Rb^+ Flux through hERG Channels Affects the Potency of Channel Blocking Drugs: Correlation with Data Obtained Using a High-Throughput Rb^+ Efflux Assay

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The nonradioactive Rb^+ efflux assay has become a reliable and efficient high-throughput hERG screening method, but it is limited by its low sensitivity for potent hERG blockers. Using the patch clamp technique, the authors found that the low sensitivity is due in part to the use of Rb^+ as the permeating cation in the assay. The affinities of the drugs measured by patch clamp technique in the presence of Rb^+ were 3- to 10-fold lower than when measured by the same method in the presence of K^+ ions. The apparent affinity of the drugs decreased even further when monitored by the Rb^+ efflux assay. It was also observed that Rb^+ had minimal effects on the activation properties of channels while there was a significant change in the half-inactivation potential. This voltage shift reduces hERG channel inactivation at efflux assay potentials, and will reduce the affinity of hERG-blocking drugs that bind to inactivated states of the channel. In combination with the effects of elevated extracellular ion concentrations, it is likely that Rb^+ modulation of hERG channel inactivation is largely responsible for the reduced drug potencies observed in the Rb^+ efflux assay. (*Journal of Biomolecular Screening* 2004:588-597)

Key words: hERG, Rb^+ efflux assay, rubidium, Q-T interval, patch clamp

INTRODUCTION

THE HUMAN ETHER-A-GO-GO RELATED GENE (hERG) was originally cloned from a hippocampal cDNA library[1] but is highly expressed in the human heart.[2,3] The gene encodes the α-subunit of an inwardly rectifying K^+ channel,[4] and in the heart, this is an important constituent of a rapid delayed rectifier current, I_{Kr}, that regulates cardiac action potential repolarization.[2,5-7] Long QT syndrome (LQTS) is characterized by prolongation of the QT interval in the electrocardiogram resulting from a slow repolarization of cardiac action potentials and is associated with a serious multifocal ventricular arrhythmia, *torsades de pointes*.[8] Reduction of I_{Kr} current by mutation of the hERG gene or by drug blockade can cause congenital and acquired LQTS, respectively,[2,3] and unfortunately, acquired LQTS is not an uncommon side effect of a number of commonly used over-the-counter and prescription medications.[9-12] The susceptibility of the hERG channel to block by so many drugs has led to intense scrutiny of new drugs by regulatory agencies. This in turn has intensified the interest of pharmaceutical companies in high-throughput screening methods to detect, early in the drug development process, compounds that block the hERG channel.

Electrophysiological techniques such as patch clamp comprise the most powerful and accurate methods for the functional analysis of ion channels and their interactions with drugs. At present these methods can, if automated, handle no more than several hundreds of samples per day, which is inadequate for the large number of candidate drugs in compound libraries.[13] The nonradioactive Rb^+ screening assay[14,15] is a high-capacity functional analysis method specifically developed for screening large numbers of compounds on potassium channels. However, there is little information available to compare how this assay method performs in comparison to the standard patch clamp methods. Using a stably transfected cell line expressing hERG channels in high numbers, we have compared the IC_{50} values determined using the Rb^+ efflux assay method to the values obtained using classical patch clamp recordings. We find that there is a consistent reduction in the potency of hERG-blocking drugs measured using the efflux assay when compared to patch clamp data. We suggest that this is in large part the result of the use of Rb^+ rather than K^+ in the efflux assay and the effects of this permeant ion on hERG channel inactivation.

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MATERIALS AND METHODS

Cell culture

A human embryonic kidney cell line, HEK293, stably expressing the hERG channel was used. Cells were dissociated for passage by using trypsin-EDTA and were maintained in minimum essential medium (MEM), 10% fetal bovine serum, penicillin-streptomycin, and 1 mg ml^{-1} gentamicin in an atmosphere of 5% CO_2 in air.

Materials

Cell culture supplies were purchased from Invitrogen. Astemizole, terfenadine, flecainide, quinidine, and lidocaine were obtained from Sigma-Aldrich (Mississauga, ON). Cisapride was purchased from Apin Chemicals (Oxfordshire, UK), dofetilide was obtained from Pfizer (Kent, UK), and E-4031 was obtained from Calbiochem (San Diego, CA, USA). Compounds A-G in Figure 1 were developed by Cardiome Pharma (Vancouver, Canada), and all the drugs were dissolved in 100% DMSO. The chemical reagents used to make the solutions were purchased from Sigma.

Rb^+ efflux assay solutions

All the solutions, with the exception of the 150 mM K^+ solution, were provided by Aurora Biomed Inc. (Vancouver, Canada) as described by Terstappen (1999). Briefly, cells were loaded with Rb^+ using the Rb^+ load buffer, which contained (mM): RbCl, 5.4; NaCl, 150; $CaCl_2$, 2; NaH_2PO_4, 0.8; $MgCl_2$, 1; glucose, 5; HEPES, 25; pH 7.4. Rb^+ was removed from the extracellular fluid using the Rb^+ wash buffer (same as above, but containing 5.4 mM KCl instead of RbCl). Cells were depolarized using the open-channel buffer, which consisted of (mM): KCl, 150; $CaCl_2$, 2; NaH_2PO_4, 0.8; $MgCl_2$, 2; glucose, 5; HEPES, 5; pH 7.4. To analyze Rb^+ concentration of the intracellular fluids, cells were lysed using 1% Triton X-100 solution (lysis-buffer).

Electrophysiology solutions

The pipette solution contained (mM): KCl or RbCl, 130; EGTA, 5; $MgCl_2$, 1; HEPES, 10; $Na^+{}_2ATP$, 4; GTP, 0.1, and was adjusted to pH 7.2 with KOH or RbOH. The bath solution contained (mM): KCl or RbCl, 5; NaCl, 135; $MgCl_2$, 1; sodium acetate, 2.8; and HEPES, 10; and was adjusted to pH 7.4 with NaOH. For recordings in the presence of high extracellular K^+ concentration, the bath solution contained (mM): KCl, 150; $MgCl_2$, 1; sodium acetate, 2.8; and HEPES, 10; and was adjusted to pH 7.4 with KOH.

