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Mapping the Block of a Cloned Human Inward Rectifier Potassium Channel by Dofetilide

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SUMMARY

Dofetilide, a methanesulfonanilide derivative, is a potent class III antiarrhythmic drug. Like other members of this class of K^+ channel blockers, the sites in the channel to which the drug binds are unknown, although high and low affinity binding has been reported in cardiomyocytes. The most sensitive K^+ channel target for dofetilide seems to be I_{Kr}, the rapid component of the repolarizing delayed rectifier K^+ current. However, block of other K^+ channels occurs at higher concentrations and is of special interest in regard to toxicity. Recently, we have demonstrated that hIRK, a cloned inward rectifier K^+ channel (IRK) isolated from human atrium and expressed heterologously in *Xenopus oocytes*, is blocked by dofetilide. We report the localization of a site that is critical for dofetilide block in hIRK. We used chimeric constructs between hIRK and ROMK1, a related inward rectifier that is drug resistant. Substitution of hIRK-M2, the second putative transmembrane spanning segment of IRKs, with ROMK1-M2 increased unblocking of dofetilide by 10–20-fold in hIRK. Site-directed mutagenesis further pinpointed the effects to a single hydrophobic residue (I177) in M2. A reduction in hydrophobicity by the point mutation I177C increased recovery from block >10-fold (1.17 sec in wild-type to 0.112 sec at −80 mV at physiological K^+ concentrations), leading us to suggest that hydrophobic interactions are essential for dofetilide block in hIRK. A similar mechanism may explain dofetilide block in other ion channels, including I_{Kr}.

Repolarization of the cardiac action potential is controlled by several potassium currents (1), including transient outward current during the initial phase, the delayed rectifier current with its rapid component (I_{Kr}) and its slow component (I_{Ks}) (2) during the plateau phase, and the inward rectifier potassium current, which produces final repolarization. The inward rectifier potassium current also determines the diastolic membrane potential and excitability (3). Class III antiarrhythmic drugs prolong the cardiac action potential by blocking repolarizing potassium currents (4). Among the new generation of class III drugs is dofetilide (Fig. 1), which belongs to the methanesulfonanilides and is very potent in prolonging the cardiac action potential probably by blocking I_{Kr} (5–7). Although I_{Kr} is the highest affinity target for methanesulfonanilides, block of other currents has been reported at higher concentrations (7–9). This was supported by a binding study with radiolabeled dofetilide in guinea pig cardiomyocytes in which high and low affinity binding was reported (10). Binding to targets other than I_{Kr} is of particular interest in regard to unwanted side effects and toxicity.

Recently, we demonstrated that dofetilide blocks cloned human inward rectifier potassium channel hIRK (11) heterologously expressed in *Xenopus* oocytes (9). The block is strongly voltage and time dependent with an IC_{50} of 533 nM at 40 mV and 20°. The block is accompanied by a reduction in mean channel open time and is reduced by extracellular K^+. Block is rapid when dofetilide is applied to the cytoplasmic surface of hIRK and ineffective in whole oocytes. Our interpretation of these results is that at physiological pH, dofetilide crosses the membrane of cardiomyocytes in a neutral form and blocks open channels as a protonated molecule (9). Recovery from block is very slow, even at hyperpolarized potentials (τ = 1.17 sec at −80 mV), and with repetitive depolarizations, incomplete recovery from block during diastole causes use dependence (9).

In addition to hIRK, dofetilide blocks IRK1, an inward rectifier potassium channel that is 70% identical to hIRK (12). In contrast, ROMK1, an inward rectifier potassium channel cloned from rat kidney outer medulla (13) that is only 39% identical to hIRK, is only weakly blocked, and the block is present only at very positive potentials (80 and 100 mV). Unblocking at hyperpolarized potentials is virtually instantaneous. By constructing chimeric exchanges with ROMK1, we localized block to M2. We then used site-directed mutagenesis to identify a single hydrophobic amino acid as a

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ABBREVIATIONS: I_{Kr}, delayed rectifier current rapid component; IRK, inward rectifier potassium channel; hIRK, human inward rectifier potassium channel; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; I_{KACh}, acetylcholine-regulated potassium channel in atrium.

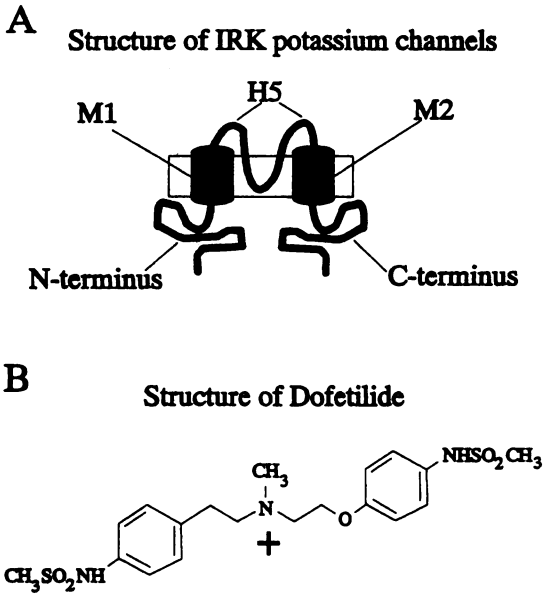

Fig. 1. A, Structure of IRKs. IRKs are thought to contain only two putative transmembrane domains, M1 and M2, which are linked by an H5 loop (12, 13). B, Chemical structure of dofetilide. At physiological pH 7.4, the amine group is 28.5% protonated (5) (+).

major determinant of the dofetilide block of hIRK. It is likely that hydrophobic interactions will also be the basis for dofetilide block of other ion channels, including I_{Kr}.

