Role of the activation gate in determining the extracellular potassium dependency of block of HERG by trapped drugs

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Abbreviations: diLQTS, drug-induced long QT syndrome; LQTS, long QT syndrome; HERG, human-ether-a-go-go-related gene; TEA, Tetraethylammonium; DMSO, dimethyl sulfoxide; 0K, 0 mM (no added) potassium; 2K, 2 mM potassium; 20K, 20 mM potassium; 20Cs, 20 mM cesium; 20Rb, 20 mM rubidium; 20NH₄, 20 mM ammonium; 40TEA, 40 mM tetraethylammonium

**Introduction**

Long QT syndrome (LQTS) is a potentially lethal cardiac arrhythmia characterized by a prolonged QT interval on an electrocardiogram. One form of long QT syndrome, referred to as drug induced long QT syndrome (diLQTS)\(^1\) has been shown to primarily result from a reduction in \(I_{Kr}\), a potassium current important in repolarizing the cardiac action potential, by a large number of diverse pharmaceutical compounds.\(^2\) Reduction in \(I_{Kr}\) can result from block of the human ether-a-go-go related potassium channel HERG (human-ether-a-go-go related gene). In some cases long QT syndrome can result in the lethal arrhythmia torsade de pointes, a rapid heart rate and severely compromised cardiac output. Many patients requiring medication present with serum potassium abnormalities due to a variety of conditions including gastrointestinal dysfunction, renal and endocrine disorders, diuretic use and aging. Extracellular potassium influences HERG channel inactivation and can alter block of HERG by some drugs. However, block of HERG by a number of drugs is not sensitive to extracellular potassium. In this study, we show that block of WT HERG by bepridil and terfenadine, two drugs previously shown to be trapped inside the HERG channel after the channel closes, is insensitive to extracellular potassium over the range of 0 mM to 20 mM. We also show that bepridil block of the HERG mutant D540K, a mutant channel that is unable to trap drugs, is dependent on extracellular potassium, correlates with the permeant ion and is independent of HERG inactivation. These results suggest that the lack of extracellular potassium dependency of block of HERG by some drugs may in part be related to the ability of these drugs to be trapped inside the channel after the channel closes.

Hypokalemia is a common medical condition \(^4\) which can lead to dangerous arrhythmias\(^4\) and is a known risk factor for long QT syndrome (LQTS).\(^2\) Although the driving force on the potassium ion is increased in low extracellular potassium, paradoxically, HERG current amplitude is reduced in low extracellular potassium. This reduction in HERG current amplitude in low extracellular potassium has been studied by a number of different investigators.\(^5-8\) A number of mechanisms have been proposed to explain the decrease in HERG current amplitude in low extracellular potassium, including an increase in block by extracellular sodium\(^6\) an increase in the rate of inactivation,\(^8\) a decrease in single channel conductance,\(^7\) and a decrease in cell surface channel density.\(^9\)

Hypokalemia has also been implicated as a risk factor for drug induced long QT (diLQTS)\(^1\) although the mechanisms that underlie this risk have not been studied extensively. One possible explanation for the increase in the risk for diLQTS in low extracellular potassium is an increase in drug blockade of HERG in low extracellular potassium. It has been shown that block of HERG by a number of different drugs, including quinidine and cisapride is reduced with an increase in extracellular potassium.\(^8,10,11\) However additional studies have shown that block of...
HERG by other drugs (for example dofetilide) is not sensitive to extracellular potassium.\textsuperscript{12,13}

It seems reasonable to ask whether there is a relationship between the extracellular potassium dependency of block of HERG by a drug and the mechanism by which a drug blocks HERG. A number of different mechanisms have been proposed to explain block of HERG by a diverse set of compounds. A number of drugs including methanesulfonalides (MK-499, dofetilide) and propafenone have been shown to be trapped within the inner vestibule upon closure of the activation gate.\textsuperscript{14-16} Other drugs, in particular quinidine and chloroquine, have been shown to slow channel closing.\textsuperscript{17,18} Finally, although the vast majority of HERG blockers are sensitive to mutations in the HERG channel at residue F656\textsuperscript{19,20} a few compounds do not show large reductions in block of HERG channels with mutations at residue F656 (i.e., fluvoxamine, dronedarone, amiodarone).\textsuperscript{21,22}