Nonradioactive Rb-efflux assay procedure and analysis

Forty thousand to 50,000 cells, determined by a hemocytometer, were seeded into noncoated 96-well cell culture microplates (Falcon, Boston, MA, USA) and allowed to incubate for 24 h at 37 °C in an atmosphere of 95% air supplemented with 5% CO_2. After discarding the medium, 198 µL of open channel buffer and 2 µL of drug solution (stocks 30 nM-300 mM, depending on the appropriate concentration range for the compound) were added to each well, except the control wells. After allowing 2.5 h of incubation, the media were replaced by a mixture of 198 µL Rb^+ load buffer and 2 µL of drug and allowed to incubate for 3 h at 37 °C. Cell layers were then quickly washed 3 times with a mixture of 198 µL of Rb^+ wash buffer and 2 µL of drug, to remove extracellular Rb^+. Subsequently, a mixture of 198 µL channel opening buffer and 2 µL of drug were added to the wells, except the control wells, to activate the hERG channels. After incubation for 5 min, the supernatant was carefully removed and collected. Cells were lysed by addition of 200 µL of lysis-buffer. Samples were then stored at 4 °C until analysis on the ICR 8000.

The rubidium content of the cell supernatant and cell lysate was determined using the ICR 8000 (Aurora Biomed, Vancouver, Canada). Prior to analysis of each plate, the machine was calibrated using 0, 0.5, 1, 2, and 5 ppm Rb^+ standard solutions provided by Aurora Biomed (Vancouver, Canada). The analysis of plates would only be performed if the R^2 for the standard solutions was greater than 0.998.

After completion of analysis of both cell supernatant and cell lysates, the Rb^+ efflux ratio was measured using the following equation:

$$Rb^+ \text{ Efflux Ratio} = ([Rb^+]_e/([Rb^+]_e + [Rb^+]_i)), \quad (1)$$

where $[Rb^+]_e$ and $[Rb^+]_i$ are the Rb^+ concentrations in the extracellular and intracellular fluid, respectively.

During our experiments, we noted an endogenous Rb^+ leak current in the hERG-expressing HEK 293 cell line. Repeated measures indicated that the leak current comprised 20% to 30% of the efflux ratio. To determine the IC_{50} of any given drug, the Rb^+ efflux ratios were corrected for the endogenous Rb^+ leak current, which was determined for wells on each plate by measuring Rb^+ efflux in the absence of activating solution. The Rb^+ efflux ratios at any drug concentration were then normalized to the maximum Rb^+ efflux ratio that was obtained for each plate in the presence of high K^+ activating solution and absence of drug compounds. The IC_{50} was then calculated using NFIT (University of Galveston, TX, USA) using the following equation:

$$y = 1/(1 + (K/x)\text{^}n), \quad (2)$$

where y = normalized efflux ratio, x = drug concentration, K = IC_{50}, and n = Hill coefficient.

Electrophysiological procedures and analysis

Glass coverslips to which cells had adhered were removed from the incubator immediately prior to experiments and were placed in a saline-filled recording chamber mounted on the stage of an inverted phase contrast microscope. The bath solution was con-

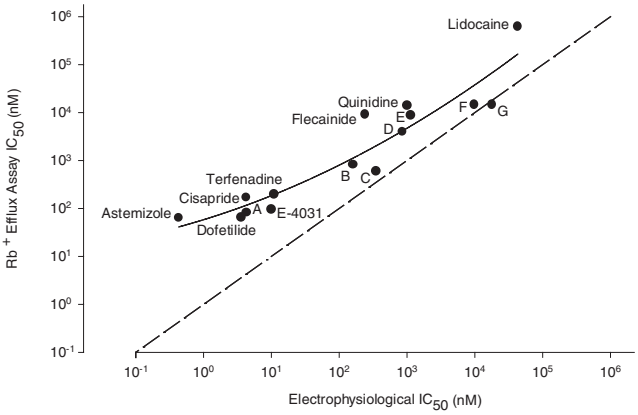

FIG. 1. Correlation of the electrophysiological IC_{50}s with the Rb^+ efflux assay IC_{50}s. Drug potencies on the hERG channel were obtained using either the Rb^+ efflux assay or patch clamp with K^+ as the permeating cation. Data were obtained for 15 compounds. IC_{50}s were determined from fitting the Hill equation to 4 determinations with 6 or 7 concentrations for each compound. The unbroken line is a nonlinear least-squares best fit through data points and has an R^2 value of 0.83. The broken line is the line of identity and is for reference only.

stantly flowing. Whole-cell current recording and data analysis were performed using an Axopatch 200B amplifier and pClamp8 software (Axon instruments, Foster City, CA, USA). Patch electrodes fashioned from thin-walled borosilicate glass (World Precision Instruments, FL, USA) had a resistance of 1.5-2.5 MΩ when filled with the pipette solutions.

To asses the potency of compounds in the presence of 5 mM K^+_o/130 mM K^+_i or 5 mM Rb^+_o/130 mM Rb^+_i, whole-cell hERG currents were activated by 4-s depolarizing pulses to +20 mV from a holding potential of –80 mV, and tail currents were recorded during 6-s repolarizing steps to –50 mV. To examine the effect of high extracellular K^+ concentration on the potency of compounds, whole-cell hERG currents were elicited by 4-s depolarizing pulses to +20 mV from a holding potential of –80 mV, and tail currents were recorded during 8-s repolarizing steps to –80 mV. Pulses were applied every 11 s. Drug effects were quantitated by measuring the reduction of the peak tail current in the presence of increasing concentrations of compounds. The concentration of compound was increased when tail current amplitude reached a steady level at each concentration. The IC_{50} values were then calculated by fitting the dose-response relationships with the Hill equation (2).

Activation curves were determined by recording hERG currents elicited by a 4-s depolarization from a holding potential of –80 mV, increasing in 10 mV increments from –80 mV to +110 mV, followed by a 6-s repolarization to –50 mV. Peak tail currents were normalized to the maximum current and plotted versus the prepulse potential. The normalized data for each cell were then fit with a single Boltzmann function:

$$y = 1/1(1 + \exp(V_{1/2} - V)/k)), \quad (3)$$

where y is the current normalized with respect to the maximal current, $V_{1/2}$ is the half-activation potential, V is the test voltage, and k is the slope factor.

The voltage dependence of hERG inactivation was studied using a triple-pulse protocol. hERG currents were 1st activated by a 500-ms depolarization to +60 mV. Recovery from any inactivation that occurred was induced by a brief 20-ms hyperpolarizing pulse to –100 mV. The 3rd pulse to a range of potentials (–100 to +90 mV) allowed activated channels to inactivate again. Inactivation was measured during the 3rd pulse as the residual current at 100 ms. For each cell, the residual current was normalized to the maximum before inactivation, plotted as a function of potential and fitted to a negative Boltzmann function (3); note that in the case of inactivation, $V_{1/2}$ corresponds to the half-inactivation potential. All data analysis was done with Clampfit (Axon Instruments), and curve fitting with NFIT. Data are shown as means ± SEM. Statistical significance was determined using Student's *t*-test.

