Isolation and characterization of $I_{Kr}$ in cardiac myocytes by Cs$^+$ permeation

Shetuan Zhang

Institute of Cardiovascular Sciences, St. Boniface General Hospital Research Centre, and Department of Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada

Submitted 22 June 2005; accepted in final form 8 October 2005

Zhang, Shetuan. Isolation and characterization of $I_{Kr}$ in cardiac myocytes by Cs$^+$ permeation. *Am J Physiol Heart Circ Physiol* 290: H1038–H1049, 2006. First published October 14, 2005; doi:10.1152/ajpheart.00679.2005.—Isolation of the rapidly activating delayed rectifier potassium current ($I_{Kr}$) from other cardiac currents has been a difficult task for quantitative study of this current. The present study was designed to separate $I_{Kr}$ using Cs$^+$ in cardiac myocytes. Cs$^+$ have been known to block a variety of K$^+$ channels, including many of those involved in the cardiac action potential such as inward rectifier potassium current $I_{K1}$ and the transient outward potassium current $I_{to}$. However, under iso- tonic Cs$^+$ conditions (135 mM Cs$^+$), a significant membrane current was recorded in isolated rabbit ventricular myocytes. This current displayed the voltage-dependent onset of and recovery from inactivation that are characteristic to $I_{Kr}$. Consistently, the current was selectively inhibited by the specific $I_{Kr}$ blockers. The biophysical and pharmacological properties of the Cs$^+$-carried human ether-a-go-go-related gene (hERG) current were very similar to those of the Cs$^+$-carried $I_{Kr}$ in ventricular myocytes. The primary sequence of the selectivity filter in hERG was in part responsible for the Cs$^+$ permeability, which was lost when the sequence was changed from GFG to GYG, characteristic of other, Cs$^+$-impermeable K$^+$ channels. Thus the unique high Cs$^+$ permeability in $I_{Kr}$ channels provides an effective way to isolate $I_{Kr}$ current. Although the biophysical and pharmacological properties of the Cs$^+$-carried $I_{Kr}$ are different from those of the K$^+$-carried $I_{Kr}$, such an assay enables $I_{Kr}$ current to be recorded at a level that is large enough and sufficiently robust to evaluate any $I_{Kr}$ alterations in native tissues in response to physiological or pathological changes. It is particularly useful for exploring the role of reduction of $I_{Kr}$ in arrhythmias associated with heart failure and long QT syndrome due to the reduced hERG channel membrane expression.

cesium; rapidly activating delayed rectifier potassium current; potassium channel; human ether-a-go-go-related gene; patch clamp

THE RAPIDLY ACTIVATING delayed rectifier potassium current ($I_{Kr}$) is an important current for cardiac repolarization (36, 37). The human *ether-a-go-go*-related gene, hERG, encodes the pore-forming subunits of the channel that conducts $I_{Kr}$ (35, 44). Reduction in $I_{Kr}$ due to the genetic defects in hERG or drug blockade prolongs cardiac action potential and causes inherited or acquired forms of long QT syndrome (LQTS), a cardiac electrical disorder that can lead to fatal arrhythmias and sudden death (21). $I_{Kr}$ may also contribute to the electrical remodeling under pathological conditions. Presently, much of the available data for $I_{Kr}$ function and drug modulation has been obtained using cell lines expressing recombinant hERG channels. Studying cloned channels overcomes difficulties caused by the existence of multiple channel types in cardiac cells. As well, the detailed structure-based analyses of channel function and drug-channel interactions can only be achieved in cloned channels. However, it has been found that K$^+$ channels incorporate modulatory (β) subunits such as minK, miRP1 (1, 28), kinase anchoring proteins (12), cytoskeletal elements, and other proteins in their higher-order structures. Because these components are different between expressing cell lines and cardiomyocytes, it is necessary to directly study native $I_{Kr}$. Quantitative study of $I_{Kr}$ is particularly important in determining its contributions to electrical remodeling under pathological conditions such as heart failure. However, because $I_{Kr}$ displays slow activation but fast and voltage-dependent inactivation kinetics, its appearance is always complicated by the existence of many other K$^+$ currents, especially those with either a faster activation or a slower inactivation gating, such as the transient outward K$^+$ current $I_{to}$. The general approach of recording $I_{Kr}$ includes detection of the fast recovering tail currents and/or subtracting “$I_{Kr}$ blocker”-sensitive component (5, 38, 48). $I_{Kr}$ recorded by the present method is usually small. Contamination of other currents such as the slowly activating delayed rectifier K$^+$ current ($I_{Ks}$) that run down during recording is particularly problematic for quantitative $I_{Kr}$ studies under pathological conditions. Presently, the difficulties in isolating $I_{Kr}$ in cardiac myocytes from other ionic currents have hampered studies of native $I_{Kr}$.

In studies of the effects of extracellular cations on hERG gating properties, a high Cs$^+$ permeability in hERG channels has been noticed (39, 51, 54). However, a detailed study of Cs$^+$ permeation through hERG channels has not been conducted, and the mechanism for the high Cs$^+$ permeability is not known. It is also not known if Cs$^+$ permeates native $I_{Kr}$ and generates the current similar to that in hERG-expressing HEK-293 cells. By using the whole cell patch-clamp and site-directed mutagenesis techniques, the present study revealed that native $I_{Kr}$ displays a unique high permeability to Cs$^+$. Furthermore, Cs$^+$-carried $I_{Kr}$ current in rabbit ventricular myocytes had similar biophysical and pharmacological properties to those of hERG channels expressed in HEK-293 cells. The modified GFG signature motif in the selective filter of hERG contributes to the high Cs$^+$ permeability of the channel. Because Cs$^+$ is known to block many other K$^+$ channels involved in the cardiac ventricular action potential, such as the inward rectifying potassium current $I_{K1}$ (14), recording Cs$^+$-carried $I_{Kr}$ provides a simple and efficient way to isolate and study this current in cardiac myocytes. This finding would be particularly useful in quantitatively studying native cardiac $I_{Kr}$ under physiopathological conditions. An abstract report of this work has appeared (53).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
MATERIALS AND METHODS

