Potassium channels play important roles in vital cellular signaling processes such as cardiac rhythm. The function of ion channels is determined by a combination of channel gating (opening versus closing) and channel numbers (channel density) in the plasma membrane. Although the function and modulation of preexisting ion channels have been extensively studied, information about the stability of ion channels in the plasma membrane is limited. In particular, it is not known whether and how the gating states of a channel affect the channel’s stability in the plasma membrane.

The rapidly activating delayed rectifier K⁺ channel (I_{Kr}) is important for repolarization of cardiac action potentials and is encoded by the human ether-a-go-go-related gene (HERG). Reduction of I_{Kr}, attributable to mutations in HERG or drug block, slows repolarization and causes long QT syndrome (LQTS), a cardiac electric disorder that predisposes affected individuals to the potentially lethal arrhythmia, torsades de pointes. A decrease in serum K⁺ concentration (hypokalemia) is known to precipitate LQTS. A reduction in extracellular K⁺ concentration ([K⁺]o) acutely decreases the amplitude of I_{Kr} in cardiomyocytes and the HERG current (I_{HERG}) in expression systems. Although this low [K⁺]o-induced reduction in I_{Kr} has previously been attributed to accelerated inactivation gating, a quantitative analysis suggested that modulation of inactivation is a minimal component for low [K⁺]o-induced I_{HERG} reduction. We previously demonstrated that a reduction in [K⁺]o chronically decreases the cell surface density of I_{Kr} in rabbit ventricular myocytes and of the HERG channel stably expressed in human embryonic kidney (HEK) cells. We showed that when HERG-expressing HEK cells were cultured at different [K⁺]o for a week, [K⁺]o regulated I_{HERG} with an EC_{50} of 2.1 mmol/L. We also showed that feeding rabbits with a low K⁺-diet reduced serum [K⁺] to 2.4 mmol/L (from 4.7 mmol/L), prolonged QT intervals by 33% and reduced I_{Kr} expression levels in ventricular myocytes. Hypokalemia is clinically defined as a serum [K⁺] below 3.5 mmol/L and has been reported in humans at levels as low as 1.2 mmol/L. Thus, the low [K⁺]o-induced reduction in HERG expression levels is clinically relevant. However, it is unknown how cells sense [K⁺]o and regulate expression levels of HERG channels in the plasma membrane in response to [K⁺]o. In the present study, we demonstrate that the presence of extracellular K⁺ ([K⁺]o) is
a prerequisite for both the function and stability of HERG channels in the plasma membrane. Under low $K^{+}_o$ conditions HERG channels entered a novel nonconducting state, which is different from either the voltage dependent inactivated state or drug-blockade state. Subsequently, the nonconducting channels were internalization and degraded. Using permeant cations and site-directed mutagenesis, we identified the amino acid residues located in the pore region of the HERG channel which are involved in the $K^{+}_o$ dependence of both the function and membrane stability of the channel. Identification of the link between a gating state and the membrane stability of a voltage-gated channel, HERG, extends our understanding of ion channel function and regulation.

Methods

Molecular Biology

HERG cDNA was obtained from Dr Gail A. Robertson (University of Wisconsin, Madison). Point mutations of HERG were generated using the overlap extension PCR technique. A HEK 293 cell line stably expressing WT HERG channels (HERG-HEK cells) was obtained from Dr Craig January (University of Wisconsin-Madison). Green fluorescent protein (GFP)-tagged HERG (HERG-GFP) in pEGFP-N2 vector was obtained from Dr Zhengfeng Zhou (Oregon Health and Science University). The mutant HERG cDNA was transfected to HEK 293 cells using Lipofectamine 2000 (Invitrogen). Stable cell lines were generated using G418 for selection and cells were cultured in Minimum Essential Medium (MEM) (Invitrogen) supplemented with 10% FBS and 200 μg/ml G418 (Sigma-Aldrich). For patch experiments with transiently expressed channels, GFP cDNA (pIRE2-EGFP, Clontech, Mountain View, Calif) was cotransfected for selection of transfected cells. A custom 0 mmol/L $K^+$ MEM (Invitrogen) was used as a baseline medium, which contains identical components as the regular MEM except $K^+$.

Patch Clamp Recording Method

The whole-cell patch clamp method was used to record the HERG current ($I_{HERG}$). The pipette solution contained (in mmol/L) 135 KCl, 5 EGTA, 1 MgCl$_2$, and 10 HEPES (pH 7.2 with KOH). A Tyrode’s solution was used as the standard bath solution which contained (in mmol/L) 135 NaCl, 5 KCl, 10 HEPES, 10 glucose, 1 MgCl$_2$, and 2 CaCl$_2$ (pH 7.4 with NaOH). The 0 mmol/L $K^+$ bath solution contained otherwise identical compositions but no KCl. Cells were superfused in a <0.5-mL chamber mounted on an inverted microscope (Ti2000, Nikon Canada). Solutions were changed by switching the perfusates at the inlet of the chamber. A complete change of bath solutions was achieved within 5 seconds to the perfusion upstream of the chamber, whereas the bath solution was sucked away from the downstream accordingly. All patch clamp experiments were performed at room temperature (22±1°C).