RESULTS

The nonradioactive Rb^+ efflux assay is potentially a convenient and powerful screening assay for hERG channels, as they readily conduct Rb^+, and a little Rb^+ is present in biological fluids to interfere with measurements.[15] We have compared potencies for hERG channel block by a number of known and proprietary channel blockers (letters A-G in Fig. 1) using the Rb^+ efflux assay, with the electrophysiological IC_{50} values determined for hERG channels expressed in the same HEK cell line under physiological conditions (5mM K^+_o/130 mM K^+_i). The data in Fig. 1 show that the IC_{50} values determined for hERG blockers using the Rb^+ efflux assay are displaced upwards relative to the electrophysiological IC_{50} values for hERG blockers, and that this disparity is more marked for more potent blockers. When the electrophysiological IC_{50} values are in the submicromolar range, the ability of the Rb^+ efflux assay to predict the IC_{50} values deteriorates. Using 5 of the agents that performed poorly in the efflux assay, dofetilide, astemizole, cisapride, terfenadine, and E-4031, we have investigated potential mechanisms for the disparity in potencies between the Rb^+ efflux assay and patch clamp values.

First, we constructed concentration-response relationships using the Rb^+ efflux assay for all the drugs indicated in Fig. 1. The relationships for cisapride (Fig. 2A), astemizole (Fig. 2B), dofetilide (Fig. 2C), and terfenadine (Fig. 2D) are shown and have IC_{50} values of 173, 65, 66, and 198 nM, respectively, with Hill coefficients of –1.1, –1.4, –1.5, and –2.0, respectively. The IC_{50} values were significantly greater than those determined using the patch-clamp technique (Table 1), whereas the Hill coefficients resemble those determined electrophysiologically, with the exception of terfenadine (Fig. 5). It must be noted that although we still could not accurately determine the potency of drugs on hERG, we were able to determine a drug rank order in reasonable agreement with that obtained from the patch clamp data. One obvious difference between the 2 assay systems is the use of Rb^+ in the efflux assay

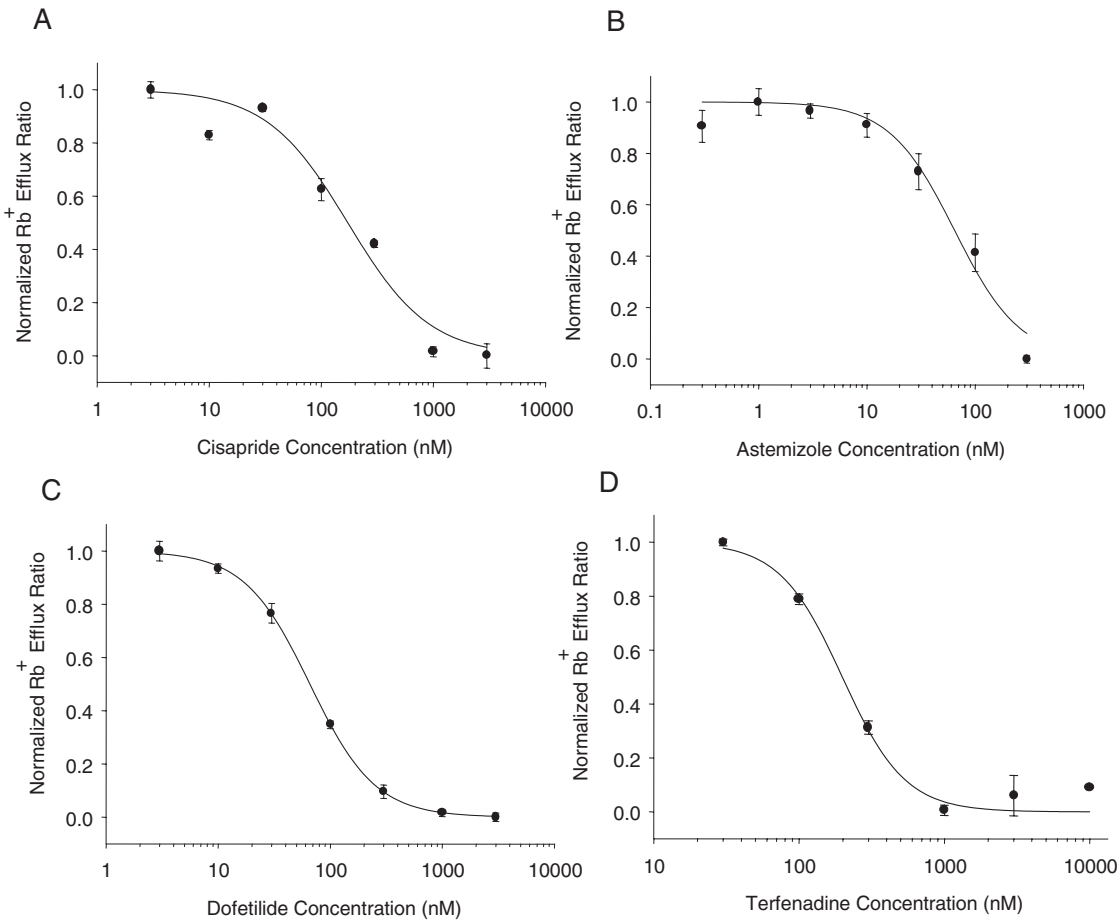

FIG. 2. Dose-response relationships determined using the Rb^+ efflux assay. Effects of cisapride (**A**), astemizole (**B**), dofetilide (**C**), and terfenadine (**D**) on hERG channels stably expressed in HEK cells. The Rb^+ efflux assay was carried out as described in Methods. Drugs were present during all steps of the experimental protocol. The Rb^+ efflux ratios were corrected for the endogenous Rb^+ leak current and then normalized to the maximum Rb^+ efflux ratio. Data were fit with the Hill equation (Methods), and the IC_{50} values and slopes, respectively, were, cisapride, 173 nM and 1.1; astemizole, 65 nM and 1.4; dofetilide, 66 nM and 1.5; terfenadine, 198 nM and 2.0. Error bars denote means ± SEM of 4 measurements.

versus the use of K^+ in the patch clamp solutions. This raised the possibility that differences in conducting ions between the assays were responsible for the discrepancy in potency measurements.

