/WT) suppressed HERG current in a dominant-negative manner, and the order of magnitude of current suppression was V630L/WT > A614V/WT > T474I/WT. In addition to decreases in slope conductance for all 3 mutants, the voltage dependence of steady-state inactivation was shifted to negative potentials for V630L/WT and A614V/WT. Consequently, channel availability at positive potentials was diminished, and inward rectification was enhanced for these 2 mutants. Thus, missense mutations of HERG caused dominant-negative suppression through multiple mechanisms. The shift in voltage dependence of HERG inactivation and the resulting enhanced inward rectification in A614V/WT and V630L/WT provide a novel mechanism for suppression of the HERG current carrying outward current during the repolarization phase of the action potential. (Circ Res. 1998;83:415-422.)

Key Words: long-QT syndrome ■ HERG mutation ■ cardiac arrhythmia

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Received December 5, 1997; accepted June 10, 1998.

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inhomogeneity of current suppression by missense mutations and would also provide information as to the structure-function relationship of the HERG channel. Thus, we chose 3 novel mutations at different sites of the HERG channel and examined the electrophysiological characteristics of current expressed in *Xenopus* oocytes. Indeed, the data suggested that the 3 missense mutations that we studied suppressed HERG channel function by multiple mechanisms. Mutations in the pore-forming (P) region suppressed HERG channel function by a novel mechanism, affecting the voltage dependence of steady-state inactivation.

**Materials and Methods**

**Molecular Biology**

The HERG cDNA clone subcloned into the BamHI-EcoRI site of a pGHI vector was a gift from Dr Gail A. Robertson, the University of Wisconsin, Madison. Three missense mutations (T474I, A614V, and V630L) within the P region of HERG were made using Altered Sites II in vitro mutagenesis systems (Promega), and mutant HERG cDNAs were subcloned into a pGHI vector. The WT HERG construct and mutant HERG constructs were confirmed by DNA sequence analyses using an automated sequencer (373 DNA sequencing system, Perkin-Elmer). WT HERG cDNA and mutant HERG cDNAs were linearized by digestion with NotI, and cRNAs were synthesized in vitro using T7 RNA polymerase with the mCAP RNA capping kit (Stratagene).

**Oocyte Handling and Electrophysiology**

*Xenopus* oocyte preparation and handling were carried out as described previously. In brief, oocytes were removed from *Xenopus laevis* (Hamamatsu Seibutsu, Hamamatsu, Japan) under anesthesia and washed in Ca²⁺-free OR-2 solution containing (mmol/L): NaCl 100, KCl 2, MgCl₂ 1, HEPES 5, and Tris 5 (pH 7.6 with HCl). Stage V and VI *Xenopus* oocytes were defolliculated by treatment with 2% microinjection (Drummond Scientific Co). Injected oocytes were incubated for 3 to 6 days at 12°C to 18°C in modified Barth’s solution containing (mmol/L): NaCl 96, KCl 2, MgCl₂ 2.6, CaCl₂ 0.18, and HEPES 5 (pH 7.6 with NaOH). Oocytes were kept in current-clamp mode for at least 5 minutes before switching to voltage-clamp mode. Only oocytes exhibiting a resting potential negative to ~40 mV were used. A P/4 or P/6 method was used to subtract leak and capacitative currents, unless otherwise indicated. All pulse protocols are described in the figure legends. To examine permeability of K⁺ relative to Na⁺ on expressed currents, [K⁺], was varied from 2 to 5 and to 10 mmol/L by replacing with equimolar Na⁺.

**Data Analyses**

pCLAMP software was used to measure current amplitudes. To determine the voltage dependence of HERG current activation, a least squares algorithm on Origin software or Microsoft Excel was used to fit tail current amplitudes (Itail) to a Boltzmann function in the following form: $I_{tail} = I_{max} - I_{min} \left[1 + \exp\left(\frac{V_{1/2} - V_{test}}{k}\right)\right]$, where $I_{tail}$ is peak $I_{test}$, $V_{test}$ is the test potential, $I_{max}$ is the voltage at which $I_{tail}$ is half of $I_{max}$, and $k$ is the slope factor.