Western Blot Analysis

Whole-cell lysates from HERG-expressing HEK 293 cells after various treatments were used for Western blot analysis as we showed previously.

Immunofluorescence Microscopy

To study HERG internalization, cell surface HERG channels were labeled with an anti-Kv11.1 antibody (Sigma) for 20 minutes at room temperature (22±1°C). Unbound antibody was then washed away. The cells were then cultured and fixed with 4% ice-cold paraformaldehyde for 15 minutes, permeabilized with 0.2% Triton X-100, and blocked with 5% BSA in PBS. The labeled HERG were stained with Alexa fluor 488-conjugated secondary antibody. For membrane staining, the cells were incubated with Alexa flor 594–conjugated wheat germ agglutinin (2.5 μg/mL, Invitrogen) for 1 minute at room temperature. Images were acquired using a Leica TCS SP2 Multi Photon confocal microscope.

All data are expressed as the means±SEM. A 1-way ANOVA or 2-tailed Student t test was used to determine statistical significance between the control and test groups. A probability value of 0.05 or less was considered significant.

Results

Extracellular $K^+$ Is Required for HERG Channel Function

Chronic reduction in $[K^+]_o$, decreases the expression level of plasma membrane HERG channels. We hypothesized that $K^+_o$ is required for the HERG channel to maintain its proper conformation for functioning and membrane stability. Thus, removal of $K^+_o$ would lead the channel to an altered state that is not functional and unstable in the plasma membrane. To test this notion, we examined the acute effects of $K^+_o$ removal on the activity of HERG channels stably expressed in HEK 293. The HERG current ($I_{HERG}$) was elicited by a depolarizing step to 50 mV, followed by a repolarizing step to −50 mV. As shown in Figure 1A, changing $[K^+]_o$ from 5 to 0 mmol/L reduced $I_{HERG}$ in a frequency-dependent manner. $I_{HERG}$ was reduced by 46±7% with a time constant of 169±11 seconds (n=7) when the pulse interval was 5 s, and $I_{HERG}$ was reduced by 83±3% with a time constant of 45±9 seconds (n=10) when the pulse interval was 15 seconds. Furthermore, when the pulse interval was 60 s, $I_{HERG}$ was completely eliminated after 2 pulses (n=10).

To understand the nature of the nonconducting state of HERG channels induced by 0 mmol/L $K^+_o$, we studied $I_{HERG}$ recovery after re-exposing cells to the 5 mmol/L $K^+$. The HERG-HEK cells were exposed to 0 mmol/L $K^+$ for 10 minutes, and then $I_{HERG}$ recordings were started in 0 mmol/L $K^+$ with a pulse interval of 15 seconds. Changing bath solution to 5 mmol/L $K^+$ Tyrode’s solution led to a recovery of $I_{HERG}$ with a single exponential time constant of
207.8±7.7 seconds (n=6, Figure 1B). It took 6 minutes for \( I_{\text{HERG}} \) to recover to a level similar to \( I_{\text{HERG}} \) in control cells exposed to 5 mmol/L K\(^+\) (1.37±0.06 nA, n=6; versus 1.52±0.12 nA in control, n=7, \( P > 0.05 \), Figure 1B). This slow recovery from the nonconducting state in 5 mmol/L K\(^+\) indicates that after K\(^+\) removal HERG channels must have experienced a substantial conformational change which is different from the fast, voltage-dependent inactivation of the channel.

From the data in Figure 1, we conclude that the loss of HERG channel conductivity within minutes of exposure to 0 mmol/L K\(^+\) solution is attributable to the entry of membrane-resident channels into a reversible nonconducting state. The inverse dependence of entry into this state on frequency of channel activation implies that K\(^+\) efflux through the channel prevents entry of the channel into the nonconducting state.\(^1^6\)

Nonconducting State Leads to Degradation of the Cell Surface HERG Channels

We hypothesized that entry into the nonconducting state on K\(^+\)\(_{\text{o}}\) removal destabilizes HERG channels in the plasma membrane. To test this hypothesis, we investigated the correlation between the entry into the nonconducting state and the reduction in expression levels of mature HERG channels in 0 mmol/L K\(^+\)\(_{\text{o}}\). Exposure of HERG-HEK cells to 0 mmol/L K\(^+\)\(_{\text{o}}\) MEM for 10 minutes or more completely eliminated \( I_{\text{HERG}} \) (Figure 2A). On the other hand, reexposure of these cells to 5 mmol/L K\(^+\) Tyrode’s solution resulted in recovery of \( I_{\text{HERG}} \) from the nonconducting state of the channel. Nevertheless, exposure to 0 mmol/L K\(^+\) induced a time-dependent reduction in \( I_{\text{HERG}} \) recorded in the 5 mmol/L K\(^+\) (Figure 2B), which is underlain by the decreased HERG expression levels. HERG from cells cultured in normal MEM displayed 2 bands with the molecular masses of 135- and

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**Figure 1. Acute effects of removal of K\(^+\)\(_{\text{o}}\) on \( I_{\text{HERG}} \).**