Cardiac myocyte isolation. Experimental protocols for animal studies were approved by the Animal Care Committee of the University of Manitoba, following the guidelines established by the Canadian Council on Animal Care. Myocytes from the left ventricles of New Zealand white rabbit hearts were isolated using an enzymatic dissociation method (2). In brief, hearts were excised from anesthetized male rabbits (2.5–3.0 kg) and mounted on a Langendorff apparatus. The hearts were flushed at 20 ml/min with Ca\(^{2+}\)-free HEPES-buffered saline (HBS). This solution contained (in mM) 132.0 NaCl, 10.0 HEPES, 1.2 MgCl\(_2\), 10.0 glucose, 4.0 KCl, 60.0 taurine, and 0.25% bovine serum albumin (pH 7.4). The heart was then perfused with HBS plus collagenase (1.8 \(\times\) 10\(^5\) U/l, Worthington, Lakewood, NJ) for 10–15 min. After the collagenase was washed with Ca\(^{2+}\)-free HBS for 5 min, the left ventricle was minced in 100 \(\mu\)M Ca\(^{2+}\) HBS and then shaken for 20 min. The cells were filtered through cheesecloth and centrifuged at 70 g for 2 min. The pellet was suspended in 200 \(\mu\)M Ca\(^{2+}\) HBS for 10 min, centrifuged at 70 g for 2 min, and then resuspended in 1.8 mM Ca\(^{2+}\) HBS.

Molecular biology. We created the F627Y hERG and Y315F KvLQT1 mutations. The rationale for designing these mutations is as follows. Sequence alignment of homologous pore region of K\(_{\text{vLQT1}}\) channels indicates that while most K\(^{+}\) channels possess a signature motif of GYG in the selectivity filter of the channel, hERG possesses a GFG sequence. Because hERG displays a high Cs\(^{+}\) permeability (39, 54), F627Y mutation was made to test whether the Phe-627 residue contributes to the high Cs\(^{+}\) permeability in hERG. Conversely, Y315F was made to test whether the Cs\(^{+}\) permeability can be increased in the mutant KvLQT1 + minK channel. hERG cDNA in pCDNA3 was obtained from Dr. Gail A. Robertson [Univ. of Wisconsin-Madison (44)]. KvLQT1 and minK cDNAs in pSP64 were obtained from Dr. Michael C. Sanguinetti [Univ. of Utah, Salt Lake City, UT (34)]. The point mutations were introduced via PCR using overlap extension (19). The first round of PCRs was performed using the forward priming primer-forward mutant primer and the reverse priming primer-forward mutant primer. The resulting PCR products were used as templates and amplified by flanking primers in a second round of PCR. The final PCR products were amplified in Zero Blunt Vector (Invitrogen, Burlington, ON, Canada). For F627Y hERG, the forward and reverse flanking primers were designed to cover two unique restriction sites, BstEI at nucleotide 2038 and Shfl at nucleotide 3093. The final PCR products were digested with BstEI and Shfl (New England Biolabs, Mississauga, ON, Canada) and ligated to the hERG-expressing plasmid digested with the same two restriction enzymes. For the Y315F KvLQT1 mutation, the forward and reverse flanking primers were designed to cover two unique restriction sites, XhoI at nucleotide 365 and BgII at nucleotide 1203 in pSP64. The final PCR products were digested with XhoI and BgII (New England Biolabs) and ligated to pSP64-KvLQT1 plasmid digested with the same two restriction enzymes. The Y315F KvLQT1, wild-type (WT), KvLQT1, and WT minK in pSP64 vector were subcloned into the pCDNA3 vector using restriction enzymes HindIII and BamHI. The mutations were verified by using a high-throughput 48 capillary ABI 3730 sequencer (UCDNA Services, Univ. of Calgary, Calgary, AB, Canada).

The F627Y hERG and WT and mutant KvLQT1 + minK channels were transiently expressed in HEK-293 cells (American Type Culture Collection, Rockville, MD). These cells were seeded at 5 \(\times\) 10\(^5\) cells/60-mm-diameter dish. For F627Y hERG, the cells were transfected using 10 \(\mu\)l Lipofectamine (Invitrogen, Burlington) with 4 \(\mu\)g hERG mutant expression vector. For WT and mutant KvLQT1 + minK, the cells were transfected using 10 \(\mu\)l Lipofectamine with 2 \(\mu\)g pCDNA3-KvLQT1 and 8 \(\mu\)g pCDNA3-minK. After 24–72 h, \(\sim\)50% of cells expressed characteristic currents. Nontransfected HEK-293 cells contain a small-amplitude background K\(^{+}\) current that is usually <100 pA on a depolarizing pulse to 50 mV. Thus the effects of overlapping endogenous currents of HEK-293 cells on the expressed current are minimal (59).

The HEK-293 cell line stably expressing hERG channels was obtained from Dr. Craig T. January [Univ. of Wisconsin (59)], where hERG cDNA (44, 45) was subcloned into BamHI/EcoRI sites of the pCDNA3 vector (Invitrogen, Carlsbad, CA). The cells were cultured in glutamine-containing MEM supplemented with 10% fetal bovine serum and 0.5 mg/ml G418 to select for transfected cells. The cells were harvested from the culture dish by trypsinization and stored in standard MEM medium at room temperature for electrophysiological study. Cells were studied within 8 h of harvest.