Inactivating currents and currents recovering from inactivation were fitted to a single- or a double-exponential function using a least squares algorithm on pCLAMP software, and deactivating currents were fitted to a double-exponential function on Origin software.

Steady-state inactivation was analyzed as described previously. Briefly, the corrected steady-state inactivation (see Figure 5F) curves were fitted with a Boltzmann function in the following form: $I(I_{max} - I_{min}) = 1/[1 + \exp(V_{test} - V_{1/2})/k]$, where $I$ is the amplitude of inactivating current corrected for deactivation, $I_{max}$ is the maximum of $I$, $V_{min}$ is the minimum of $I$, $V_{1/2}$ is the prepulse of test potential, and $V_{test}$ is the voltage at which $I$ is half of $I_{max}$, and $k$ is the slope factor.

All average values are expressed as SEM. Multiple comparisons among groups were performed by ANOVA with the Tukey-Kramer method (SAS version 6, GLM procedure with Tukey option). A value of P<0.05 was considered significant.

**Results**

**Concentration-Dependent Current Expression by Injection of WT cRNA**

First, in order to make quantitative analysis feasible, we determined the range where expressed current amplitude and the amount of cRNA injected exhibited a linear relationship.

**Figure 1. Schematic representation of sites of naturally occurring LQT2 mutations in HERG and artificial mutations that affect HERG inactivation.** 

© indicates the mutations that have been reported in LQT2; ○, positions 620 and 631, which affect the rate of inactivation. Mutations that we studied are indicated by the box surrounding the amino acid substitution. Mutations for which electrophysiological studies have been performed are indicated in italics.
For this purpose, various amounts of WT cRNAs were injected into *Xenopus* oocytes, and amplitudes of expressed currents were compared. Even when the same amount of cRNA was injected, expressed current amplitude differed substantially among different batches of oocytes. Thus, comparisons of quantitative data were performed using data obtained from the same batches of oocytes in the analyses. Similar to the report by Sanguinetti et al., the current amplitude of oocytes in which 3.0 ng WT cRNA was injected was roughly twice as large as that of oocytes in which 1.5 ng cRNA was injected (Figure 2). Voltage dependence of activation was not different between the currents recorded from oocytes injected with 1.5 and 3.0 ng cRNA. Therefore, using those amounts of cRNA, we performed the following experiments of quantitative analysis.

**Currents Were Not Expressed by Injection of Mutant cRNA Alone**

We injected various amounts of T474I cRNA, A614V cRNA, or V630L cRNAs into *Xenopus* oocytes. Although we injected up to 20 ng of each cRNA into *Xenopus* oocytes, amplitudes of membrane currents in any of these preparations were not different from those in H2O-injected oocytes (Figure 3).

**Expressed Currents by Coinjection of WT Plus Mutant cRNA**

Romano-Ward syndrome is an autosomal-dominant form of LQTS, and in LQT2, one allele contains normal HERG and the other allele has mutant HERG. Thus, we injected the same amounts (1.5 ng) of WT and each mutant HERG cRNA together into oocytes and examined the characteristics of the expressed current. The current amplitudes of each coinjected oocyte were compared with those with 1.5 ng WT cRNA alone (Figure 4). The current-voltage (I-V) relationships during test depolarization showed a bell shape with a current peak between −20 and −10 mV in WT alone. The I-V curves recorded from oocytes coinjected with WT and each of 3 mutants were also bell-shaped. Voltage at peak amplitude was slightly shifted (<5 mV) to negative potentials in oocytes co injected with WT cRNA plus A614V cRNA and those with WT cRNA plus V630L cRNA (Figure 4E). The amplitude of steady-state currents measured at depolarization pulses to −20 mV was 813 ± 670 nA (n = 8) in oocytes injected with 1.5 ng T474I cRNA plus 1.5 ng WT cRNA (T474I/WT). The value was 489 ± 44 nA (n = 10) with 1.5 ng A614V cRNA plus 1.5 ng WT cRNA (A614V/WT). It was 156 ± 16 nA (n = 10) with 1.5 ng V630L cRNA plus 1.5 ng WT cRNA (V630L/WT). All 3 values were significantly smaller than...
that with 1.5 ng WT cRNA (WT1.5) alone (1134±78 nA) (n=10), and the order of current amplitude was WT1.5>T474I/WT>A614V/WT>V630L/WT (Figure 4 and Table). The amplitude of the tail currents measured at −70 mV after a depolarizing test pulse to +20 mV was 993±68 nA (n=8) for T474I/WT, 710±49 (n=10) for A614V/WT, and 252±22 (n=10) for V630L/WT. All 3 values were significantly smaller than that in oocytes injected with WT alone (1285±77 nA) (n=10) (Figure 4 and Table). Despite the same amount of WT cRNA (1.5 ng) injected, the current amplitudes during depolarizing pulses and tail currents were smaller than those of WT cRNA alone when each of the mutant cRNAs (1.5 ng) was co-injected with WT cRNA. The activation curves obtained from the tail current amplitude on repolarization to −70 mV from test potentials are shown in Figure 4F. The half-activation voltage, \( V_{1/2} \), was not different among WT and WT with 3 different mutants. The slope factor, however, was slightly smaller in A614V/WT and V630L/WT than in WT alone (Figure 4F and Table). These data suggest that all 3 mutants suppress HERG channel currents in a dominant-negative manner.