**A**, Frequency-dependent reduction in \( I_{\text{HERG}} \) after cell exposure to 0 mmol/L K\(^+\) solution. **Top**, Superimposed \( I_{\text{HERG}} \) traces recorded at a pulse interval of 5, 15, or 60 seconds after changing K\(^+\) from 5 to 0 mmol/L. **Bottom**, Time-dependent reduction of \( I_{\text{HERG}} \) in 0 mmol/L K\(^+\) at different pulsing frequencies. The amplitudes of HERG tail currents at −50 mV were plotted against the recording time and fitted to a single exponential function. **B**, Recovery of \( I_{\text{HERG}} \) from 0 mmol/L K\(^+\)-induced nonconducting state in 5 mmol/L K\(^+\). The amplitude of \( I_{\text{HERG}} \) was plotted as a function of time and fitted to a single exponential function.

**Figure 2. Extracellular K\(^+\) is required for the function and plasma membrane expression of HERG channels.**

**A**, The time-dependent effects of exposure to 0 mmol/L K\(^+\) \(_{\text{MEM}}\) on \( I_{\text{HERG}} \) recorded in 0 mmol/L K\(^+\) \(_{\text{MEM}}\). **B**, The time-dependent effects of exposure to 0 mmol/L K\(^+\) \(_{\text{MEM}}\) on \( I_{\text{HERG}} \) recorded in 5 mmol/L K\(^+\). **C**, The time-dependent effects of exposure to 5 or 0 mmol/L K\(^+\) \(_{\text{MEM}}\) on HERG protein expression levels. **D**, The time courses of 0 mmol/L K\(^+\) exposure–induced reductions of \( I_{\text{HERG}} \) recorded in 0 or 5 mmol/L K\(^+\) Tyrode’s solution and of the 155-kDa band density of HERG proteins. The intensity of the 155-kDa band at each time point was normalized to the initial value and plotted as a function of time.
they were progressively internalized in 0 mmol/L K\(^+\) MEM. Addition of 5 mmol/L Rb\(^+\) to the 0 mmol/L K\(^+\) MEM effectively prevented HERG internalization. Similar results were observed in each set of 5 independent experiments.

155-kDa (Figure 2C). The 155-kDa band represents the mature, fully glycosylated form of functional HERG channels in the plasma membrane, and the 135-kDa band represents the immature, core-glycosylated form residing in the endoplasmic reticulum.\(^{14,17}\) Exposure of HERG-HEK cells to 0 mmol/L K\(^+\) MEM resulted in a time-dependent reduction in the 155-kDa band density (Figure 2C) which paralleled the reduction in \(I_{\text{HERG}}\) (Figure 2D).

To further investigate the correlation between the nonconducting state and the plasma membrane instability of HERG channels and to gain insight into the role of K\(^+\) in HERG function and its stability in the plasma membrane, we studied the effects of permeant (eg, Rb\(^+\) and Cs\(^+\)) and nonpermeant (eg, Li\(^+\)) cations on HERG channels. We used 0 mmol/L K\(^+\) MEM containing 140 mmol/L Na\(^+\) as a baseline solution and added 5 mmol/L K\(^+\), Rb\(^+\), Cs\(^+\), or Li\(^+\) to it to examine the role of these cations in maintaining HERG channel function. Under normal conditions, Na\(^+\) does not permeate through HERG channels. A ratio of Na\(^+\) to K\(^+\) permeability (\(P_{\text{Na}}/P_{\text{K}}\)) of 0.008 has previously been reported.\(^{14}\) Unlike many other K\(^+\) channels, the HERG channel allows Cs\(^+\) to permeate with a \(P_{\text{Cs}}/P_{\text{K}}\) of 0.36.\(^{18}\) Using a biionic strategy\(^{18}\) of recording reversal potentials with 135 mmol/L K\(^+\) present in the pipette solution and 135 mmol/L Rb\(^+\) or Li\(^+\) present in the bath solution in whole-cell recordings, we determined a \(P_{\text{Rb}}/P_{\text{K}}\) of 0.92±0.05 (n=6), and a \(P_{\text{Li}}/P_{\text{K}}\) of 0.009±0.001 (n=7). Thus, Rb\(^+\) and Cs\(^+\), but not Li\(^+\), permeate through HERG channels. Figure 3A (top row) shows \(I_{\text{HERG}}\) recorded in the indicated incubation solutions. Whereas a 10-minute exposure to 0 mmol/L K\(^+\) MEM completely eliminated \(I_{\text{HERG}}\), addition of either 5 mmol/L Rb\(^+\) or Cs\(^+\) to the medium maintained \(I_{\text{HERG}}\) to an extent similar to 5 mmol/L K\(^+\). On the other hand, addition of 5 mmol/L Li\(^+\) did not maintain \(I_{\text{HERG}}\) (Figure 3A, top row). Thus, permeant cations, but not nonpermeant cations, effectively prevented HERG channels from entering nonconducting state. To determine the relationship between the nonconducting state and membrane instability, we cultured cells in various conditions for 6 hours. We then recorded \(I_{\text{HERG}}\) in 5 mmol/L K\(^+\) (Figure 3A, middle row). Compared to the exposure of cells to 5 mmol/L K\(^+\) MEM, exposure to 0 mmol/L K\(^+\) or 5 mmol/L Li\(^+\) MEM for 6 hours eliminated \(I_{\text{HERG}}\). In contrast, exposure to 5 mmol/L Rb\(^+\) for 6 hours did not reduce \(I_{\text{HERG}}\) at all, and exposure to 5 mmol/L Cs\(^+\) only reduced \(I_{\text{HERG}}\) by 19±4% (Figure 3A, lower row). We also examined HERG expression levels in cells cultured with various cations solutions. As shown in Figure 3B, HERG from cells cultured in normal (5 mmol/L K\(^+\)) MEM displayed 2 bands, the 155- and 135-kDa bands.\(^{14,17}\) Exposure to 0 mmol/L K\(^+\) MEM for 6 hours drastically decreased the intensity of the mature form of HERG proteins (155-kDa band). Addition of 5 mmol/L Rb\(^+\) or Cs\(^+\), but not Li\(^+\), effectively prevented the 0 mmol/L K\(^+\) exposure–induced reduction in the intensity of 155-kDa HERG band (Figure 3B). Thus, like K\(^+\), both Rb\(^+\) and Cs\(^+\) prevented HERG channels from entering nonconducting state and preserved HERG expression levels.