In this paper we show that block of HERG by two drugs, bepridil and terfenadine, is not sensitive to extracellular potassium. This is in contrast to a previous report in which we showed in an identical expression system, that block of HERG due F656\textsuperscript{19,20} a few compounds do not show large reductions in block of HERG by either quinidine or cisapride. Bepridil showed a much greater difference in block of WT HERG with block of the HERG mutant D540K between 0 mM K and so was used for the remainder of the experiments in block of the HERG mutant D540K by both bepridil and terfenadine, shows a similar extracellular potassium dependency to block of WT HERG by bepridil or terfenadine. Block of WT HERG by 1 \textmu M bepridil or 1 \textmu M terfenadine is similar in both 0 mM and 20 mM extracellular potassium. This is in contrast to the reduction in block of WT HERG by either quinidine or cisapride with an increase in extracellular potassium from 0 to 20 mM.\textsuperscript{10}

Results

Block of HERG mutant D540K by bepridil and terfenadine. Figure 1 shows experiments illustrating the effect of extracellular potassium on block of WT HERG by either bepridil or terfenadine. Block of WT HERG by 1 \textmu M bepridil or 1 \textmu M terfenadine is similar in both 0 mM and 20 mM extracellular potassium. Thus block of the HERG channel after channel closure,\textsuperscript{16} whereas quinidine and cisapride cannot be trapped in the channel after channel closure.\textsuperscript{16,17} In order to determine if the lack of extracellular potassium dependent block of WT HERG by bepridil or terfenadine is related to the ability of these drugs to be trapped in the channel after the channel closes, block of the HERG mutant D540K by bepridil and terfenadine in low and high extracellular potassium was measured. The HERG mutant D540K shows an unusual gating behavior in that it opens both with membrane depolarization (similar to WT HERG) and with membrane hyperpolarization (unlike WT HERG).\textsuperscript{23} As a result, the HERG mutant D540K cannot trap drugs at negative voltages since the activation gate does not close.\textsuperscript{15} The results of block of D540K by bepridil and terfenadine are shown in Figure 2 and show that block of the HERG mutant D540K by bepridil and terfenadine is reduced with elevated extracellular potassium. Thus block of the HERG mutant D540K by both bepridil and terfenadine, shows a similar extracellular potassium dependency to block of WT HERG by quinidine or cisapride. Bepridil showed a much greater difference in block of the HERG mutant D540K between 0 mM K and 20 mM K and so was used for the remainder of the experiments described below. Figure 3 shows dose response curves comparing block of WT HERG with block of the HERG mutant D540K by bepridil in 0K and 20K.

Block of D540K by bepridil in different extracellular solutions. Previously we have shown that block of WT HERG by
quinidine and cisapride, two drugs that are not trapped inside the channel after channel closure, correlates with the permeant ion and not with inactivation, at least in the range of extracellular potassium from 0 to 20 mM. If the potassium dependency of block of WT HERG by bepridil and terfenadine is related to the ability of these drugs to be trapped inside the channel, then block of D540K by these drugs should be dependent on the permeant ion in the same way that block of WT HERG by quinidine or cisapride is dependent on the permeant ion. To address this we measured block of D540K in the presence of different extracellular cations with different permeabilities through the HERG channel.