**Conductance and Rectification Properties**

To delineate the underlying mechanisms for HERG current suppression in these mutants, we examined slope conductances and rectification properties of expressed currents. For this purpose, we studied the fully activated \( I-V \) relationships by applying various test potentials after a depolarizing conditioning pulse (Figure 5). The slope conductance of expressed currents was measured as a slope of the \( I-V \) curves between −130 and −110 mV. The value of the slope conductance was 44.1±3.7 \( \mu S \) (n=8) for T474I/WT, 32.2±1.2 \( \mu S \) (n=8) for A614V/WT, and 30.4±2.1 \( \mu S \) (n=9) for V630L/WT. All 3 values were significantly smaller than that of WT 1.5 (56±3.4 \( \mu S \) ) (n=8) (Table).

\( I-V \) curves showed inward rectification properties for WT1.5 and WT plus each of the 3 mutants, and the magnitude of inward rectification was apparently stronger for A614V/WT and V630L/WT than for WT1.5 or T474I/WT (Figure 5). Inward rectification is suggested to be a reflection of reduced channel availability at depolarized potential compared with hyperpolarized potentials, and channel availability can be assessed by examining steady-state inactivation.23 Thus, we examined steady-state inactivation using a dual-pulse protocol as described previously.22 The steady-state inactivation for A614V/WT was shifted in its voltage dependence to a negative potential to −96 mV and that for V630L/WT was shifted to −109 mV compared with that for WT1.5 (−87 mV) (Figure 5F and Table). The slope factor of steady-state inactivation was also augmented slightly for

| Parameters of Activation and Steady-State Inactivation in Currents Expressing WT HERG and Coexpressing WT Plus Each Mutant HERG |
|----------------------|----------------------|----------------------|----------------------|
|                      | Activation           | S-S Inactivation     |
|                      | Test, nA | Tail, nA | \( V_{1/2} \), mV | \( k \), mV | SC, \( \mu S \) | Test, nA | Tail, nA | \( V_{1/2} \), mV | \( k \), mV |
| WT1.5                | 1134±78  | 1285±77  | −32.4±1.1   | 9.6±0.4  | (n=10) | 56.3±3.6     | −87.0±3.7  | 27.3±1.3  | (n=8) |
| T474I/WT             | 813±70*  | 993±68*  | −29.3±0.3  | 9.7±0.3  | (n=8)  | 44.1±3.7*   | −89.7±2.6  | 28.5±1.2  | (n=7) |
| A614V/WT             | 489±44†  | 710±49†  | −29.3±0.8† | 7.9±0.2†| (n=10) | 32.2±1.2†   | −96.3±2.9† | 27.6±0.8† | (n=7) |
| V630L/WT             | 156±16*† | 252±22*† | −31.8±0.9  | 7.0±0.1* | (n=10) | 30.4±2.1*† | −109.2±1.7*†| 30.3±0.5*‡| (n=6) |

*Test indicates amplitude of steady-state current measured at depolarizing test pulse to −20 mV; tail, amplitude of tail current measured at −70 mV after depolarizing test pulse to +20 mV; SC, slope conductance; S-S, steady-state; WT1.5, oocytes injected with 1.5 ng WT cRNA; T474I/WT, oocytes co-injected with 1.5 ng T474I cRNA and 1.5 ng WT cRNA; A614V/WT, oocytes co-injected with 1.5 ng A614V cRNA and 1.5 ng WT cRNA; and V630L/WT, oocytes co-injected with 1.5 ng V630L cRNA and 1.5 ng WT cRNA.