To further investigate the ability of permeant ions to maintaining HERG stability in the plasma membrane, we treated live HERG-HEK cells with an anti-HERG antibody targeting an extracellular domain of the HERG channel. The culture media were then changed to antibody-free, 5 mmol/L K\(^+\) MEM, 0 mmol/L K\(^+\) MEM, or 0 mmol/L K\(^+\)
plus 5 mmol/L Rb⁺-MEM. After various periods of culture, cells were permeabilized and incubated with Alexa fluor 488–conjugated (green) secondary antibody to observe the fate of the surface-labeled HERG channels. Compared to cells cultured in 5 mmol/L K⁺-MEM, those in 0 mmol/L K⁺-MEM displayed accelerated internalization and degradation of HERG channels. After 4 hours of culture in 0 mmol/L K⁺-MEM, HERG channels were absent from the plasma membrane (Figure 3C). However, 5 mmol/L Rb⁺ effectively maintained HERG channels in the plasma membrane (Figure 3C).

**Amino Acid Residues in the Pore Region Are Involved in the K⁺o Dependence of the HERG Channel**

The fact that permeant cations (eg, K⁺, Rb⁺, and Cs⁺) maintain HERG integrity suggests that the K⁺-interacting site(s) is located in the channel permeation pathway. We used point mutations to investigate the site(s) in HERG that are involved in the K⁺o dependence of HERG function and membrane stability. We examined the link between the nonconducting state and the membrane instability in mutant channels on exposure to 0 mmol/L K⁺o. Based on the assumed homology with the solved crystal structure of the bacterial KcsA K⁺ channel,19 we used an alanine-scanning strategy to generate point mutations in the pore helix and selectivity filter of the HERG channel (Figure 4A and 4B). In addition, to exclude the possibility that extracellular cations may interact with the negatively charged amino acid residues in the uniquely long extracellular S5-pore linker that forms the turret of the HERG channel, we mutated all 4 negatively charged amino acid residues (E575A, D580A, D591A, and D609A). Two aromatic residues in S6 (Y652 and F656) are the targets for high affinity binding of various drugs to HERG channels.20 The S641 in S6 participates in HERG inactivating gating.21 Thus, we made mutant channels of S641A, Y652A, and F656T in S6 and studied the effects of these mutations on the K⁺o dependency of the channel (Figure 4). Because some mutant channels did not express current, alternative mutations were used for analysis. Removal of negatively charged amino acid residues in the S5-P linker (E575A, D580A, D591A, and D609A) did not affect the channel K⁺o dependency (Figure 4). As well, mutations in S6 such as S641A, Y652A, and F656T did not alter the K⁺o dependency of the channel (Figure 4). Specifically, similar to WT channels, on exposure to 0 mmol/L K⁺o, these mutant channels became nonconductive, and after overnight culture in 0 mmol/L K⁺-MEM, no current could be recorded in the 5 mmol/L K⁺ bath solution. In contrast, point mutations in the pore helix and selectivity filter regions such as T618A, S621A, S624A, F627Y, N629D, and V630A effectively eliminated the overnight 0 mmol/L K⁺o exposure–induced reduction in IHERG (Figure 4). We also examined the S624T mutation. The amplitude of the S624T HERG current was also not altered by overnight culture in 0 mmol/L K⁺ (480.9±68.4 pA in 0 mmol/L K⁺, n=22, versus 453.0±58.7 pA in 5 mmol/L K⁺, n=18, P>0.05). Thus, both S624A and

For S620T, N629D, V630A, and S631A, the currents at the end of a depolarizing step to 50 mV were used for measuring the current amplitudes. For the D609A, F617W, T618A, S621A, S623A, and S641A mutant channels, the currents were recorded in a 135 mmol/L K⁺ containing bath solution, and the inward tail currents on the repolarization steps to −80 mV after a 4-second, 50-mV depolarization were used. For each mutant channel, 5 to 11 cells were tested from at least 3 independent experiments. N.E. indicates no expression; T.S., too small current for meaningful analysis. *P<0.05 or **P<0.01 compared with the reduction in WT HERG current.