We answered this question by comparing drug potencies in patch clamp experiments after replacing the K^+ in the extracellular and intercellular solutions with Rb^+, to mimic the conditions in the Rb^+ efflux assay. Data in Figs. 3 and 4 show K^+ and Rb^+ patch clamp data for hERG channel block by 2 potent hERG blockers (cisapride and astemizole). In K^+ (Fig. 3A), a slow rising current was apparent upon depolarization to +20 mV from a holding potential of –80 mV, and a slow deactivating tail current was observed upon repolarization to –50 mV. In Rb^+, however, the rising current during the initial depolarization was different from that in K^+ due to the presence of a sharp initial peak. The peak tail current amplitude recorded under control conditions in the presence of Rb^+ (Fig. 3B, panel a) was also greater than that in the presence of K^+ (Fig. 3A). Peak initial current amplitudes were 25 ± 2 and 170 ± 17 pA/pF in the presence of K^+ and Rb^+, respectively ($P < 0.0001$, $n = 20$), whereas peak tail current amplitudes were 70 ± 6 and 200 ± 20 pA/pF ($P < 0.0001$, $n = 20$). The change in the hERG current amplitude suggests that the species of conducting cation can significantly modulate channel behavior. The diary of each experiment is shown below (Figs. 3C, D), with each point representing the peak tail current amplitude during the 2nd step of the voltage clamp protocol. The graph shows the effect of increasing concentrations of cisapride on tail currents with either K^+ (Fig. 3C) or Rb^+ (Fig. 3D) as the conducting cation. It is clear that in the presence of Rb^+ an increased concentration of cisapride is required to create the same level block as that of the K^+ current. For example, addition of 10 nM cisapride caused a 70% reduction in the peak tail current in the presence of K^+, whereas a 30% reduction was observed in the presence of Rb^+ ions. Figure 4 illustrates a similar experiment us-

Table 1. Summary of IC_{50} Values Determined Using Patch-Clamp Technique or Rb^+ Efflux Assay

	Electrophysiological IC_{50} (nM)		*Rb^+ Efflux Assay IC_{50} (nM)*	*Potency Ratio*		
	K^+_o/K^+_i	Rb^+_o/Rb^+_i		*Rb^+ Patch Clamp/K^+ Patch Clamp*	*Rb^+ Efflux Assay/K^+ Patch Clamp*	*Rb^+ Efflux Assay/Rb^+ Patch Clamp*
Cisapride	4.3 ± 0.3	22 ± 2	173	5.1	40	7.8
Astemizole	0.43 ± 0.09	3.5 ± 0.4	65	8.1	150	18
Dofetilide	3.8 ± 0.2	26 ± 2	66	6.8	17	2.5
Terfenadine	11 ± 2	29 ± 5	198	2.6	19	6.9
E-4031	11 ± 3	115 ± 17	95	11	8	0.81

Note. The electrophysiological IC_{50}s were determined using patch clamp with 5 mM external cation and 130 mM internal cation, either Rb^+ or K^+. These are denoted as K^+_o/K^+_i or Rb^+_o/Rb^+_i. The Rb^+ efflux assay was carried out as described in Methods. The patch clamp potencies obtained with the different permeant cations methods are compared to those obtained using the Rb^+ efflux assay as expressed by ratios in columns 5 and 6.

ing the highly potent agent astemizole. As for cisapride, astemizole exhibited a reduced ability to block the hERG current in the presence of Rb^+. It is apparent for both these agents that little change in tail current kinetics occurs during the application of different concentrations of drug.

The relationships in Figure 5 summarize the effect of Rb^+ on the potency of cisapride (A), astemizole (B), dofetilide (C), and terfenadine (D), in blocking hERG currents. The peak tail amplitude at each drug concentration was normalized to the maximum peak tail current and plotted against the drug concentration. In the presence of Rb^+, the IC_{50} values increased by 3- to 10-fold (Table 1). From these data, it is apparent that although Rb^+ reduces the potency of drugs used to block hERG channels, it does not affect the number of putative binding sites on the channel, as the slopes of the relations remain close to 1.0.

Table 1 summarizes the IC_{50} values for 5 blockers determined using 5 mM K^+_o/130 mM K^+_i and 5 mM Rb^+_o/130 mM Rb^+_i in patch clamp experiments and the IC_{50} values obtained using the Rb^+ efflux assay. In general it can be seen that substitution of Rb^+ for K^+ in the patch clamp experiments results in ~ 10-fold reduction in blocker potency. The table also compares potency ratios using these different techniques. When Rb^+ is used as the conducting ion in patch clamp experiments, the drug potencies are much closer to those obtained using the efflux assay, with potency ratios between 0.81 and 18. From this table, it appears that the difference between drug potencies obtained using the Rb^+ efflux assay and K^+ in the patch clamp can be partly explained by the use of Rb^+ as opposed to K^+ as the conducting ion. However, it should be noted that real

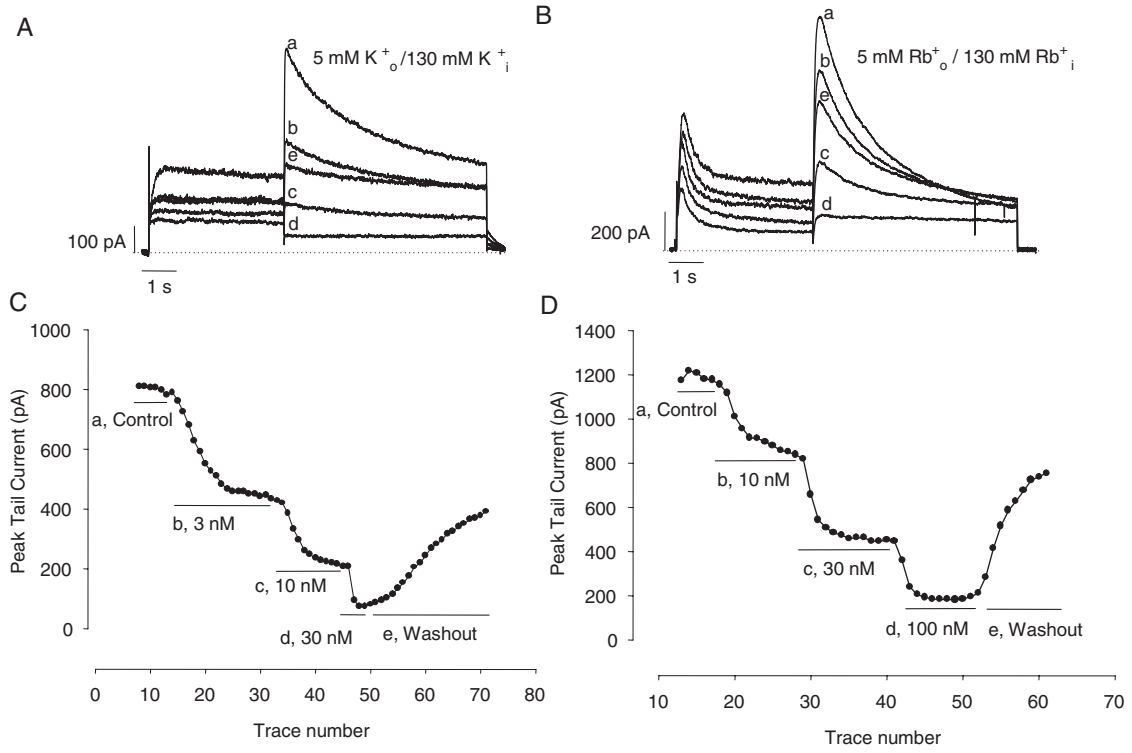

FIG. 3. Effect of cisapride on hERG Rb^+ and K^+ currents. Drug effects were quantitated by measuring the reduction of the peak tail current in the presence of increasing concentrations of compound (Methods). **A** and **B**, Traces obtained at different concentrations of cisapride in 5 mM K^+_o/130 mM K^+_i (**A**) or 5 mM Rb^+_o/130 mM Rb^+_i (**B**). The dotted line represents the zero current level. **C** and **D**, Time course of cisapride action on hERG K^+ current (**C**) and Rb^+ current (**D**). The amplitude of peak tail currents was plotted against the trace number. Letters on record at each concentration correspond to traces plotted above in **A** and **B**.