\*P<0.05, T474I/WT, A614V/WT, and V630L/WT vs WT1.5; †P<0.05, A614V/WT and V630L/WT vs T474I/WT; and ‡P<0.05, V630L/WT vs A614V/WT.
V630L/WT compared with WT1.5 (27.3 mV for WT1.5 versus 30.3 mV for V630L/WT) (Figure 5F and Table). Thus, at the same depolarized potential, channel availability was diminished for A614V/WT and V630L/WT, resulting in enhanced inward rectification.

Inactivation, Recovery From Inactivation, and Deactivation

The inactivation time course of expressed currents was analyzed by applying brief hyperpolarizing pulses to allow the HERG channel to recover from inactivation after an initial long depolarizing pulse, and then depolarizing test pulses were applied to record inactivating currents (Figure 6A). The time course of fast inactivating currents could be fitted by a single-exponential function. Recovery from inactivation was measured using the same pulse protocol shown in Figure 5D. Recovery from inactivation was observed as the time-dependent initial increase in current amplitude at potentials between −50 and −130 mV. Tail currents could be fitted by a double-exponential function, and the fast component was defined as the time constant of recovery from inactivation. For V630L/WT, the time constants for inactivation and recovery from inactivation were significantly decreased at all potentials, whereas those for T474I/WT or A614V/WT were not altered compared with those of WT1.5 (Figure 6B).

Figure 6. Time courses of inactivation and recovery from inactivation of expressed currents in oocytes coinjected with WT plus each mutant cRNA. A, To examine the inactivation time course, a conditioning pulse to +40 mV for 900 milliseconds from a holding potential of −80 mV was followed by a hyperpolarizing pulse to 100 mV for 15 milliseconds, and subsequent depolarizing test pulses between −40 and +40 mV in 10-mV steps were applied. The inset illustrates the voltage protocol. Representative current recordings were those in oocytes injected with 1.5 ng WT cRNA. Inactivation time constants (τ) were measured by fitting inactivating currents during test pulses at each potential with a single-exponential function. B, τ values representing inactivation time constants and time constants of recovery from inactivation for expressed currents in oocytes injected with WT1.5, T474I/WT, A614V/WT, and V630L/WT were plotted as a function of test potential (Vt). Recovery from inactivation was measured using the same pulse protocol shown in Figure 4D. Tail currents between −130 mV and −50 mV in 10-mV increments could be fitted by a double-exponential function, and the fast component of time constants was defined as a time constant of recovery from inactivation. Since expressed currents at potentials near the reversal potential could not be accurately fitted by exponential function, time constants at −100 and −90 mV were omitted. *P<0.05 for time constant between V630L/WT and WT1.5.
To analyze the deactivation time course, long hyperpolarizing test pulses were applied after a depolarizing conditioning pulse (Figure 7A). Deactivating currents during test pulses could be fitted to a double-exponential function. At all test potentials, neither fast nor slow time constants of deactivation for T474I/WT, A614V/WT, or V630L/WT were different from those for WT1.5 (Figure 7B and 7C).

**Ion Permeability**

Among the 3 mutants we studied, A614V and V630L are mutations in the P region of the HERG channel. Thus, we examined whether the ion permeability of expressed currents was altered in oocytes injected with A614V/WT or V630L/WT. The permeability of $K^+$ relative to $Na^+$ was evaluated by measuring the reversal potential of expressed currents in oocytes bathed in solution containing different concentrations of $K^+$ (2, 5, and 10 mmol/L) with a supplement of $Na^+$ (Figure 8). Although the reversal potentials at each [K$^+$] showed a slight positive shift (<5 mV) in oocytes injected with A614V/WT or V630L/WT compared with WT1.5, the slope of the reversal potential versus [K$^+$] and [Na$^+$], was not much different from that of WT1.5 (52.7±0.7 mV for WT1.5 versus 52.5±0.8 mV for T474I/WT, 52.3±0.8 mV for A614V/WT, and 51.7±1.2 mV for V630L/WT) (Figure 8). This indicates that the permeability of $K^+$ relative to $Na^+$ in the expressed currents with T474I/WT, A614V/WT, or V630L/WT was not much different from that with WT1.5.