**Figure 4. Effects of point mutations in the HERG channel on the K⁺o dependence of channel function. A, Alignment of HERG and KcsA channels in the S5-P linker, P-loop, and S6 regions. B, Estimated locations of the HERG mutations (red) based on the KcsA model. C, Representative WT and mutant HERG currents recorded in 5 mmol/L K⁺ from cells cultured in 5 or 0 mmol/L K⁺-MEM for 12 hours. The currents were evoked by depolarizing steps to voltages between −70 and +70 mV in 10 mV increments, followed by a repolarizing step to −50 mV. D, Relative reduction of WT or mutant IHERG after overnight culture in 0 mmol/L K⁺-MEM. IHERG from cells cultured in 0 K⁺-MEM was normalized to IHERG cultured in 5 mmol/L K⁺. For most mutant channels, the 5 mmol/L K⁺ bath solution was used and the tail currents at −50 mV were used for measuring the current amplitudes.**
S624T HERG currents did not decrease on exposure to 0 mmol/L K⁺ medium.

There was a direct link between the nonconducting state and plasma membrane instability of HERG channels on exposure to 0 mmol/L K⁺. For example, in contrast to WT HERG channels, the S624A mutant channel current did not decrease after the cells were exposed to 0 mmol/L K⁺ for up to 6 hours (Figure 5A). Consequently, neither the current (Figure 5A) nor intensity of the 155-kDa form of the S624A channel (Figure 5B) decreased after 6 hours of exposure to 0 mmol/L K⁺ MEM. Furthermore, after the cells were cultured in 0 mmol/L K⁺ MEM for 3 hours, the antibody-labeled S624A HERG channels primarily remained in the plasma membrane, whereas the antibody-labeled WT HERG channels were completely internalized (Figure 5C). The correlation between the nonconducting state and membrane instability was also confirmed in the additional mutant channels including D580A, F619M, S624T, F627Y, and Y652A. The mutant channels whose current remained on 0 mmol/L K⁺ exposure were also stable in the plasma membrane after overnight culture in 0 mmol/L K⁺ MEM (Figure 5D and 5E). Thus, under 0 mmol/L K⁺ conditions, the nonconducting state also preceded internalization and degradation of the cell surface mutant HERG channels.

The Nonconducting State of HERG Channels in 0 mmol/L K⁺ Is Not Dependent on the Inactivation Gating of the Channel and Is Different From E4031-Blocked State

To investigate the role of inactivation in the K⁺,o dependence of IHERG, we examined the relationships between mutation-induced changes in inactivation gating and mutation-induced changes in K⁺,o dependence of IHERG. Figure 6A (a) shows the acute effects of exposure to 0 mmol/L K⁺,o on the currents of WT, S624A, F627Y, and S631A HERG channels. Currents were evoked by a depolarizing step to 50 mV followed by a repolarizing step to −50 mV. The pulse interval was 15 seconds. The tail currents of WT, the S624A, and the F627Y were at −50 mV were used for analysis. The maximum currents at 50 mV for S631A were used for analysis. The current of each channel recorded after 5 minutes superfusion in 0 mmol/L K⁺ Tyrode’s solution was normalized to the initial current on each test voltage. The currents were evoked by a 4-second depolarization to 50 mV followed by a 5-second repolarization to −50 mV with a pulse-interval of 15 seconds. E, WT or mutant HERG expression levels from cells cultured in 5 or 0 mmol/L K⁺ MEM for 12 hours. In A through E, each experiment was independently performed 3 to 5 times.

The effects of mutations on HERG inactivation were investigated. The voltage dependence of steady-state inactivation of WT and mutant HERG channels were analyzed. A 500-ms pulse to +50 mV was used to inactivate HERG channels. The cell membrane was then clamped to −100 mV to allow channels to recover from inactivation for 10 ms which does not allow significant channel deactivation. The cell was then clamped to voltages between −60 mV and +130 mV for WT, S624A and S627Y, and to voltages between 0 and 210 mV for the S631A to induce the voltage-dependent inactivation. The ratios of the currents measured at 100 ms to the instantaneous current on each test voltage were plotted and fitted to a Boltzmann function (Figure 6A, c and d). The midpoints of the steady-state inactivation curve (V_half) were −62.4±1.7 mV (n=6) for WT, −47.1±1.3 mV (n=11) for S624A, −178.3±15.3 mV (n=6) for F627Y, and 107.8±0.4 mV (n=5) for S631A HERG channels. The slope factors for these channels were
levels in 5 or 0 mmol/L K<sup>+</sup> and examined the chronic effects of E4031 on HERG expression. We examined the chronic effects of E4031 on HERG expression. We examined the chronic effects of E4031 on HERG expression.