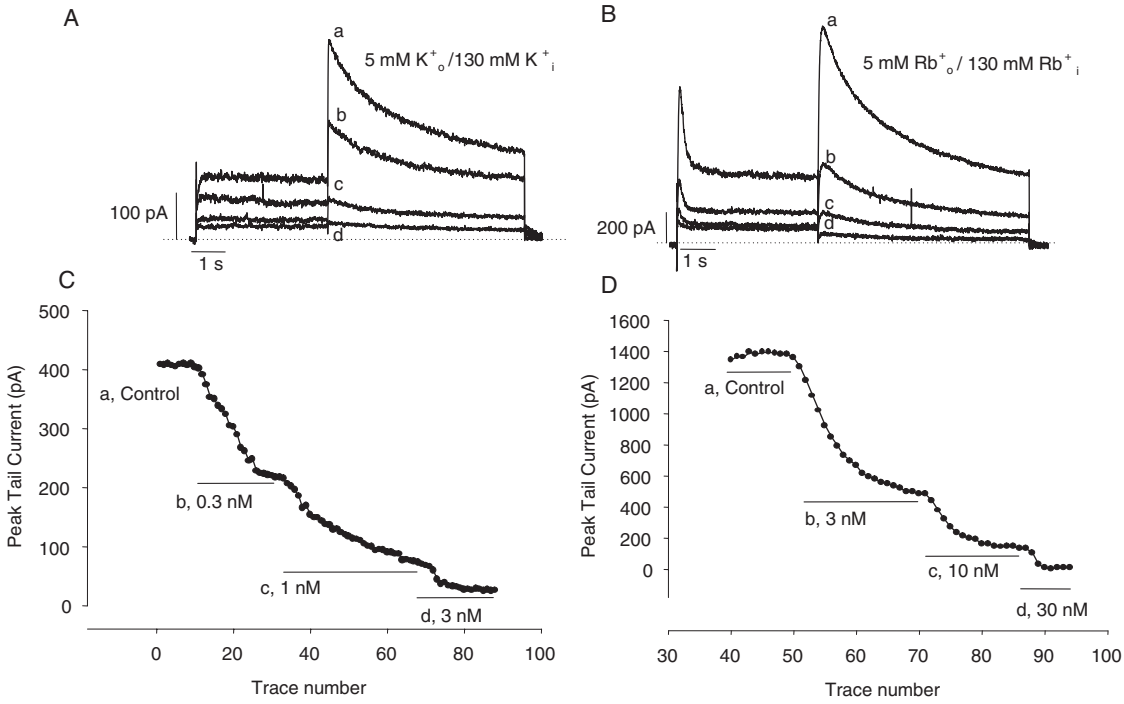

FIG. 4. Effect of astemizole on hERG Rb^+ and K^+ currents. Drug effects were quantitated by measuring by the reduction of the peak tail current in the presence of increasing concentrations of compound (Methods). **A** and **B**, Traces obtained at different concentrations of astemizole in 5 mM K^+_o/130 mM K^+_i (**A**) or 5 mM Rb^+_o/130 mM Rb^+_i (**B**). The dotted line represents the zero current level. **C** and **D**, Time course of astemizole action on hERG K^+ current (**C**) and Rb^+ current (**D**). The amplitude of peak tail currents was plotted against the trace number. Letters on record at each concentration correspond to traces plotted above in **A** and **B**.

differences remain between the potencies determined using the 2 methods. Additional reasons for these differences are considered in the Discussion.

To determine if a change in the hERG channel kinetics of activation and/or inactivation could also be partly responsible for the difference in drug potencies with K^+ or Rb^+, we examined the channel kinetics in the 2 solutions. Data on the hERG half-activation potential ($V_{1/2}$) in Rb^+ are shown in Figure 6A. An outward current was activated at voltages positive to –40 mV, and current amplitude increased to reach a maximum at 0 mV. With further depolarization, current amplitude decreased again. This is explained by the inwardly rectifying property of hERG channels. A similar trend was observed for K^+ current, although the current spikes on activation were not observed (see also Fig. 3). The peak tail currents were normalized to the maximal value, plotted against the prepulse potential, and fitted to a Boltzmann equation (Fig. 6B). The $V_{1/2}$ and slope factor (k) in 5 mM K^+_o/130 mM K^+_i were –28 ± 1.3 mV and 5.9 ± 0.20 mV, respectively. The corresponding values in 5 mM Rb^+_o/130 mM Rb^+_i were –20 ± 2.3 mV and 7.6 ± 0.6 mV. Therefore, replacement of K^+ ions resulted in only small changes in the activation kinetics of hERG.

It has been reported that the presence of Rb^+ in the extracellular solution results in slowing of hERG inactivation.[16] Inclusion of Rb^+ in both intracellular and extracellular solutions in our experiments seemed to have a similar effect (Fig. 7). The inactivation relationship was measured using the protocol described by Zhang et al.[16] In brief, the cell was depolarized to +60 mV for 500 ms from the holding potential of –80 mV, to activate and inactivate hERG channels. The cell was then hyperpolarized briefly to –100 mV for 20 ms to allow recovery from inactivation with minimal deactivation. This was followed by step test pulses in the range –100 mV to +90 mV. The amount of inactivation was measured during the 3rd pulse as a ratio of peak instantaneous current to that measured 100 ms later. This ratio was normalized to the maximum value, plotted against the 3rd pulse potential, and fitted with a Boltzmann equation. A direct comparison of 3rd-pulse data in Fig. 7A (K^+ current) and 7B (Rb^+ current) shows that Rb^+ modulates hERG inactivation, because there are much larger tail currents when Rb^+ is the conducting ion. The substitution of K^+ with Rb^+ resulted in a shift of the half-inactivation potential (Fig. 7C) to more positive potentials by ~ +30 mV (from –33 to 0 mV), without much change in the slope factor (–17 ± 0.8 mV to –14 ± 0.6 mV, $n = 6$).