Figure 4 shows reversal potential measurements in bionic conditions for WT HERG and for both the depolarization activated (D540K Dep) and hyperpolarization activated (D540K Hyp) HERG mutant D540K channels for the cations, TEA, NH₄⁺, Cs and Rb. The permeability ratio (P_cation/P_K) are given in Table 1. Both WT HERG and the HERG mutant D540K show the same relative permeability sequence (P_K ≈ P_Rb > P_Cs > P_NH₄ > > > P_TEA). This permeability ratio sequence is in agreement with previously published permeability ratios for the WT HERG channel.²⁴⁻²⁶

Figure 5 shows block of the HERG mutant D540K by 1 μM bepridil in extracellular solutions of 0K, 20K, 20NH₄⁺, 20Cs and 40TEA. Compared with block in 0K, block of D540K by bepridil was reduced in both extracellular potassium and extracellular cesium, but was not reduced with either extracellular NH₄⁺ or extracellular TEA. Figure 6 shows a dose response curve for block of D540K by bepridil in 0K, 20K, 20Cs and 40TEA. Given the permeability sequence (P_K ≈ P_Rb > P_Cs > P_NH₄ > > > P_TEA), these data are consistent with a correlation between block of D540K by bepridil and the permeant ion.

Inactivation and permeability properties of WT HERG and the HERG mutant D540K. It is possible that the differences in block of HERG by bepridil in different extracellular solutions (i.e., K⁺, NH₄⁺, Cs⁺ and TEA) are due to an effect of the different extracellular cations on inactivation. To address this, the effects of various extracellular cations on both the inactivation time constant and the fraction of channels in the inactivated state were measured. Figure 7 shows a comparison between WT HERG and the HERG mutant D540K of the effects of extracellular K⁺, NH₄⁺, Cs⁺, Rb and TEA on both the time constant and the fraction of channels in the inactivated state. For both WT HERG and the HERG mutant D540K, both the time constant and the fraction of channels in the inactivated state depended qualitatively in the same way on the extracellular cation, especially at +20 mV, the voltage used in the experiments in this paper to assess drug blockade. However, the extracellular cations tested showed a greater effect on the fraction of channels in the inactivated state for D540K compared with WT HERG. A comparison of the fraction of inactivated channels in 0K with the fraction of inactivated channels in the other solutions (in particular 20 NH₄⁺, 20K, 20Rb, 20Cs, 40TEA) shows a greater reduction in the fraction of inactive channels for the HERG mutant D540K compared with WT HERG. This could be due to the protocol used to measure the fraction of channels in the inactivated state: for example, in 20K, there were always some D540K channels open at all voltages measured, whereas in 0K there is a small voltage range around −80 mV where there were virtually no open channels. Therefore the current during the pulse to +20 mV (voltage used to assess the fraction of inactive channels) in 20K could be due to both depolarization activated and hyperpolarization activated channels. If this was the case, then the protocol used to measure the fraction of channels in the inactivated state could have underestimated the fraction of depolarization activated channels in the inactivated state in 20K, as well as possibly the fraction of inactivated channels in the other solutions tested.
summarized in Figure 8 measuring current every 6 sec after drug addition. Representative −120 mV, by holding the membrane potential at -120 mV and in the presence of different extracellular cations was measured at HERG we took advantage of the fact that D540K channels do to attempt to minimize any effects of inactivation on block of WT HERG by bepridil is due to WT HERG channels entering into a unique conformation in 0K that is distinct from the conformations in 20K, and which has a lower affinity for bepridil. In other words, block of WT HERG by bepridil is actually dependent on extracellular potassium in a similar way to block of WT HERG by quinidine (i.e., block is reduced with increased extracellular potassium) but because WT HERG channels enter into a unique conformation in 0K, block by bepridil is less than what it would be if WT HERG channels did not enter into this unique confirmation. This might explain why there is no difference in block of WT HERG by bepridil in 0K vs. 20K.