40.5±2.3 mV (n=6) for WT, 45.8±1.9 mV (n=11) for S624A, 69.3±7.9 mV (n=6) for F627Y, and 27.9±0.3 mV (n=5) for S631A. These results are consistent with the previous reports and indicate that compared to WT channels, the voltage dependence of steady-state inactivation is greatly shifted to the hyperpolarized potentials for the F627Y, slightly shifted to the depolarized potentials for the S624A, and drastically shifted to the depolarized potentials for the S631A. If inactivation is responsible for the reduced I<sub>HERG</sub> in low K<sup>+</sup>o, we would expect that mutant channels with a diminished inactivation (depolarized shift of the steady-state inactivation, eg, S631A) would exhibit a reduced sensitivity to 0 mmol/L K<sup>+</sup> exposure, whereas mutant channels with enhanced inactivation (hyperpolarized shift of the steady-state inactivation, eg, F627Y) would display an increased sensitivity to 0 mmol/L K<sup>+</sup> exposure. However, there was no correlation between the modified inactivation gating and the modified K<sup>+</sup> dependency in these mutant channels (Figure 6A). These results indicate that the voltage-dependent HERG inactivation is not responsible for the 0 mmol/L K<sup>+</sup>o exposure–induced diminution of I<sub>HERG</sub>.

A drug-blocked state is not conductive and may also affect the folding of mutant HERG channels. We examined the chronic effects of E4031 on HERG expression levels in 5 or 0 mmol/L K<sup>+</sup>. E4031 blocks HERG with an IC<sub>50</sub> of 7.7 mmol/L. When HERG-HEK cells were cultured in 5 mmol/L K<sup>+</sup> with 5 μmol/L E4031 for 6 hours, both 155- and 135-kDa bands of HERG proteins were present. Thus, E4031-blocked state did not destabilize HERG localization in the plasma membrane. Furthermore, culturing cells in 0 mmol/L K<sup>+</sup> with 5 μmol/L E4031 for 6 hours still eliminated the 155-kDa HERG band and I<sub>HERG</sub> (recorded in 5 mmol/L K<sup>+</sup> with E4031 washout). Therefore, E4031 did not prevent 0 mmol/L K<sup>+</sup> exposure–induced HERG degradation. We propose that 0 mmol/L K<sup>+</sup>o exposure causes HERG channels to enter a nonconducting state which triggers internalization of the channel. To verify that 0 mmol/L K<sup>+</sup>o–induced HERG internalization is a energy-dependent process, HERG internalization was examined at a reduced temperature which is known to impair endocytosis of various membrane receptors. In contrast to cell culture at 37°C, culture of HERG-HEK cells at 22°C in 0 mmol/L K<sup>+</sup> MEM for 6 hours did not reduce the 155-kDa band of HERG proteins or I<sub>HERG</sub> that has recovered from the nonconducting state under the 5 mmol/L K<sup>+</sup> condition (Figure 6B). Thus, a reduced temperature culture at 22°C inhibited the 0 mmol/L K<sup>+</sup>o–induced HERG internalization.

Culture of cells for 6 hours in 0 mmol/L K<sup>+</sup> MEM completely eliminated WT (GFP tagged) HERG current but did not affect the S624T current (Figure 6C). To investigate the stoichiometry required for internalization and degradation of HERG channels in 0 mmol/L K<sup>+</sup>, S624T stable cell line was transfected with GFP plasmid. Only GFP-positive cells were selected for electrophysiological analysis. Culture of cells for 6 hours in 0 mmol/L K<sup>+</sup> MEM completely eliminated the current of the channel presumably formed by WT and S624T subunits (Figure 6C).

**Ubiquitin Modification Is Involved in WT and but Not the S624T Mutant HERG Channels Under Low K<sup>+</sup> Conditions**

Ubiquitin (Ub) plays an essential role in the selective degradation of many membrane proteins. We studied Ub modification of WT or S624T mutant channels. Figure 7A shows confocal images of HERG (green) and Ub (red) distributions in WT or the S624T HERG-expressing HEK cells after 4 hours of culture in 0 mmol/L K<sup>+</sup> MEM. WT HERG channels were essentially internalized and colocalized with Ub. In contrast, S624T HERG channels were...
primarily present in the plasma membrane, and did not colocalize with Ub. The role of Ub in the HERG degradation was investigated by overexpressing Ub in cells stably expressing WT or S624T HERG channels. At 36 hours after transfection, we exposed the Ub-transfected cells to 0 mmol/L K+ for 2 hours. We then examined HERG expression levels in cells with or without Ub overexpression. Overexpression of Ub promoted 0 mmol/L K+-induced reduction of the mature form of WT HERG channels (Figure 7B). However, overexpression of Ub did not lead to any reduction of the S624T mutant channel proteins under the same conditions (Figure 7B). Interestingly, under our experimental conditions, Ub overexpression preferentially reduced the expression levels of the 155-kDa form of WT HERG, because the expression levels of the 135-kDa form of WT HERG and actin were minimally affected (Figure 7B). These data indicate that the mature form of WT HERG channels is particularly susceptible to Ub under 0 mmol/L K+ conditions. We also examined the effects of overexpression of GFP-tagged Ub (GFP-Ub) on the currents from cells stably expressing WT or S624T mutant HERG channels. At 36 hours after transfection of cells with GFP-Ub plasmid or GFP-plasmid as a control, cells were exposed to 0 mmol/L K+ MEM for 2 hours. The cells were then collected and \( I_{HERG} \) was recorded in 5 mmol/L K+. Compared to GFP overexpression (control), GFP-Ub overexpression significantly decreased the WT HERG current but did not affect the S624T HERG current (Figure 7C). These results indicate that at low [K+]o, Ub facilitates the degradation of WT, but not S624T mutant HERG channels.