**Discussion**

We characterized novel missense mutations (T474I, A614V, and V630L) found in Japanese LQT2 families using a heterologous expression system in *Xenopus* oocytes. In oocytes injected with T474I, A614V, or V630L cRNA alone, recorded currents were not any larger than currents recorded in H$_2$O-injected oocytes. At least 3 potential explanations for this finding can be given. Homomultimers formed from each of the mutant subunits could not be properly targeted to plasma membrane, or they could be targeted to the plasma membrane but failed to operate as a functional channel. The third possible explanation is that mutant subunits could not coassemble, thereby failing to form homotetramers.

Injection of each mutant cRNA together with WT cRNA resulted in dominant-negative suppression, in agreement with the data by Sanguinetti et al.18 Severity of suppression of channel function varied among different mutants. When it was assessed by amplitude of outward currents at positive potentials, the order was WT1.5>T474I/WT>A614V/WT>V630L/WT. In order to clarify the underlying mechanism for dominant-negative suppression and to explain the different levels of severity of suppression among the 3 mutants, we examined slope conductances, inward rectification properties, and kinetics of expressed currents in oocytes in which mutant and WT cRNA had been injected. Slope conductance values were significantly lower for all 3 types of coinjected oocytes than for WT1.5. These findings may indicate that heterotetrameric channels have either lower single-channel conductance or fewer numbers of functional channels, which may be due to less effective membrane targeting or greater susceptibility to protein degradation than WT1.5.
in WT1.5. To differentiate these 2 possibilities, recording of single-channel current and/or quantification of subunits properly targeted to plasma membrane by Western blotting may be required.

Since for T474I/WT the reduction in slope conductance was the only affected property, dominant-negative suppression in this mutant was exclusively explained by this mechanism. For A614V/WT and V630L/WT, in addition to reduction in slope conductance, the voltage dependence of the steady-state inactivation was shifted to negative potentials (−96 mV for A614V/WT and −109 mV for V630L/WT compared with −87 mV for WT1.5). For V630L/WT, the slope factor for the steady-state inactivation was also slightly augmented. The degree of inward rectification of the HERG channel can be determined by the availability of channel opening at each membrane potential,11,23,25,26 and channel availability can be assessed by the degree of steady-state inactivation.23,26,28,29 Substitution of 631 Ser (serine at position 631) to cysteine (Cys) accelerated the rate of inactivation, and substitution of 631 Ser to alanine (Ala) and a double substitution of 628Gly (glycine at position 628) to Cys and 631 Ser to Cys completely abolished HERG inactivation.23,26 HERG channel inactivation shows several unique properties different from classical C-type inactivation in the Shaker B channel. Importantly, HERG inactivation is voltage dependent and is stronger at more depolarized potentials, which gives an inward rectifying property to the HERG channel.23,24,30 The data showing that the mutation at 630 Val strongly altered the voltage dependence of HERG inactivation confirm that the region from 629Gly to 631Ser may be a part of the inactivation gate and suggest that the residue 630 Val may be somehow related to the voltage dependence of HERG inactivation. A recent study31 reported that substitutions of Ser at position 620 to threonine or Cys, which is considered to be located in deeper parts of the pore, interfered with C-type inactivation; thus, further and more systematic approaches are required to clarify the molecular basis providing the voltage dependence to the HERG inactivation.

Acknowledgments
We thank Gail A. Robertson (University of Wisconsin, Madison) and K. Hirai for helpful discussion, J.C. Makielski (University of Wisconsin, Madison) for reading and commenting our manuscript, T. Nakayama for statistical analyses, T. Ogura and T. Terai for technical assistance, and Y. Sugamoto for data analyses.

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Circ Res. 1998;83:415-422
doi: 10.1161/01.RES.83.4.415

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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