We attempted to address the potential conformational changes that might occur in 0K by measuring the change in HERG current amplitude after perfusion to 0K as well as the change in HERG current amplitude after recovery from 0K. Figure 10A shows the time constant of current decrease after perfusion from 5K to either 0K, 20K or 20 NH₄ for both WT and D540K. The data in Figure 10A show that, in agreement with a previous report, the time constant after addition of 0K for both WT and D540K is slow (much slower than the bath exchange), consistent with WT HERG channels entering a non-conducting conformational state, distinct from inactivation. In contrast to the change seen after perfusion from 5K to 0K, perfusion from 5K to either 20NH₄ or 20K, results in a rapid change in current amplitude, suggesting that the HERG channel does not enter into a unique non-conducting conformational state in either 20K or 20NH₄. Figure 10B show similar results for the time constant of recovery from either 0K, 20K or 20NH₄ back to the 5K solution. Figure 11A shows that block of WT HERG is the same in 0K and 20NH₄, which indicates that block of WT HERG by bepridil does not correlate with the time constants measured in the different solutions in Figure 10. Block of the HERG mutant D540K is also similar in 0K and 20NH₄ (see Figure 5). Since block by bepridil is the same in 0K and 20NH₄ for both WT HERG and the HERG mutant D540K, this suggests that any conformational state that occurs in 0K does not impact block of HERG by bepridil. Consistent with this, Figure 11B shows that the voltage dependence of block of the HERG mutant D540K by bepridil over the voltage range of -120 mV to -80 mV is similar in both 0K and 20K, suggesting a similar bepridil binding site in 0K and 20K.

Discussion

The data presented in this paper support three conclusions concerning the extracellular potassium dependency of block of

| WT | D540K Dep | D540K Hyp |
|----|----------|----------|
| TEA | 0.02 | 0.02 | 0.01 |
| NH₄ | 0.15 | 0.21 | 0.18 |
| Cs | 0.33 | 0.39 | 0.41 |
| Rb | 1.2 | 1.07 | 1.04 |

Permeability ratios using bionic conditions for wild type HERG (WT), the depolarization activated HERG mutant D540K (D540K Dep) and the hyperpolarization activated HERG mutant D540K (D540K Hyp). Permeability ratios were measured as described in Figure 4.

Block of hyperpolarization activated HERG mutant D540K.

To attempt to minimize any effects of inactivation on block of HERG we took advantage of the fact that D540K channels do not inactivate after opening at negative voltages. Block of D540K in the presence of different extracellular cations was measured at −120 mV, by holding the membrane potential at -120 mV and measuring current every 6 sec after drug addition. Representative experiments are shown in Figure 8 for 0K and 20K and the results summarized in Figure 9. These data show that block of D540K is highest in 0K and 20NH₄ and lowest in 20K and 20Cs. This demonstrates a correlation between drug block of D540K in the open state (the hyperpolarization activated open state) and the permeant ion, suggesting that the observed decrease in block of D540K by bepridil in 20 mM extracellular potassium is not dependent on inactivation.

WT HERG and the HERG mutant D540K in 0K. One of the mechanisms to explain the decrease in HERG current in low extracellular potassium proposes that, in the absence of external potassium, WT HERG channels enter into a non-conducting conformation, distinct from inactivation. It is possible that the observed lack of extracellular potassium dependency of block of WT HERG by bepridil is due to WT HERG channels entering into a unique conformation in 0K that is distinct from the conformations in 20K, and which has a lower affinity for bepridil. In other words, block of WT HERG by bepridil is actually dependent on extracellular potassium in a similar way to block of WT HERG by quinidine (i.e., block is reduced with increased extracellular potassium) but because WT HERG channels enter into a unique conformation in 0K, block by bepridil is less than what it would be if WT HERG channels did not enter into this unique confirmation. This might explain why there is no difference in block of WT HERG by bepridil in 0K vs. 20K.
Figure 4. Estimation of WT and D540K permeability. For both D540K and WT, oocytes were depolarized to +30 mV for 2 sec, hyperpolarized to −160 mV for 10 msec, then depolarized to voltages between −150 and +50 mV for 400 msec. Oocytes containing WT were held at −100 mV and oocytes containing D540K were held at −80 mV. The current at the beginning of the last pulse was plotted as a function of voltage and the reversal potential was obtained by a linear regression of the 3–4 points near where the current reversed sign. D540K Dep, depolarization-activated D540K current; D540K Hyp, hyperpolarization-activated D540K current. Current was sampled at either 10 or 200 μsec and filtered at 1 kHz. Error bars represent standard error of the mean. n ranges from 3 to 13 oocytes depending on the solution.