**Discussion**

Our data demonstrated a link between HERG gating states and its membrane stability. On exposure to 0 mmol/L K+ solutions, HERG channels entered a nonconducting state within minutes. Subsequently, nonconducting channels were completely internalized within 4 hours. The HERG channel is extremely sensitive to extracellular K+ (Figures 1 and 2). At the resting state, closure of the activation gate, which is located between the selectivity filter and the cytoplasm,27,28 effectively prevents intracellular K+ from accessing the permeation pathway. However, channel opening would allow intracellular K+ to reach the regulatory site(s). More frequent opening would lead to more K+ accumulation at the site, which explains the frequency-dependent reduction of \( I_{HERG} \) in 0 mmol/L K+ (Figure 1).

The 0 mmol/L K+-induced nonconducting state is different from the voltage-dependent inactivation state. First, there was no correlation between the mutation-induced changes of the steady-state inactivation and the mutation-induced changes of the K+ dependence of \( I_{HERG} \) (Figure 6A). Second, after reexposure to 5 mmol/L K+ solution, recovery from 0 mmol/L K+-induced nonconducting state was slow and is not consistent with HERG recovery from inactivation (Figure 1B). Furthermore, the 0 mmol/L K+--induced nonconducting state destabilized HERG plasma membrane localization. The 0 mmol/L K+-induced nonconducting state is different from the E4031-blocked state; E4031 did not destabilize HERG plasma membrane localization. E4031 also did not affect 0 mmol/L K+-induced HERG degradation. On the other hand, a reduced temperature culture (22°C) of cells pre-
vented 0 mmol/L K\textsuperscript{o} induced HERG degradation. A reduced temperature culture inhibits energy-dependent endocytic process.\textsuperscript{25} We believe the nonconducting state induced by 0 mmol/L K\textsuperscript{o} triggers an energy-dependent endocytic process of the channel.\textsuperscript{25} Because HERG channels still entered a nonconducting state at room temperature (22°C) in 0 mmol/L K\textsuperscript{o} during the whole-cell patch clamp experiments (Figure 1A), culturing at 22°C likely inhibited the energy-dependent endocytic process of the channel.

We propose that the HERG channel requires K\textsuperscript{o} occupancy to the external pore mouth to maintain proper conformation for function and membrane stability. The HERG channels lacking K\textsuperscript{o} occupancy undergo a conformational change that triggers channel internalization and degradation via Ub modification (Figure 8). The nonconducting state on exposure to 0 mmol/L K\textsuperscript{o} reflects such a conformational change. Like K\textsuperscript{o}, permeant ion Rb\textsuperscript{+} or Cs\textsuperscript{+}, but not nonpermeant ion Na\textsuperscript{+} or Li\textsuperscript{+}, stabilizes HERG channels and thus prevents HERG from entering the nonconducting/unstable conformation (Figure 3). The direct link between permeability and the ability to maintain HERG membrane stability for external permeant cations (K\textsuperscript{o}, Rb\textsuperscript{+}, and Cs\textsuperscript{+}) suggests that permeant ions interact with site(s) in the channel permeation pathway to stabilize HERG channels. Indeed, point mutations in the selectivity filter and pore helix eliminated the K\textsuperscript{o} requirement for HERG channel function and membrane stability (Figures 4 and 5).

The K\textsuperscript{o} dependence of the HERG channel may be related to the unique flexibility of the channel. Most K\textsuperscript{o} channels possess a GYG signature sequence for K\textsuperscript{o} selectivity filter and double tryptophans (WW) at the N-terminal end of the pore loop (Figure 4A, bold letters). In KcsA, the side chains of the 2 W and the Y residues from each subunit form a sheet from 12 aromatic amino acid residues for a single channel. This sheet is placed as a cuff around the selectivity filter diameter.\textsuperscript{19} Amino acid interactions such as hydrogen bonds between the nitrogen atoms of the WW and the hydroxyls of Y, as well as the extensive van der Waals contacts within the sheet of the 4 subunits, function as a layer of springs that are stretched radially outward to hold the channel pore.\textsuperscript{19} In HERG, the selectivity filter has a GFG sequence, and the WW are replaced by YF. As a result, HERG may have a more flexible selectivity filter region.\textsuperscript{29} Other factors such as the uniquely long extracellular S5-P linker could also contribute to HERG flexibility.\textsuperscript{29} The overall flexibility of the HERG channel would be expected to depend on more complex and delicate amino acid residue interactions. Occupancy with K\textsuperscript{o} may somehow enhance HERG stability and as such be required for HERG function.