In the Rb^+ efflux assay, hERG channels were activated by using a 150 mM K^+ solution. To see if there was an effect of elevated extracellular [K^+] on the affinity of drugs measured under patch clamp, we examined the potency of cisapride on hERG in the presence of 150 mM extracellular K^+. In the presence of an elevated external [K^+] (Fig. 8A), a slowly deactivating inward tail current was

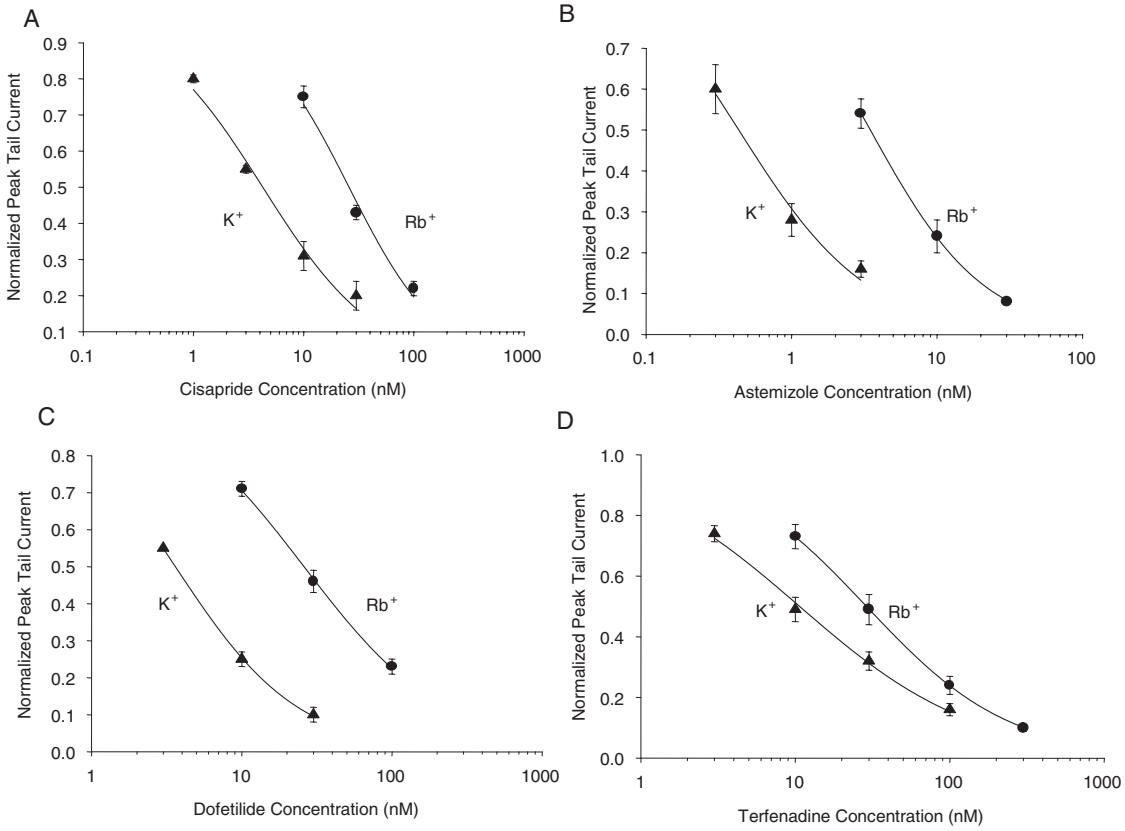

FIG. 5. Patch clamp dose-response relationships using K^+ or Rb^+ as the charge carrier. Dose-response relationships obtained from tail currents measured during patch clamp experiments in 5 mM K^+_o/130 mM K^+_i (triangles) are compared with those obtained in the presence of 5 mM Rb^+_o/130 mM Rb^+_i (circles). **A**, The IC_{50} values and Hill coefficients were 4.3 nM and 0.83, respectively, for cisapride in K^+ ($n = 3$), and 22 nM and 0.83 in Rb^+ ($n = 3$). **B**, For astemizole, the IC_{50} values and Hill coefficients were 0.43 nM and 0.96 in K^+ ($n = 7$) and 3.5 nM and 1.1 in Rb^+ ($n = 3$). **C**, For dofetilide, the IC_{50} values and coefficients were 3.8 nM and 1.0 in K^+ ($n = 3$) and 26 nM and 0.91 in Rb^+ ($n = 3$). **D**, For terfenadine, the IC_{50} values and coefficients were 11 nM and 0.76 in K^+ current ($n = 4$) and 28.9 nM and 0.94 in Rb^+ ($n = 4$).

observed in control conditions (Aa) upon repolarization to –80 mV. The diary of the experiment is shown below (Fig. 8B), with each point representing the peak inward tail current amplitude during the repolarizing step of the voltage clamp protocol. The graph shows the effect of increasing concentrations of cisapride on tail currents (Fig. 8C). Individual current tracings obtained at different cisapride concentrations as indicated in panel B are shown above in panel A. The concentration-response relation for cisapride is shown in Fig. 8C and shows that the IC_{50} value for cisapride was increased 4-fold from 4.3 nM in 5 mM K^+_o/130 mM K^+_i to 18 mM in the presence of a high extracellular [K^+]. It is clear from this result that the presence of high $[K^+]_o$ modified the cisapride block of hERG channels.

DISCUSSION

In this study, we have shown that drug potencies measured in the Rb^+ efflux assay are somewhat higher than those recorded using the patch clamp technique with K^+ as the conducting ion (Figs. 1, 2, and Table 1). Our hypothesis is that this is related to the different cations (Rb^+ vs. K^+) used in the 2 assays. Indeed, when we patch clamped hERG currents using Rb^+ as the conducting cation, instead of K^+, blocking drug potencies were much closer to those obtained using the efflux assay (Figs. 3-5, Table 1). Along with changes in drug potency, we also saw that the presence of Rb^+ (5 mM Rb^+_o/130 mM Rb^+_i) affected the inactivation of hERG currents (Figs. 3, 4). There were spikes of current on depolarization, larger tail currents and a significant shift in the half-inactivation potential from –33 mV to –0.9 mV (Fig. 7), whereas the effect on the half-activation potential was insignificant (Fig. 6).