Figure 5. Bepridil block of D540K in the presence of extracellular cations. Summary of block of D540K by 1 μM bepridil in the following extracellular solutions: 0K, 20K, 20NH₄, 20Cs and 40TEA. Data were collected as in Figures 1 and 2. The voltage protocol shown in the figure was repeated every 6 sec and blockade assessed as described in the materials and methods section. Error bars indicate standard error of the mean; n values are as follows bepridil 0K(6), 20K(3), 20NH₄(4), 20Cs(6), 40TEA(4) An asterisk indicates a statistically significant difference between the 0K solution and solution X (X = 20K, 20NH₄, 20Cs, 40TEA) (p < 0.05).
the cardiac potassium channel HERG by different compounds: (1) Block of HERG by bepridil and terfenadine, drugs that can be trapped in the HERG channel after channel closure, is not significantly different in low and high extracellular potassium; (2) Not all compounds block HERG with the same extracellular potassium dependency, at least over the concentration range of 0 to 20 mM; (3) The lack of a potassium dependency of block of HERG by bepridil and terfenadine involves the ability of these drugs to be trapped inside the channel after channel closure.

A number of reports have suggested that block of HERG by some drugs is sensitive to extracellular potassium whereas block of HERG by other drugs is not sensitive to extracellular potassium. In particular block of HERG by dofetilide has been shown to be independent of extracellular potassium over a concentration range from 2 mM to 50 mM. In contrast block of HERG by quinidine and cisapride is reduced with an increase in extracellular potassium and also that this block is dependent on the permeant ion. In addition, the observed difference in extracellular potassium dependency of block of HERG by bepridil and terfenadine compared with block by quinidine and cisapride could be due to differences that occur during any of the voltages in the pulsing protocol used to assess drug blockade of HERG (i.e., +20, −60, −130 mV for the protocol used in the experiments described in this manuscript). If the difference in potassium dependency is related to the trapping mechanism for bepridil, then it seems unlikely that the difference in potassium dependency of block is due to differences that occur during the pulse to +20 mV because drugs are not trapped at positive voltages. However, it has been shown, at least for dofetilide, that block continues during the pulse to negative voltages. Thus it is possible that an increase in extracellular potassium is able to reduce block at negative voltages by drugs that are not trapped, but not able to reduce block at negative voltages by drugs that are trapped, since it is at negative voltages that the activation gate closes and traps the drug. Trapped drugs are therefore not as sensitive to extracellular potassium as drugs that are not trapped because extracellular potassium is not able to destabilize a trapped drug at negative voltages, but is able to destabilize a drug that is not trapped at negative voltages. This is directly relevant to the cardiac action potential since the HERG channel opens during the repolarization phase.

Drug trapping may only account in part for the observed difference in extracellular potassium dependency of block of HERG by bepridil and terfenadine compared with quinidine and cisapride. It may be that, in addition, differences in the relative affinity of bepridil and terfenadine compared with quinidine and cisapride for the inactive state over the open state, could also contribute to observed differences in potassium sensitivity of block of HERG by these drugs. For example, if bepridil had a lower affinity for the inactivated state compared with the open state, then block in 0K might be reduced due to the increase in inactivation in 0K, but increased due to a lack of permeant ion knock-off effect. The net effect would be the observed lack of extracellular potassium...
demonstrates that the non-conducting conformational change measured in 0 mM K is not seen in 20 mM NH₄, possibly because NH₄, an ion that shows some permeability through the HERG channel (PNH₄/PK = 0.15), binds to one or more sites in the HERG channel at or near the selectivity filter and prevents collapse of the selectivity filter. Since block of either WT HERG or the HERG mutant D540K by bepridil is similar in 0 mM K and 20 mM NH₄ (see Figure 11), this suggests that the drug binding site has not been altered by any conformational change in 0 mM K. In addition, the similarity in the voltage dependence of block of the HERG mutant D540K channels by bepridil between 0K and 20K (Fig. 11B) is consistent with a similar bepridil-binding site in 0K compared with 20K. Finally, block of WT HERG by quinidine and cisapride is increased in 0 mM K, suggesting that potassium dependency of block of HERG by bepridil in 0K. This paper does not address this issue directly. We previously reported that there is not a large difference in the fraction of WT HERG channels in the inactivated state at +20 mV between 0K and 20K, suggesting that any drug blocking differences between 0 and 20K are not due to inactivation. Further experiments looking at the potassium dependency of inactivation deficient mutants could help address this issue.