Within the pore helix and the selectivity filter, mutations at approximately every third amino acid position, T618A, S621A, S624A, F627Y, N629D, and V630A eliminated K\textsuperscript{o} dependency of HERG channels (Figure 4). The exact locations of these residues and their interactions with the nearby residues are not known. It is possible that the mutations in the permeation pathway change the channel flexibility to such an extent that K\textsuperscript{o} is no longer required for HERG to maintain its proper conformation. For example, S624 in HERG corresponds to T75 in KcsA, which constitutes the innermost selectivity filter position of the channel and contributes to the potential ring of hydrogen bonds encircling the inner selectivity filter.\textsuperscript{22} F627 in HERG corresponds to Y78 in KcsA, which forms hydrogen bonds with nearby nitrogen atoms of the W. Thus, S624T or F627Y may allow formation of hydrogen bonds in the mutant channel and thus lead to a more rigid channel conformation. We showed that WT/S624T channels displayed K\textsuperscript{o} dependency similar to WT channels (Figure 6C), suggesting that the mutation in each subunit is required to enhance the channel stability. Degradation of WT HERG but not the S624T mutant channel was enhanced by Ub overexpression under 0 mmol/L K\textsuperscript{o} conditions (Figure 7). Thus, it is likely that the 0 mmol/L K\textsuperscript{o}-induced conformational change leads to exposure of a previously hidden Ub site(s), allowing Ub modification of the channel, which is...
known to trigger internalization and degradation of various membrane proteins. Genetic analyses have identified $\approx$200 LQT2-associated HERG mutations that cause the loss of function phenotype with multiple mechanisms. A number of long QT HERG mutations are in the pore region. Further investigation of the effects of $[K^+]_o$ on the clinically relevant mutant HERG channels would be interesting.

We previously showed the internalized HERG channels localize in the multivesicular bodies and lysosomes. The clathrin-mediated endocytosis represents a classical endocytic pathway for membrane proteins including channels such as chloride channel CFTR (cystic fibrosis transmembrane conductance regulator) and the gap junction channel connexin. We previously showed that HERG channels did not colocalize with clathrin during the internalization, and knockdown of clathrin using clathrin siRNA did not affect 0 mmol/L $K^+$-induced reduction of the 155-kDa HERG band. We also showed that both proteasome inhibitor lactacystin and lysosome inhibitor bafilomycin inhibited HERG degradation. Thus, both the proteasome and the lysosome are involved in the process. The molecular mechanisms for low $K^+$-induced internalization of HERG warrant further investigation.

In summary, our present study links a gating conformational state with membrane stability of HERG channels in a novel way; surface HERG channels exposed to 0 mmol/L $K^+$ adopt a nonconducting state and become ubiquitinated, internalized, and degraded. Permeant ions from the outside of the membrane prevent entry into the nonconducting state and thus maintain HERG channels in the plasma membrane. This finding extends our understanding of ion channel biology and has implications for the regulation of ion channel function.

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Disclosures

None.

References

27. Massaeli et al K$^+$ Is a Prerequisite for HERG Integrity 1081


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**What Is Known?**

- A reduction in potassium levels in the blood (hypokalemia) exacerbates cardiac electric disorders, long QT syndrome (LQTS), and tachyarrhythmias.
- We previously demonstrated that hypokalemia induces endocytosis of a cardiac potassium channel, HERG (human ether-a-go-go-related gene), whose dysfunction contributes to LQTS.

**What New Information Does This Article Contribute?**

- Here we report that, at low extracellular potassium concentrations ([K\(^+\)]\(_o\)), the HERG channel enters into a novel nonconducting state.
- Entry into this nonconducting state triggers endocytosis of HERG channels.
- Amino acid residues in the channel permeation pathway are involved in the extracellular K\(^+\) dependence of HERG function and membrane stability.

**Novelty and Significance**

Electric signals generated by a cardiac potassium channel encoded by the *HERG* gene are essential for normal heart rhythm. Dysfunction of the HERG channel contributes to LQTS and sudden cardiac death. We previously demonstrated that a reduction in [K\(^+\)]\(_o\) causes endocytosis and, thus, loss of function of HERG channels. The aim of this study was to elucidate how a reduction in [K\(^+\)]\(_o\) triggers HERG channel endocytosis. We discovered that at low [K\(^+\)]\(_o\), the HERG channel enters a novel nonconducting state. Entry into this nonconducting state subsequently triggers rapid internalization and degradation of the cell surface HERG channels. Thus, our data demonstrate, for the first time, a direct link between a functional state and the plasma membrane stability of the HERG channel. By mutating specific amino acid residues in the channel, we also identified the sites in the channel that are involved in the K\(^+\)o dependence of HERG channels. These findings extend our understanding of ion channel function and have implications with respect to designing new ways for intervening in channel regulation.
Extracellular K+ Is a Prerequisite for the Function and Plasma Membrane Stability of HERG Channels
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