Electrophysiological procedures and analysis. The whole cell patch-clamp method (54–57) was used to record currents in rabbit ventricular myocytes and HEK-293 cells that express specific channels. For hERG and I\(_K\), the pipette solution contained (in mM) 135 CsCl or KCl, 10 EGTA, 1 MgCl\(_2\), and 10 HEPES. The pH was adjusted to 7.2 with CsOH or KOH. The bath solution contained (in mM) 135 CsCl, 10 HEPES, 10 glucose, and 1 MgCl\(_2\). For hERG current recorded at 135 K\(^{+}\) bath solution, CsCl was replaced by KCl. For recordings in the 5 mM Cs\(^{+}\), NaCl was used as the substitute for the rest of CsCl. The pH of the bath solutions was adjusted to 7.4 with CsOH, KOH, or NaOH. For KvLQT1 + minK channels, the pipette solution contained (in mM) 150 CsCl or KCl, 0.5 MgCl\(_2\), 5 EGTA, and 10 HEPES with pH 7.2 by CsOH or KOH. The bath solution contained (in mM) 150 CsCl or KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), and 10 HEPES with pH 7.4 by CsOH or KOH. All chemicals were obtained from Sigma. Throughout the text the subscripts “i” and “o” denote intra- and extracellular ion concentrations, respectively. In the preliminary experiments with rabbit ventricular myocytes, a depolarizing step to \(-50 mV\) started to evoke an inward current. As depolarizing voltages became positive, outward currents appeared and inactivated in a voltage-dependent manner. On repolarization to the holding potential of \(-80 mV\), a tail current was seen. While addition of calcium channel blocker nifedipine (10 \(\mu\)M) had no effect on the outward pulse current or the tail current, it did eliminate a rapidly activating and inactivating inward current induced by voltage steps between \(-30 mV\) and 0 mV. Because L-type Ca\(^{2+}\) channels have been shown to have some Cs\(^{+}\) permeability (16), this rapidly activating and inactivating inward current may be related to L-type Ca\(^{2+}\) channels. To exclude the interference from L-type Ca\(^{2+}\) channels, 10 \(\mu\)M nifedipine (Sigma) was included and Ca\(^{2+}\) was excluded in the bath solutions in all experiments with rabbit ventricular myocytes. For comparison, Ca\(^{2+}\) was also excluded in the bath solutions for hERG current recordings and 10 \(\mu\)M nifedipine had no effects on hERG currents.

Conductance-voltage relationship was fitted to a single Boltzmann function, \(y = I/(1 + \exp[V_{1/2} - V/k])\), where \(y\) is the current normalized with respect to the maximal tail current, \(V_{1/2}\) is the half-maximum activation voltage, \(V\) is the voltage to activate the channel, and \(k\) is the slope factor (in mV) reflecting the steepness of the voltage dependence of gating. Concentration-dependent effects were quantified by fitting data to the Hill equation, \(I_{\text{drug}}/I_{\text{control}} = I_{\text{control}}(1 + (D/I_{\text{IC50}})^H)\), where \(I_{\text{drug}}\) is the current in the presence of drugs, \(I_{\text{control}}\) is the control current in the absence of drugs, \(D\) is the drug concentration, \(I_{\text{IC50}}\) is the drug concentration for 50% block, and \(H\) is the Hill coefficient. Data analysis and curve fitting were done using Clampfit (Axon Instruments) and Origin (Microcal Software). Data are given as means \(\pm\) SE. All experiments were performed at room temperature (23 ± 1°C).

RESULTS

Cs\(^{+}\) current recorded in rabbit ventricular myocytes. Under isotonic Cs\(^{+}\) solutions (135 mM Cs\(^{+}\)/135 mM Cs\(^{-}\)), significant currents were recorded by the whole cell clamp method in isolated rabbit ventricular myocytes. Figure 1A shows a family of Cs\(^{+}\) currents obtained from a single myocyte. The cell was clamped at a holding potential of \(-80 mV\) and depolarized in

\(\begin{align*}
\text{AJP-Heart Circ Physiol} & \quad \text{VOL 290} \quad \text{MARCH 2006} \quad \text{www.ajpheart.org} \\
\text{H1039}
\end{align*}\)
10-mV incremental steps to voltages between −70 and +70 mV for 4 s to activate the current. The cell was then clamped back to −80 mV to record the tail current. Depolarizing pulses to −50 mV started to induce inward currents. Depolarizations to voltages > 0 mV (outward driving force) induced outward currents that inactivated in a voltage-dependent manner; stronger depolarizations induced faster inactivation. After strong depolarizations, the inward tail currents on returning to the holding potential (−80 mV) displayed a rising phase. C: current (I)-voltage (V) relationships of the maximal current during depolarization (▴) and the current at the end of depolarizing steps (●, n = 6). D: activation curve of the Cs⁺ current. Amplitudes of the tail currents on repolarizations to −80 mV shown in A were normalized to the largest tail currents and plotted against depolarizing voltages. Data were fitted to a Boltzmann function (●, n = 6).

To elucidate the nature of the Cs⁺ current in rabbit ventricular myocytes, the properties of the current were investigated (Fig. 2). To estimate the time course of Cs⁺ current activation, the time to reach the peak current amplitude (T_peak) of each current trace was measured (Fig. 2A). To study the time course of Cs⁺ current inactivation, a triple-pulse protocol (Fig. 2B) was used (40, 41). In this protocol, the membrane was initially depolarized to +60 mV for 500 ms to inactivate the channels. A 20-ms repolarizing step to −100 mV was applied to recover inactivated channels to the open state. Before deactivation, the membrane was depolarized to different voltages to evaluate the voltage dependence of inactivation. The decay of the currents was fitted to a single-exponential function to obtain inactivation time constants (τ_inact). To study the recovery from inactivation of the Cs⁺ current, a double-pulse protocol was used (Fig. 2C). The channels were inactivated by a depolarizing pulse to +60 mV for 500 ms. Repolarizations from +60 mV to voltages between −10 and −120 mV were applied to evaluate the recovery, which appeared as a rising phase (hook) on repolarization. The time constants of recovery (τ_rec) from inactivation were measured as a single-exponential fit to the rising phase (greater than −80 mV) or as the fast time constant of a double-exponential fit (less than or equal to −80 mV) to the tail currents (35, 40, 41, 44). Deactivation of the Cs⁺ current in rabbit ventricular myocytes was also analyzed. The current decay after recovery was fitted to a single exponential to obtain deactivation time constants (τ_deact). All these parameters, including T_peak, τ_inact, τ_rec, and τ_deact, were plotted against the test voltages and shown in Fig. 2, D–F (n = 6 cells). Like all voltage-gated K⁺ channels, the Cs⁺ currents in rabbit ventricular myocytes displayed voltage-dependent activation and deactivation. Significantly, the Cs⁺ currents in rabbit...
ventricular myocytes also displayed strong voltage dependence of onset of and recovery from inactivation, which are characteristic to \( I_{Kr} \) currents. The following experiments were performed to obtain additional evidence that the Cs\(^+\) current indeed represents \( I_{Kr} \).