One potential concern with the results presented in this paper is the possibility of unique conformational changes associated with the 0K solution that might alter channel blockade. We attempted to address this question by showing that there was no correlation between block of HERG by bepridil and any unique conformational states associated with 0K. Figure 10 demonstrates that the non-conducting conformational change measured in 0 mM K is not seen in 20 mM NH₄, possibly because NH₄, an ion that shows some permeability through the HERG channel (PNH₄/PK = 0.15), binds to one or more sites in the HERG channel at or near the selectivity filter and prevents collapse of the selectivity filter. Since block of either WT HERG or the HERG mutant D540K by bepridil is similar in 0 mM K and 20 mM NH₄ (see Fig. 11), this suggests that the drug binding site has not been altered by any conformational change in 0 mM K. In addition, the similarity in the voltage dependence of block of the HERG mutant D540K channels by bepridil between 0K and 20K (Fig. 11B) is consistent with a similar bepridil-binding site in 0K compared with 20K. Finally, block of WT HERG by quinidine and cisapride is increased in 0 mM K, suggesting that

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Figure 7. Estimation of HERG inactivation. For both (A and B) oocytes were held at −100 mV, depolarized to +20 mV for 1 sec, hyperpolarized to −100 mV for 25 msec, then depolarized to +20 mV for 400 msec. Current during the second pulse to +20 mV was fit to a single exponential function and the current extrapolated back to the beginning of the pulse to +20 mV. (A) Fraction of inactive channels: the fraction of inactive channels was calculated using a slight modification of the method described in the literature as 1−Iₑ/Ip where Iₑ is the current extrapolated back to the beginning of the second pulse to +20 mV and Ip is the steady-state current at the end of the first pulse to +20 mV. An identical protocol was used with depolarizing pulses to +10, 0 and −10 mV. As long as the two depolarizing pulses in the protocol are the same, the fraction of inactive channels is 1−Iₑ/Ip. In most cases current was sampled at 200 μsec and filtered at 1 kHz. In some cases the current was sampled at 10 μsec. Error bars represent standard error of the mean; n ranges from 3–8 oocytes for each solution. (B) Inactivation time constant: The current resulting from the 2nd depolarizing pulse in the protocol described in (A) was fitted with a single exponential function.
any conformational change in 0K does not impact channel block by either quinidine or cisapride. A report using alanine scanning mutagenesis demonstrated a similar binding site for cisapride (not trapped) and terfenadine (trapped) in normal extracellular potassium. In combination with the data showing an increase block of WT HERG by cisapride in 0K, this suggests that drugs, trapped or not, will not necessarily be sensitive to any unique conformation changes that occur in 0K.

Zhang et al. have shown that a number of point mutations near the pore of HERG render the HERG channel insensitive to extracellular potassium. It is possible that, unlike the WT HERG channel, the HERG mutant channel D540K is insensitive to extracellular K, even though residue D540 is not a pore residue (residue 540 is located in the S4-S5 linker). In this case, if the lack of potassium dependency of block of HERG by trapped drugs was due to any conformational change that occurred in 0K, this lack of potassium dependency might not occur with the D540K mutant since the D540K mutant would not enter into a unique non conducting state. Figure 11 indicates that both the WT HERG channel and the HERG mutant D540K behave in similar way in 0K, 20K or 20NH4. Taken together these results indicate little impact on channel blockade due to any unique conformational states that might occur in 0K for both the WT HERG channel and the HERG mutant D540K.