It is well understood that drug binding to the hERG channel requires activation,[4,5] but whether the block for most drugs then occurs via the inactivated state or the open state has been controversial. It has been reported that the removal of the rapid inactivation, which has been labeled as C-type inactivation,[17] resulted in the reduction of the sensitivity of hERG channels to E-4031 and

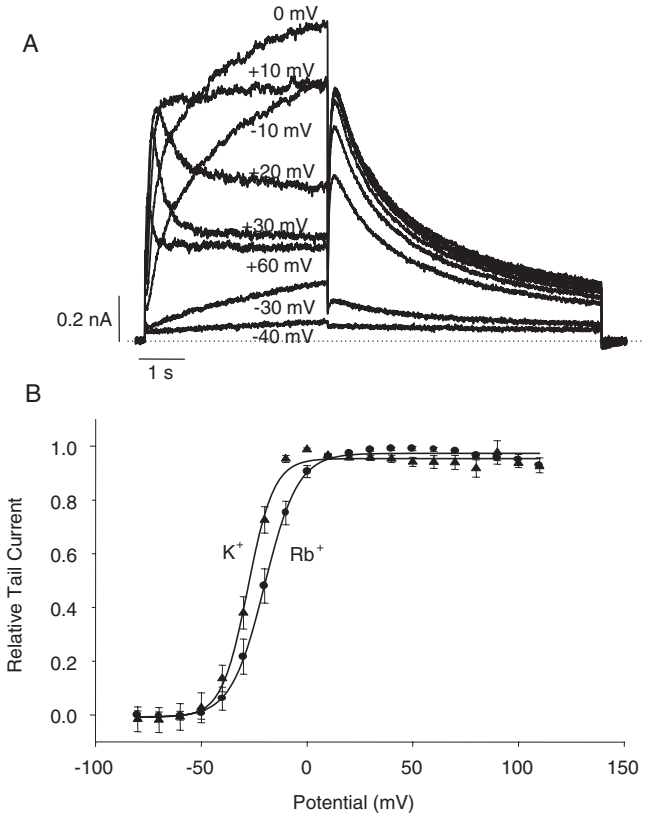

FIG. 6. Rb^+ has little effect on the voltage dependence of hERG activation. **A**, From a holding potential of –80 mV, hERG currents in 5 mM Rb^+_o/130 mM Rb^+_i were elicited by a 4-s depolarization, increasing in 10 mV increments from –80 mV to +110 mV, followed by a 6-s repolarization to –50 mV. **B**, Voltage dependence of hERG activation in the presence of K^+ (triangles) and Rb^+ (circles). The mean half-activation voltage ($V_{1/2}$) and the slope factor (k) in 5 mM K^+_o/130 K^+_i mM were –28 ± 1.3 mV and 5.9 ± 0.2 mV, respectively ($n = 9$). In 5 mM Rb^+_o/130 Rb^+_i mM, the values were –20 ± 2.3 mV and 7.6 ± 0.6 mV ($n = 6$).

dofetilide.[18,19] Mitcheson et al.[20] showed that hERG mutations that shifted the inactivation curve toward either negative or positive directions resulted in the loss of sensitivity to drugs. These results underline the importance of the C-type inactivation in high-affinity drug binding and also suggest the involvement of other factors. Furthermore, hERG inactivation is intrinsically voltage dependent (Fig. 7A), due to substantial transmembrane movement of charge.[21] Thus, it is possible that the significant conformational changes accompanying inactivation provide a high-affinity binding site for drugs. Such a role for inactivation in the binding of drugs to hERG channels provides a ready explanation for the reduced affinity of drug binding to Rb^+-conducting channels, because we have clearly shown that Rb^+ conduction impedes the rate, degree, and voltage dependence of inactivation of the channel.

The changes in channel inactivation when conducting Rb^+ rather than K^+ suggest that the species of conducting ion can significantly alter the outer pore conformation of the channel. It is also possible that these changes themselves can alter drug affinity as well as their effects on the inactivation process. In Kv2.1 channels, block by tetraethylammonium (TEA) is also modulated by the permeating ions.[22] Replacement of K^+ by Na^+ can completely prevent the action of the drug on the channel. Two possible explanations are given for this result. The 1st is that the smaller ion can pass unimpeded through the channel with TEA bound, and the 2nd (and favored explanation) is that K^+ conditions the binding site for TEA on the channel. Replacement of K^+ by Na^+ alters the specific binding pocket for the drug and reduces its affinity for the channel. In our experiments, it is likely that an analogous event is occurring but one that is less complete, due to the closer similarities between K^+ and Rb^+ than K^+ and Na^+.

In addition to the species effect of Rb^+ on the channel outer pore conformation and the inactivation curve, increasing the concentration of extracellular K^+ also affected the potency of cisapride block of hERG channels (Fig. 8). It is known that elevation of extracellular K^+ also slows hERG inactivation.[16] Because the open channel buffer in the Rb^+ efflux screening assay contained 150 mM [K^+], this could potentially reduce the sensitivity of the assay, compared with the patch clamp experiments, which were carried out at 5 mM extracellular K^+. Increased extracellular K^+ is also reported to reduce the affinity of E-4031, which has a net single positive charge, to WT hERG channels, possibly due to electrostatic repulsion.[18] Furthermore, the E_K was +3.6 mV at room temperature in the Rb^+ efflux assay, based on an unchanged 130 mM internal [K^+] during Rb^+ loading. At this potential, at any given time, only about 50% of the hERG channels are in the inactivated state (Fig. 7C), and this might also contribute to the reduction in drug sensitivity. From Table 1, electrophysiological IC_{50}s determined in the presence of Rb^+ ions are much closer to the values obtained using the Rb^+ efflux assay than those obtained using K^+ in the patch clamp measurements. Further differences that exist may be attributable to the increased $[K^+]_o$ as discussed above.

CONCLUSION

We have shown in patch clamp experiments that the use of different conducting ions, K^+ or Rb^+, causes significant changes in the potencies of a number of drugs that block hERG channels. Rb^+ replacement of K^+ results in a significant slowing of hERG channel inactivation, as a result of the positive shift in the voltage dependence of the inactivation relationship. Because many drugs that block hERG channels bind preferentially to inactivated states of the channels, block of Rb^+-conducting channels is reduced. Conditioning of the drug binding site by the conducting ion species and differences in the concentrations of extracellular ions present during the experiments are also partly responsible for the differences in potencies of drug block measured between standard patch clamp experiments and the Rb^+ efflux assay. Thus, a major limitation of the Rb^+ efflux assay is to accurately predict the potency of drugs with high affinity for hERG. Although the Rb^+ efflux assay is not able to accurately predict the potency of high-affinity hERG