Cs\(^+\) current recorded in hERG-expressing HEK-293 cells. In the same ionic conditions used in rabbit ventricular myocytes (135 mM CsCl in the pipette and bath solutions), the Cs\(^+\) current was recorded from HEK-293 cells that stably express hERG channels (59). Fig. 3A shows that hERG generated a...
Cs⁺ current that behaved strikingly similar to the Cs⁺ current recorded in cardiac myocytes. To observe the characteristic fast recovery of inactivated hERG channels to the open state before deactivation, the portion of the tail current in the dotted box in Fig. 3A was expanded in Fig. 3B. After strong depolarizations, the inward tail currents on returning to the holding potential of −80 mV displayed an initial rising phase, indicative of the recovery of inactivated channels. I-V relationships of the peak outward and inward currents on depolarizations and the steady-state currents at the end of 4 s depolarizing steps were analyzed in Fig. 3C. To obtain the activation (g-V) curve of the Cs⁺ hERG current, the peak amplitudes were determined by extrapolating the tail current traces to the moment of the voltage change by tail current fitting. The peak amplitudes were normalized to the maximum value and plotted against the depolarizing voltages. The data points were fitted to a Boltzmann function (Fig. 3D, n = 8 cells). The V½ and k were −41.2 ± 2.0 and 6.4 ± 0.4 mV, respectively (n = 8 cells). These values are not different from those in rabbit ventricular myocytes (P > 0.05). The average current density measured by tail current amplitude at −80 mV following a voltage step to 20 mV, where the Cs⁺ current is fully activated, was 52.5 ± 3.3 pA/pF (n = 8 cells). Consistent with our previous finding (57), the hERG Cs⁺ current was not affected by Ca²⁺ channel blocker nifedipine (10 μM, n = 3, data not shown). It should be noted that no Cs⁺ current could be recorded in HEK-293 cells that do not express hERG channels (data not shown, n = 5). Therefore, the Cs⁺ current recorded in hERG-expressing HEK 293 cells represents the Cs⁺ hERG current.

The properties of the Cs⁺ hERG current were analyzed (Fig. 4). The activation time course of the current was estimated by Tpeak. The time constants τinact (Fig. 4B) and τrec and τdeact of the Cs⁺ current (Fig. 4C) in hERG-expressing HEK 293 cells were evaluated in manners as described in detail in Fig. 2. The values of Tpeak, τinact, τrec, and τdeact were plotted against test voltages in Fig. 4. D–F (n = 8 cells). The biophysical properties of the hERG Cs⁺ current were very similar to those of the Cs⁺ current in rabbit ventricular myocytes. To further study the nature of the Cs⁺ current in rabbit cardiomyocytes, its responses to changes in external Cs⁺ concentration and its drug sensitivity were studied in parallel with those of the hERG Cs⁺ current.

Effects of extracellular Cs⁺o concentration on the inactivation time course of the Cs⁺ current. Our previous study showed that increasing extracellular Cs⁺o concentration ([Cs⁺]o) slowed hERG K⁺ current inactivation (54). Figure 5 shows the effects of [Cs⁺]o on τinact of the Cs⁺ current in rabbit ventricular myocytes and hERG-expressing HEK-293 cells. Figure 5, A and B, show the effects of increasing [Cs⁺]o on the inactivation time course of the hERG Cs⁺ current. The current decay was fitted to a single-exponential function, and τinact was plotted against the test voltages. Figure 5C shows the [Cs⁺]o voltage dependence of the τinact of the Cs⁺ current in 0 and 135 mM Cs⁺o in rabbit ventricular myocytes, and Fig. 5D shows those in hERG-expressing HEK-293 cells. Elevation of [Cs⁺]o similarly slowed the Cs⁺ current inactivation in both ventricular myocytes and hERG-expressing HEK-293 cells.

Pharmacology of the Cs⁺ currents in rabbit ventricular myocytes and hERG-expressing HEK-293 cells. The specific hERG/Kc blockers astemizole, cisapride, and E-4031 were used to study the nature of the Cs⁺ current recorded in rabbit ventricular myocytes. The effects of these compounds on Cs⁺ currents in hERG-expressing HEK-293 cells were studied in parallel for comparison. The Cs⁺ currents were elicited by depolarizing steps to voltages between −70 and +70 mV from a holding potential of −80 mV. The interpulse interval was

Fig. 4. Biophysical properties of hERG Cs⁺ currents. A: current traces during hERG channel activation under the isoticonic Cs⁺ conditions (135 mM Cs⁺; 135 mM Cs⁺o). Tpeak of each current trace on various depolarizing steps was measured to estimate the activation time course. B: voltage-dependent inactivation of the hERG Cs⁺ current. C: time- and voltage-dependent recovery from inactivation and deactivation. D: voltage dependence of Tpeak (n = 8). E: voltage dependence of time constants of inactivation (●) and recovery from inactivation (■, n = 8). F: voltage dependence of the deactivation time constants (●, n = 8).
been seen in Fig. 6, cisapride blocked the Cs$^+$ amplitude of the outward current on depolarization. This effect was similar in both ventricular myocytes and hERG-expressing HEK-293 cells. A and B: voltage-dependent hERG Cs$^+$ current inactivation in the absence and presence of 135 mM Cs$^{++}$, respectively. C: voltage dependence of the inactivation time constants of the Cs$^+$ current from ventricular myocytes in the absence ($\circ$) and presence of 135 mM Cs$^{++}$ ($\bullet$, $n = 4$ cells). D: voltage dependence of the inactivation time constants of the Cs$^+$ current from hERG-expressing HEK-293 cells in the absence ($\circ$) and presence of 135 mM Cs$^{++}$ ($\triangledown$, $n = 7$ cells).