A complete risk profile associated with the diverse set of compounds that block HERG is currently not known. It is not clear if the increased risk of diLQTS in patients with hypokalemia will be the same for all drugs. In addition, extracellular potassium may help reduce the risk of torsade de pointes in patients with LQTS. However, this might not be the case for all drugs. Experiments designed to understand the mechanism by which extracellular potassium can alter block of HERG by different
compounds can lead to both more clearly identifying risk factors for LQTS and diLQTS in different individuals and to developing methods to reduce the risk for diLQTS associated with hypokalemia.

**Materials and Methods**

**Oocyte isolation and cRNA injection.** Oocytes were harvested using standard dissociation techniques. Briefly, oocytes were removed in a Ca²⁺-free solution and enzymatically defolliculated for about 1 h with 1.5 mg/ml type 2 collagenase (LS004177-Worthington Biochemical Corporation) and then stored in ND96 (ND96–98 mM NaCl, 2mM KCl, 1.8 mM CaCl₂, 1mM MgCl₂, 5 mM Hepes). Between 25–50 ng of cRNA of either WT HERG or the HERG mutant D540K was injected into defolliculated oocytes.

**Electrophysiology.** Two electrode volume clamp experiments were performed at temperatures between 14–21°C on oocytes expressing either WT HERG or the HERG mutant D540K approximately 1–4 d after injection using a Geneclamp 500 (Axon instruments/Molecular Devices). The average current level used to assess block of HERG was the same for both oocytes expressing WT HERG and oocytes expressing the HERG mutant D540K. Before entering the bath, the solution was precooled using a CH cooling/heating module (Cell MicroControls) and the bath temperature was monitored. The CH cooling module was controlled by an analog TC2BIP 2/3 channel bipolar temperature controller (Cell MicroControls). Glass electrodes with resistances between 0.5 MΩ and 5 MΩ were filled with 3 M KCl. Data were acquired using pCLAMP software (Molecular Devices).

**Solutions.** All oocytes were initially impaled in ND96 and then the solution in which drug blockade was assessed was perfused into the chamber. All chemicals were obtained from Sigma-Aldrich. A potassium buffer was not used and 0K refers to no added potassium. All chemicals used for the 0K solution contain trace amounts of potassium contaminant. The estimated potassium concentration due to contamination is 10 μM which is lower than the potassium concentration estimated from reversal potential measurements (data not shown). This suggests that there is most likely some potassium accumulation in 0K due to potassium efflux through open HERG channels. To minimize potassium accumulation oocytes were continuously perfused at a rate of 1–3 ml/min throughout the entire experiment. Reversal potential measurements indicate that the extracellular potassium concentration with no added potassium is significantly less than 2 mM,¹⁰

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**Figure 9.** Bepridil dose response for D540K at -120 mV in different extracellular cations. Data were collected as described in Figure 8, and are plotted as the average of between 3 and 7 oocytes for each drug concentration. In some cases more than one concentration of drug was obtained in the same oocyte. Interrupted dashed line (20 NH₄); solid line (0K), dotted line (20 Cs), dashed line (20K). Error bars indicate standard error of the mean. IC₅₀ was estimated by fitting the data to 1/(1 + [IC₅₀]ⁿ/[drug]ⁿ), where [drug] is the drug concentration and n is the Hill coefficient. Estimated IC₅₀ and hill coefficient: WT: 20 NH₄ (10.30 μM, 1.19), 0K (11.19 μM, 1.61); 20Cs (44.12 μM, 1.26); 20K (32.79 μM, 1.56).