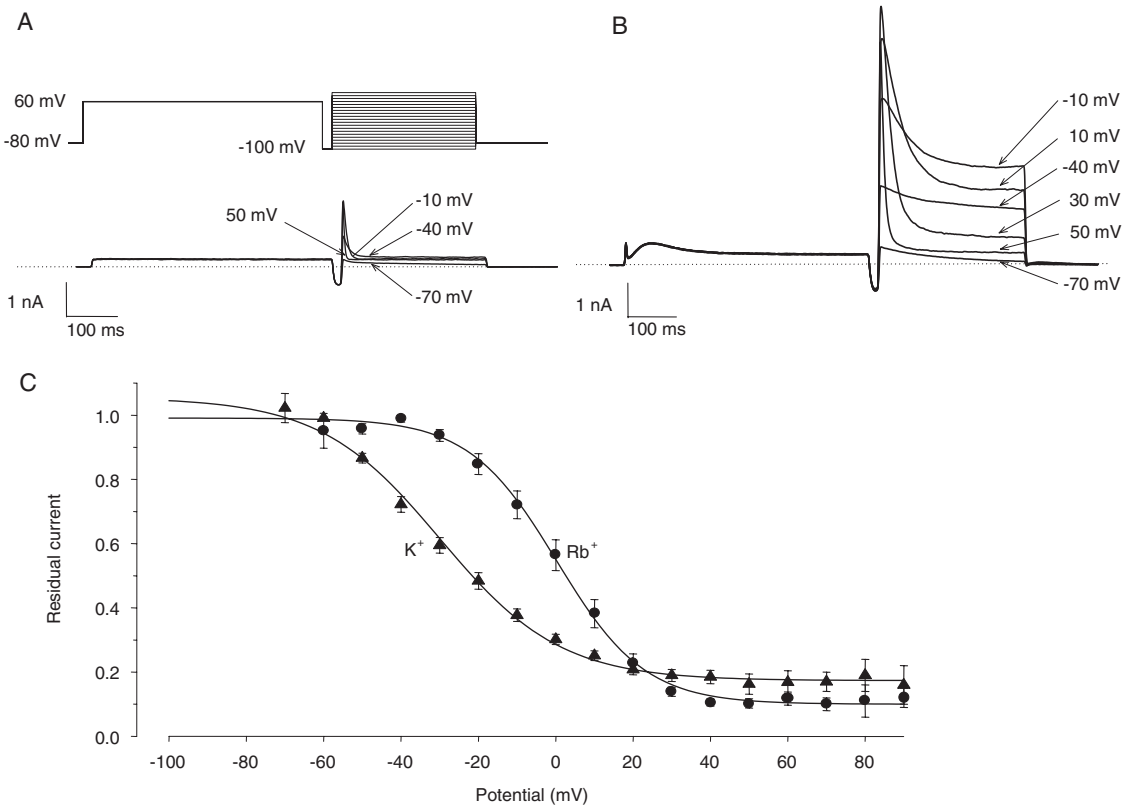

FIG. 7. Rb^+ shifted the hERG inactivation relationship to positive potentials. Inactivation was studied using a triple-pulse protocol, shown above the current traces and described in the Methods section. Current tracings were obtained in 5 mM K^+_o/130 mM K^+_i (**A**) and 5 mM Rb^+_o/130 mM Rb^+_i (**B**). **C**, Inactivation was measured during the 3rd pulse as the residual current at 100 ms. For each cell, the residual current was normalized to the maximum before inactivation, plotted as a function of potential, and fitted to a Boltzmann function. The $V_{1/2}$ and k were –33 ± 2.2 mV and –17 ± 0.8 mV for 5 mM K^+_o/130 mM K^+_i (n = 6-9), and –0.9 ± 3.6 mV and –14 ± 0.6 mV for 5 mM Rb^+_o/130 mM Rb^+_i (n = 6). The change in half-inactivation potential is highly significant, $P < 0.001$.

blockers, it does provide a rapid means of rank-ordering test compounds based on approximate potencies.

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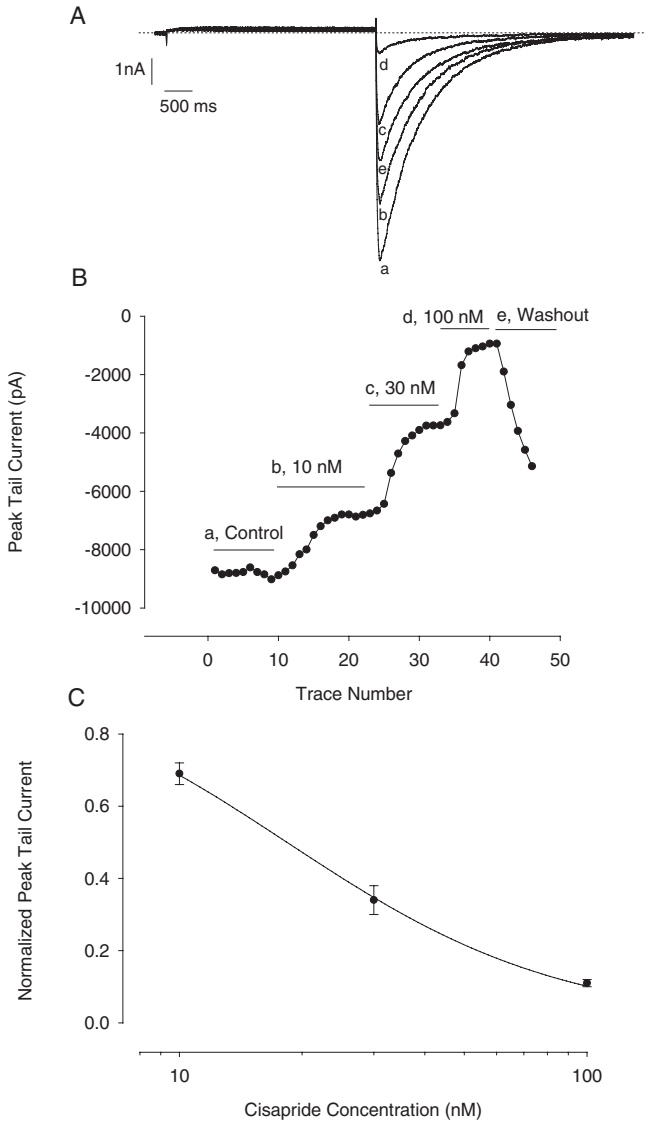

FIG. 8. High extracellular K^+ reduced the affinity of cisapride block of hERG channels. **A**, Peak tail currents were recorded at –80 mV in control (a), and in the presence of increasing concentrations of cisapride (for clamp protocol, see Methods), with 150 mM extracellular [K^+] in the bath. The dotted line represents the zero current level. **B**, Time course of cisapride action on hERG inward tail currents. The amplitude of peak tail currents was plotted against the trace number and the letters at each concentration correspond to traces plotted above in **A**. **C**, Dose-response relationship obtained from tail currents. The IC_{50} value and Hill coefficient were 18 ± 2 nM and 1.3 ± 0.1, respectively (n = 3).

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