As shown in Fig. 6A, 1 μM astemizole essentially abolished Cs$^+$ currents in both ventricular myocytes and hERG-expressing HEK-293 cells. The IC$_{50}$ and Hill coefficient for astemizole inhibition of the Cs$^+$ current in rabbit ventricular myocytes were 72.9 ± 6.4 nM and 2.0, respectively ($n = 5$). The corresponding values for astemizole block of the hERG Cs$^+$ current were 63.1 ± 5.1 nM and 2.2, respectively ($n = 4$). These values are not statistically different from each other ($P > 0.05$). As can been seen in Fig. 6B, cisapride blocked the Cs$^+$ current with unique kinetics; 1 μM cisapride almost abolished the Cs$^+$ tail current at −80 mV but had much less effect on the peak amplitude of the outward current on depolarization. This blocking feature was similar for both Cs$^+$ currents in rabbit ventricular myocytes and the hERG-expressing HEK-293 cells. The smaller effect of cisapride on the outward pulse current is due to the fast unblock of the drug on repolarization (30). With frequent depolarizations to +50 mV for 200 ms at 3 Hz, 1 μM cisapride caused use-dependent block of the outward current with a steady-state block > 80% (data not shown, $n = 3$ in rabbit ventricular myocytes; $n = 4$ in hERG-expressing HEK-293 cells). The IC$_{50}$ and Hill coefficient for cisapride were 183 ± 12 nM and 1.0 ($n = 6$) to block the tail current in rabbit ventricular myocytes and were 193 ± 11 nM and 1.1 to block the hERG Cs$^+$ tail current ($n = 7$). These values are not statistically different from each other ($P > 0.05$). The effects of E-4031 on the Cs$^+$ current are shown in Fig. 6C. Among the three compounds tested, E-4031 was the least potent for blockade of the Cs$^+$ current in both cardiomyocytes and hERG-expressing HEK-293 cells; 10 μM E-4031 blocked ~70% of the currents in both systems. The IC$_{50}$ and Hill coefficient were 3.1 ± 0.3 μM and 0.8 ($n = 6$) for the Cs$^+$ tail current in ventricular myocytes and 3.9 ± 0.5 μM and 0.9 for the hERG Cs$^+$ current ($n = 4$). These values were very similar ($P > 0.05$).

The biophysical and pharmacological properties of the Cs$^+$ currents in these two systems are compared in Table 1. Except for the slower activation, inactivation, and recovery from inactivation of the Cs$^+$ hERG current than those of the Cs$^+$ $I_{Ks}$ current, the Cs$^+$ hERG and Cs$^+$ $I_{Ks}$ current were very similar.

Cs$^+$ permeation is negligible in most K$^+$ channels. It has been known that most K$^+$ channels have a GYG signature motif for K$^+$ selectivity, whereas hERG has a GFG sequence (46). To examine whether the high Cs$^+$ permeability of hERG is related to its modified signature motif GFG, the mutant F627Y channel was created to restore the GYG sequence. The Cs$^+$ permeability relative to K$^+$ ($P_{Cs}/P_K$) was studied in WT and F627Y mutant channels. The $P_{Cs}/P_K$ was determined under bi-ionic conditions, in which 135 mM K$^+$ was present in the pipette solution and 135 mM Cs$^{++}$ was present in the bath solution. $I_{Ks}$ is another important channel for cardiac repolarization. In many species including human, $I_{Ks}$ overlaps $I_{Kf}$ in
cardiac myocytes. $I_{Ks}$ is encoded by KvLQT1 + minK. The KvLQT1 + minK channel was expressed in HEK-293 cells, and its Cs$^+$ permeability was investigated by measuring the $P_{Cs}/P_K$. The Y315F KvLQT1 mutant channel was also constructed to modify the GYG signature motif to GFG to test whether the phenylalanine in the signature motif is exclusively responsible for the high Cs$^+$ permeability of the channel.

Families of WT hERG, F627Y hERG, WT KvLQT1 + minK, and Y315F KvLQT1 + minK channel currents were elicited by 4-s depolarizing steps from the holding potential of −80 mV to between −70 and 70 mV. Because of the 135 mM Cs$^+$-induced slowing of hERG inactivation, significant outward K$^+$ currents were observed on depolarizations. These outward K$^+$ currents inactivated quickly in a voltage-dependent manner. The inward Cs$^+$ tail currents on repolarization to −80 mV indicated a significant Cs$^+$ conductance in WT hERG.

Table 1. Biophysical and pharmacological properties of Cs$^+$ currents in HEK-hERG cells and rabbit ventricular myocytes

<table>
<thead>
<tr>
<th>Channels</th>
<th>$V_{1/2}$, mV</th>
<th>$k$, mV</th>
<th>$T_{peak}$, ms</th>
<th>$\tau_{deact}$, ms</th>
<th>$\tau_{inact}$, ms</th>
<th>$\tau_{rec}$, ms</th>
<th>Astemizole</th>
<th>Cisapride</th>
<th>E-4031</th>
</tr>
</thead>
<tbody>
<tr>
<td>$I_{Kr}$</td>
<td>$-38.9 \pm 2.0$</td>
<td>$6.8 \pm 0.3$</td>
<td>$64 \pm 6$</td>
<td>$405 \pm 82$</td>
<td>$117 \pm 9$</td>
<td>$25 \pm 4$</td>
<td>$73 \pm 6$</td>
<td>$183 \pm 12$</td>
<td>$3,108 \pm 301$</td>
</tr>
<tr>
<td>hERG</td>
<td>$-42.3 \pm 2.0$</td>
<td>$6.2 \pm 0.3$</td>
<td>$108 \pm 7^*$</td>
<td>$386 \pm 38$</td>
<td>$203 \pm 21^*$</td>
<td>$37 \pm 5^*$</td>
<td>$63 \pm 5$</td>
<td>$193 \pm 11$</td>
<td>$3,907 \pm 515$</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n$ = 4–8 cells for each parameter. Currents were measured in 135 mM intracellular Cs$^+$/135 mM extracellular Cs$^+$. HEK-hERG cells, HEK-293 cells expressing human ether-a-go-go-related gene; $I_{Kr}$, rapidly activating delayed rectifier potassium current; $V_{1/2}$, voltage of half-maximal activation; $k$, slope of the activation curve; $T_{peak}$, time to peak current at 50 mV; $\tau_{deact}$, deactivation time constants measured at −80 mV; $\tau_{inact}$, inactivation time constant at 50 mV; $\tau_{rec}$, time constant of recovery from inactivation at −80 mV; IC$_{50}$, concentration for half-maximal inhibition of the peak tail current. *$P < 0.01$. 