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**Figure 10.** Time course into and recovery from 0K solution. (A) After extracellular solution change from ND96 to 5K, oocytes containing either WT HERG or D540K were repetitively pulsed as described in Figures 1 and 2 and the Materials and Methods and the current level was assessed at the beginning of the pulse to -60 mV. After a steady-state current level was reached in 5K, either 0K, 20K or 20NH₄ (solution X) was perfused into the bath. (A) shows the time constant to reach steady-state for WT and D540K in either 0K, 20K or 20NH₄ (solution X) back to 5K.

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The amount of drug block was assessed for either WT or the HERG mutant D540K using the current at the beginning of the pulse to −60 mV. The fractional drug block was calculated as 1-(Iss/Ipre), where Iss is the steady-state current level after drug addition and Ipre is the pre-drug current before addition of drug.

**Leak Subtraction.** Leak subtraction for WT HERG was obtained by subtracting the current at −60 mV before the pulse to +20 mV from the current at the beginning of the pulse to −60 mV after the pulse to +20 mV. For most experiments measuring block of the mutant D540K, leak in D540K oocytes was assessed by applying a high concentration of drug at the end of each experiment. This was not possible for some experiments in which a very high concentration of drug did not completely block all current.

**Run-up/run-down.** Repetitive pulsing of oocytes containing either WT HERG or the HERG mutant D540K often shows either channel run-up or channel run-down. To correct for either channel run-up or channel run-down oocytes were initially pulsed in drug free solution for 5–10 min until a steady-state pre-drug current level was established. The initial pre-drug run-up or run-down was fit with a single exponential and the fit extrapolated to the time when drug block reached steady-state. For run-up or run-down corrected data, the extrapolated fit of the

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**Figure 11.** Drug block in 0K. (A) Data were collected and analyzed as described in Figure 1 and are plotted as the average of between 3 and 6 oocytes. Comparison of block of WT by 1 μM bepridil in 0K, 20K and 20NH4. (B) Data were collected and analyzed as described in Figure 8 and are plotted as the average of between 3 and 6 oocytes. Comparison of block of D540K by 3 μM bepridil in both 0 mM extracellular K and 20 mM extracellular K with a holding potential of either −120 or −80 mV.

sugesting that the 0K solution is a solution with an extracellular potassium concentration significantly lower than 2 mM.

**Drugs.** All drugs were obtained from Sigma-Aldrich and were stored frozen at −80°C in a stock solution in DMSO and then thawed and diluted into the appropriate electrolyte solution each day just prior to the experiment. The concentration of stock solution varied from 10 to 60 mM.

**Data Collection and Analysis.** Block of either WT HERG or the mutant D540K by quinidine, bepridil and terfenadine was assessed using repetitive pulsing at a frequency of 0.167 Hz.

**WT HERG.** The oocytes were held at −100 mV, then pulsed briefly to −60 mV for 40 msec (to measure leak current at −60 mV before HERG channels have opened), then pulsed to +20 mV for 1 sec, then to −60 mV for 300 msec, then to −130 mV for 750 msec (to close all HERG channels prior to the next pulse). In some experiments with cesium the second pulse was to −50 mV instead of −60 mV.

**D540K.** Since the HERG mutant D540K opens with hyperpolarization (and is open at −100 mV, at least in some solutions), oocytes containing D540K were held at −80 mV (a voltage where most D540K channels are closed—data not shown), then pulsed to +20 mV for 1 sec, then to −60 mV for 300 msec and then back to −80 mV.
pre-drug current to the value at steady-state after drug addition, was used as the pre-drug current level (I_p, above). In general, for both WT and D540K, neither leak corrected nor run-down or run-up corrected data were significantly different from raw uncorrected data. This manuscript presents data that is both leak and run-down/run-up corrected. For all raw data traces shown, capacity transients were blanked for clarity. All data were analyzed using pCLAMP software (Molecular Devices).

Statistical analysis. Confidence levels were determined with SPSS software (SPSS, Inc.) or Microsoft EXCEL (Microsoft Corp.) using either an unpaired t-test or a one-way ANOVA analysis followed by the Bonferroni test. Significance was accepted at p < 0.05.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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