AJP-Heart Circ Physiol • VOL 290 • MARCH 2006 • www.ajpheart.org
channels (Fig. 7Aa). In contrast, the inward Cs\(^+\) tail currents were significantly reduced by the F627Y mutation (Fig. 7Ab). For WT KvLQT1 + minK channels, depolarizations evoked slowly activating outward K\(^+\) currents that did not inactivate. On repolarization to the holding potential of -80 mV, the inward Cs\(^+\) current was evident albeit small. The inward Cs\(^+\) current did not significantly change in the Y315F KvLQT1 + minK channels. To calculate the \(P_{Cs}/P_K\) in each of these channels, the reversal potentials were measured. The WT and mutant hERG channels were activated and inactivated by a prepulse to 60 mV for 500 ms. Repolarization to -100 mV for 10 ms allowed the inactivated channels to recover to the open state. Before deactivation, various test steps were applied to measure the reversal potential of the channel. WT and mutant KvLQT1 + minK channels were activated by a depolarizing step to +70 mV for 4 s and then repolarized to various voltages to obtain the reversal potential of the channels. Instantaneous \(I-V\) relationships of currents on the testing voltages were constructed, and the reversal potentials (\(E_R\)) for each channel were obtained. The \(P_{Cs}/P_K\) was calculated based on the modified Nernst equation, \(E_R = (RT/\gamma F) \times \ln(P_{Cs}/P_K)\) (18), where \(R\) is universal gas constant, \(T\) is absolute temperature, \(\gamma\) is valence of the ion, and \(F\) is Faraday constant. Figure 7B shows the averaged \(P_{Cs}/P_K\) for each of these channels. While the F627Y mutation in hERG significantly reduced the \(P_{Cs}/P_K\), the Y315F mutation in KvLQT1 did not alter the \(P_{Cs}/P_K\) in KvLQT1 + minK channels (\(n = 5–11\) cells in each group).

Because KvLQT1 + minK channels also displayed some permeability to Cs\(^+\), the Cs\(^+\) currents through hERG and KvLQT1 + minK channels were further investigated (Fig. 8). The hERG and KvLQT1 + minK currents were recorded in either isotonic K\(^+\) or Cs\(^+\) conditions. For hERG channels, the K\(^+\) inward tail current was large, but the outward current on depolarization was small because of the voltage-dependent inactivation. Under isotonic Cs\(^+\) conditions, as already shown in Fig. 1, there is a significant Cs\(^+\) current in both outward and inward directions (Fig. 8B). The appearance of the outward current was due to the slowed inactivation caused by high external Cs\(^+\). The inward Cs\(^+\) currents reflect the rapid recovery of the channels from the inactivated state to open state before deactivation. Because activation of the hERG current is relatively faster, reduction of the depolarizing step from 4 to 1 s did not affect the size of the Cs\(^+\) hERG current (Fig. 8C).

For KvLQT1 + minK channels under isotonic K\(^+\) conditions, the outward K\(^+\) current activated slowly and did not display inactivation. On repolarization to the holding potential of -80 mV, large K\(^+\) tail currents were recorded (Fig. 8D). Consistent with Cs\(^+\) also being able to permeate through KvLQT1 + minK channels, a small Cs\(^+\) current with kinetics similar to that of the K\(^+\) current was recorded under isotonic Cs\(^+\) conditions (Fig. 8E). However, because activation of the KvLQT1 + minK is slow, reduction of the depolarizing step from 4 to 1 s significantly decreased the Cs\(^+\) current through KvLQT1 + minK channels (Fig. 8F).

---

**Fig. 7.** Cs\(^+\) permeability in wild-type (WT) hERG, F627Y hERG, WT KvLQT1 + minK, and Y315F KvLQT1 + minK channels. A: WT hERG (a), F627Y hERG (b), WT KvLQT1 + minK (c), and Y315F KvLQT1 + minK current (d) recorded in a 135 mM intracellular K\(^+\) (K\(^+\)):135 mM Cs\(^+\), condition. The voltage protocol was the same as shown in Fig. 1A. B: averaged Cs\(^+\) permeability relative to K\(^+\) (\(P_{Cs}/P_K\)) ratios of the channels tested. **P < 0.001 compared with any of the other examined channels.
DISCUSSION

The present study demonstrated that recording Cs⁺ current represents a simple way to isolate \( I_{Kc} \) in cardiac myocytes without interference from other K⁺ currents. Four features of the Cs⁺ current in rabbit ventricular myocytes suggest it is the \( I_{Kc} \) current. First, it inactivated in a voltage-dependent manner with time constants in the millisecond range. Second, it displayed fast and voltage-dependent recovery from inactivation. Third, its inactivation rate was slowed by the elevation of extracellular Cs⁺. Fourth, it was sensitive to hERG/Kr blockers, and the IC₅₀ values for E-4031, cisapride, and astemizole were very close to those for blockade of hERG Cs⁺ current.

Isolation of \( I_{Kc} \) from other currents in cardiac myocytes has been a difficult task because of the coexistence of multiple K⁺ channels and the unique fast and voltage-dependent gating kinetics of \( I_{Kc} \). The native \( I_{Kc} \) is known to be blocked by Cs⁺ at micromolar to millimolar concentrations (14, 27). Consistently, the Kir2.1 channel is blocked by Cs⁺ (42). It has been reported that a mutation of Ser 165 in the transmembrane domain M2 to Leu (S165L) lowered Cs⁺ blocking affinity (42). In addition, the inward rectifier muscarinic K⁺ channel formed by Kir3.1/Kir3.4 is also blocked by Cs⁺ (9). \( I_{o} \) is another current that can be abolished by Cs⁺ substitution for intracellular K⁺ (3, 22). The effects of Cs⁺ on \( I_{Kc} \) current are not well known. In a study using guinea pig ventricular myocytes, Hadley and Hume (13) reported that \( I_{K} \) displayed a permeability sequence K⁺ ≥ Rb⁺ > NH₄⁺ ≥ Cs⁺ > Na⁺, with a P₅₀ of 0.15. They found that when the myocytes were dialyzed with Cs⁺, a sizable time-dependent outward current, similar to the one seen with K⁺ dialysis, was demonstrated (13). However, because the \( I_{Kc} \) in their study included both \( I_{Kc} \) and \( I_{Kr} \), it is uncertain whether Cs⁺-carried current was \( I_{Kc} \), \( I_{Kr} \), or both. When minK was expressed in \textit{Xenopus} oocytes, a voltage-dependent K⁺-selective channel activity was recorded. This channel was blocked by Cs⁺ (11). It is now understood that expression of minK in \textit{Xenopus} oocytes that possess endogenous KCNQ1 (KvLQT1) produces \( I_{Ks} \) channels (4, 34). For other KCNQ families, it was reported that heterologously expressed KCNQ2/KCNQ3 channels showed a permeation sequence of Tľ⁺ > K⁺ > Rb⁺ > NH₄⁺ ≥ Cs⁺ > Na⁺, and a conductance sequence of K⁺ > Tľ⁺ > NH₄⁺ > Rb⁺ > Cs⁺ (32). Our experiment showed that Cs⁺ also permeates through KvLQT1 + minK channels, which encode \( I_{Kc} \). However, the Cs⁺ permeability of KvLQT1 + minK is significantly less than that of hERG (P₅₀/P₆ ᵈ 0.11 vs. 0.38, \( P < 0.01 \), \( n = 11 \) for KvLQT1 + minK and 6 for hERG). Furthermore, because the activation time...
course of KvLQT1 + minK is slower than that of hERG, when 1-s depolarizing steps were used, the amplitude of the hERG Cs\(^+\) current was minimally affected, but the KvLQT1 + minK Cs\(^+\) current was essentially eliminated. Thus the I_{Kr} current can be effectively separated from I_{Ks} by recording Cs\(^+\) current with 1-s depolarizing steps.

In our experiment, the outward Cs\(^+\) current displayed voltage-dependent inactivation properties. The steady-state currents at the end of 4-s depolarizing steps were very small and did not increase with stronger depolarizations. Importantly, the similar steady-state currents at the end of 4-s depolarizing steps were also observed in hERG-expressing HEK-293 cells. The steady-state currents in both cardiac myocytes and hERG-expressing HEK-293 cells were entirely sensitive to hERG/I_{Kr} blockers. These results indicate that Cs\(^+\) current in the present study was not contaminated by I_{Ks}.

Cs\(^+\) permeation through L-type calcium channels has been reported. Monovalent ions follow the sequence Li\(^+\) > Na\(^+\) > K\(^+\) > Cs\(^+\) and are much less permeant than the divalents (16). In our experiments the L-type Ca\(^{2+}\) currents were excluded by including 10 \(\mu\)M nifedipine in a Ca\(^{2+}\)-free bath solution. Addition of 2 mM Ca\(^{2+}\) to the bath solution shifted the activation curve to the depolarized direction without affecting either inactivation-voltage relationships or the tail current amplitudes (data not shown, n = 5). Replacement of Cl\(^-\) with aspartate, a membrane-impermeant anion, in the pipette solution did not significantly change the amplitude of the Cs\(^+\) current (data not shown, n = 3). Therefore, Cl\(^-\) flux was not involved in the Cs\(^+\) current recordings. In rat atrial myocytes, it has been reported that under isotonic Cs\(^+\) (140 mM) conditions a current through the nonselective cation channels existed in the presence of 5 \(\mu\)M E-4031 and 1 \(\mu\)M nicardipine (52). In the present study, the Cs\(^+\) current from rabbit ventricular myocytes was completely abolished by 1 \(\mu\)M astemizole, 5 \(\mu\)M cisapride, or 30 \(\mu\)M E-4031. Therefore, the Cs\(^+\) current recorded in the present study was not complicated by the nonselective cation channels.

Although Cs\(^+\) generally does not permeate through voltage-gated K\(^+\) channels (15, 17), the Cs\(^+\) permeability in hERG channels was previously noticed in experiments studying the modulatory effects of extracellular Cs\(^+\) on hERG channels (39, 51, 54). Cs\(^+\) permeability in rat atrial myocytes has been thought to be due to the Cs\(^+\) permeation through I_{Kr} (52). The mechanism underlying the high Cs\(^+\) permeability in hERG/I_{Kr} channels is not known. A similar high Cs\(^+\) permeability through EAG channels has also been reported (6, 31). The pore structure of hERG and EAG is distinct from most other voltage-gated K\(^+\) channels and may be responsible for their high Cs\(^+\) permeability. Whereas most K\(^+\) channels including Shaker possess a GYG signature sequence for K\(^+\) selectivity filter, hERG and EAG have a GFG sequence (46). The Cs\(^+\) permeability through the well-studied Shaker channels is negligible (15). Our results showed that the mutation that changes GFG to GYG in hERG significantly reduced the Cs\(^+\) permeability, indicating that the GFG sequence in the selectivity filter is at least in part responsible for the unique high Cs\(^+\) permeability of hERG channels. However, in KvLQT1 + minK channels, the Y315F mutation (changing the tyrosine in the signature motif to phenylalanine) did not increase the Cs\(^+\) permeability in KvLQT1 + minK channels. Therefore, GFG sequence may not be exclusively responsible for the high Cs\(^+\) permeability of hERG channels. In addition to a tyrosine (Y) in the "signature motif" (GYG), the Shaker sequence has double tryptophans (WW) at the NH\(_2\)-terminal end of the pore-loop. According to the crystal structure of KcsA, a model K\(^+\) channel (10), hydrogen bonds are thought to be formed around the outer mouth between the nitrogen atoms of the WW and the hydroxyl group of Y of the four subunits (10). In hERG and EAG, the WW are replaced by YF, which lack nitrogens. Therefore, hydrogen bonds are missing in hERG channels. The role of these hydrogen bonds in the permeability of the channel is not known. The fact that Y315F KvLQT1 + minK did not display a high Cs\(^+\) permeability indicates that, in addition to phenylalanine in the signature motif, other components may contribute to the high Cs\(^+\) permeability of hERG channels.

The rabbit clone of ERG is 99% identical to the human sequence of ERG at the amino acid level (50). The biophysical properties of the Cs\(^+\) current and its modulation by extracellular Cs\(^+\) concentration and specific hERG blockers cisapride, E-4031, and astemizole behave strikingly similar in rabbit ventricular myocytes and in hERG-expressing HEK-293 cells. The small difference between Cs\(^+\)-carried hERG and Cs\(^+\)-carried I_{Kr} is that the former displayed slower rates of activation, onset of, and recovery from inactivation (Table 1). However, given the different environments in which the channels are expressed, the similarities between the Cs\(^+\) hERG and Cs\(^+\) I_{Kr} current are remarkable. On the other hand, it should be noted that the biophysical properties and drug sensitivities are different between the Cs\(^+\)-carried hERG/I_{Kr} and the K\(^+\)-carried hERG/I_{Kr} current. Most notably, Cs\(^+\) slows the onset of and recovery from inactivation of hERG channels but has no significant effect on hERG channel activation properties (54). As shown in Figs. 2 and 4 and Table 1, the inactivation rate of the hERG Cs\(^+\) current is ~10-fold slower than that of the hERG K\(^+\) current (49, 54), but the activation properties of the hERG Cs\(^+\) current is close to that of K\(^+\) current (49, 54). The relatively negative V_{1/2} of the hERG Cs\(^+\) current was due to the absence of external Ca\(^{2+}\) in the bath solution. Ca\(^{2+}\) is known to shift the V_{1/2} of hERG to the depolarized direction (20). Similar to hERG channels, the major difference between Cs\(^+\)-carried I_{Kr} and K\(^+\)-carried I_{Kr} is that the former displayed a slowed onset of and recovery from inactivation (36). In addition to the inactivation gating, the drug sensitivity of the Cs\(^+\) current is different from that of the K\(^+\) current (25). Whereas astemizole, cisapride, and E-4031 are reported to block the hERG K\(^+\) current with IC_{50} in the tens of nanomolar range (30, 59, 60), the present study found that IC_{50} for these three compounds to block hERG Cs\(^+\) current were 72.9 nM (astemizole), 193 nM (cisapride), and 3.9 \(\mu\)M (E-4031). The IC_{50} for blockade of the Cs\(^+\) current in rabbit ventricular myocytes by astemizole, cisapride, and E-4031 were very close to those for blockade of hERG Cs\(^+\) current. They were 63.1 nM (astemizole), 183 nM (cisapride), and 3.1 \(\mu\)M (E-4031), respectively. These results indicate that the Cs\(^+\) current in rabbit ventricular myocytes indeed represents I_{Kr} current. The reason for the difference between block of the K\(^+\) and Cs\(^+\) current is not yet known. It has been found that Phe-656 in S6 is an important site for drug binding to the hERG channel (23, 29) and its relocation underlies the C-type inactivation-facilitated drug binding. C-type inactivation reflects a stabilized P-type inactivation, and S5-S6 arrangements might be involved to affect the selectivity filter and adjacent structures. The
selectivity filter is the region modified by the permeant ions as suggested by Zhou and MacKinnon (58). The occupancy of the selectivity filter by particular permeant ions might stabilize a conformation of the S5–S6 arrangement. Therefore, the molecular state/position of the drug binding site in S5–S6 may differ between K⁺ permeation and Cs⁺ permeation, which may modify the drug sensitivity (25).

The roles of Iₖᵥ in the electrical remodeling under pathological conditions are not well understood mainly because of the difficulties in isolating pure Iₖᵥ from the other ionic currents. For example, whereas the involvement of Iₖᵥ in LQTS is well known, the role of Iₖᵥ in the prolonged action potential duration in heart failure is not clear. Studies from heart failure animal models and from human failing heart have generalized that prolongation of ventricular action potential duration is the single most consistent electrophysiological abnormality in heart failure (26, 43). In a recent study that addressed ionic current abnormalities associated with prolonged action potentials in cardiomyocytes from diseased human right ventricles, the authors (24) failed to obtain reliable currents with properties of Iₖᵥ because of the low yield and the small size of the Iₖᵥ current. Even in the animal models, it has been particularly difficult to quantitatively determine Iₖᵥ with drug-sensitive component because of the contamination from the rundown or runup of other currents (47). The present study showed that unique Cs⁺ permeation provides an effective way to isolate Iₖᵥ in cardiac myocytes. Therefore, recording Cs⁺–carried native Iₖᵥ would be particularly useful in studying its alterations and contributions to the electrical remodeling under pathological conditions such as LQTS, diabetic heart disease, cardiac ischemia, hypertrophy, and heart failure.

ACKNOWLEDGMENTS

The author thanks Dr. Craig January (Univ. of Wisconsin) for the stable hERG cell line (59); Dr. Gail Robertson (Univ. of Wisconsin) for the hERG cDNA (44); Dr. Michael Sanguinetti (Univ. of Utah) for the KvLQT1 and minK cDNA (34); Brad Ander and Dr. Anton Lukas (St. Boniface Research Centre, University of Manitoba) for providing isolated rabbit ventricular myocytes; and Jun Guo, Hongying Gang, Peter Wojciechowski, and Wentao Li in the laboratory for assistance in some of the experiments.

GRANTS

S. Zhang is a recipient of the New Investigator Award from the Heart and Stroke Foundation of Canada. The project was supported by operating grants from the Canadian Institutes of Health Research, and the Heart and Stroke Foundation of Manitoba.

REFERENCES

34. Sanguinetti MC, Curran ME, Zou A, Shen J, Spector PS, and Atkinson Sanguinetti MC and Jurkiewicz NK. 37. 32.
35. Smith PL, Baukrowitz T, and Yellen G. Scho¨nherr R and Heinemann SH. 38. 42.
49. Zhang S, Kurata HT, Kehl SJ, and Fedida D. Rapid induction of 