Materials and Methods

Electrophysiology. Macropatch currents were recorded from oocytes using pipettes made from borosilicate glass with tip openings of 10–15 μm (14). After achieving a gigaseal, macropatches were excised and positioned in the stream of a large pipette (diameter, 2 mm) to wash out the naturally occurring blocking particles, Mg^{2+} and polyamines, from the patches (15–17). Dofetilide was applied to the cytoplasmic surface of the inside-out patches by changing the solution flowing through the application pipette.

Solutions and drug administration. All measurements were performed in a bath solution (EDTA-K^+ solution) containing 100 mM KCl, 10 mM EDTA, and 10 mM HEPES, pH 7.3. The high-K^+ pipette solution contained 100 mM KCl, 2 mM $MgCl_2$, and 10 mM HEPES, pH 7.3. The extracellular low-K^+ pipette solution contained 5 mM KCl, 100 mM NaCl, 1.5 $CaCl_2$, 2 mM $MgCl_2$, and 10 mM HEPES, pH 7.3.

Dofetilide (*N*-[4-(2-{[4-(methanesulphonamino)phenoxyl]-*N*-methylethylamino)ethyl) phenyl]methanesulphonamide; Pfizer Central Research, Sandwich, Kent, UK) (structure in Fig. 1) was dissolved in distilled water, acidified to pH 3.0 by the addition of HCl in a stock solution to 10 mM, and stored at −20°. On the day of the experiments, the stock solution was diluted with the intracellular solution (EDTA-K^+ solution) to the desired concentration. All measurements were done at room temperature (20°).

Data analysis. Data were low-pass filtered at 1–2 kHz (−3 dB; four-pole Bessel filter) before digitalization at 5–10 kHz. PClamp software (Axon Instruments, Burlingame, CA) was used for generation of the voltage-pulse protocols and for data acquisition.

Removal of drug block during the hyperpolarizing test pulse was fitted by the single exponential equation $I = I_{max} \exp[-(t - t_0)/\tau] + C$, where I_{max} is the maximum current at the hyperpolarized pulse, t_0 is the start time of the repolarizing pulse, and τ is the time constant of unblocking. C is a constant (i.e., the inward current amplitude at −40 mV).

Recovery time constants were fitted by $(I_{rec} - I_0)/(I_{max} - I_0) = 1 - \exp(-t/\tau)$, where I_{rec} is the peak current after the time of recovery, I_0 is the unblocked current at the end of the test pulse, I_{max} the peak current of the test pulse, and τ is the time constant of recovery. Statistical data are given as mean ± standard deviation.

Molecular biology. Heterologous expression in *Xenopus* oocytes of cRNAs encoding hIRK, IRK1, and ROMK1 was performed as described previously (11, 15, 18). The chimeric constructs involving IRK1 and ROMK1 were prepared and described previously (18). CHM 11 and CHM 12 were constructed between IRK1 and ROMK1 by overlap extension through sequential polymerase chain reaction as described previously (19). CHM 11 consists of a ROMK1 M1 domain plus the adjoining first 26 residues of H5 transplanted into IRK1 from which the corresponding region (residues 88–131) has been moved. CHM 12 consists of a ROMK1 M2 domain plus the adjoining terminal 8 residues of H5 transplanted into IRK1 from which the corresponding region (residues 149–178) has been deleted.

The hIRK\ROMK1 chimeras involving the M2 exchanges were done by overlap extension at the junctions of M2 using sequential polymerase chain reaction (20). Single point mutations in hIRK were made by polymerase chain reaction overlap extension (21). All constructs were subcloned into the A^+-PCRII vector for expression in *Xenopus* oocytes as described previously (18).

The IC_{50} values interpreted as K_D were calculated from the half-maximal blocking concentration by fitting a dose-response curve to the normalized current values ($I_{drug}/I_{control}$). The normalized current was expressed as a function of the dofetilide concentration according to the following binding equation $I_{drug}/I_{control} = 1/(1 + X/K_D)^n$, where X is the dofetilide concentration, and n is the Hill coefficient.

Results

Dofetilide block of different IRKs. When an inside-out macropatch is excised in a 0 Mg^{2+} and 0 polyamine solution, hIRK loses most of its instantaneous inward rectification. By using a 100 mM K^+ extracellular solution, the hIRK potassium current reverses at ~0 mV. The outward current at 20 and 40 mV shows no time dependence, whereas between 60 and 100 mV a time-dependent inactivation can be observed, even after washout of 10 min (Fig. 2, *left*). This remaining inactivation is due to incomplete washout of polyamines, although there may be some contribution from a truly intrinsic gating process. During the step back to −40 mV/400 msec, the inactivation is removed immediately. When dofetilide is applied to the cytoplasmic side of the macropatch, the outward current of hIRK is blocked in a voltage and time dependent manner (Fig. 2, *right*). During the step back to −40 mV, the very slow release from block by dofetilide produces a time-dependent increase in inward current (increase in negative current) to the control value at −40 mV. This slow increase in current or unblocking is caused by the transition of channels from blocked to unblocked states at negative potentials and reflects the off rate of dofetilide from its receptor. Every test pulse is preceded by a short prepulse to −40 mV for 100 msec to monitor whether the patch was stable during the measurements. Dofetilide (10 μM) blocked the hIRK outward current by 85 ± 5.4% (five experiments) at the end of the 100-mV test pulse. The unblocking time constant at −40 mV was τ = 232 ± 57 msec (five experiments), and unblocking was concentration independent. Dofetilide (10 μM) added to the extracellular pipette solution did not block hIRK (five experiments).

IRK1, cloned from a mouse macrophage cell line (12), has the same control current properties as hIRK and is also blocked by dofetilide in excised macropatches but to a lesser extent [62 ± 5.5% (three experiments)] (Fig. 2). The block of

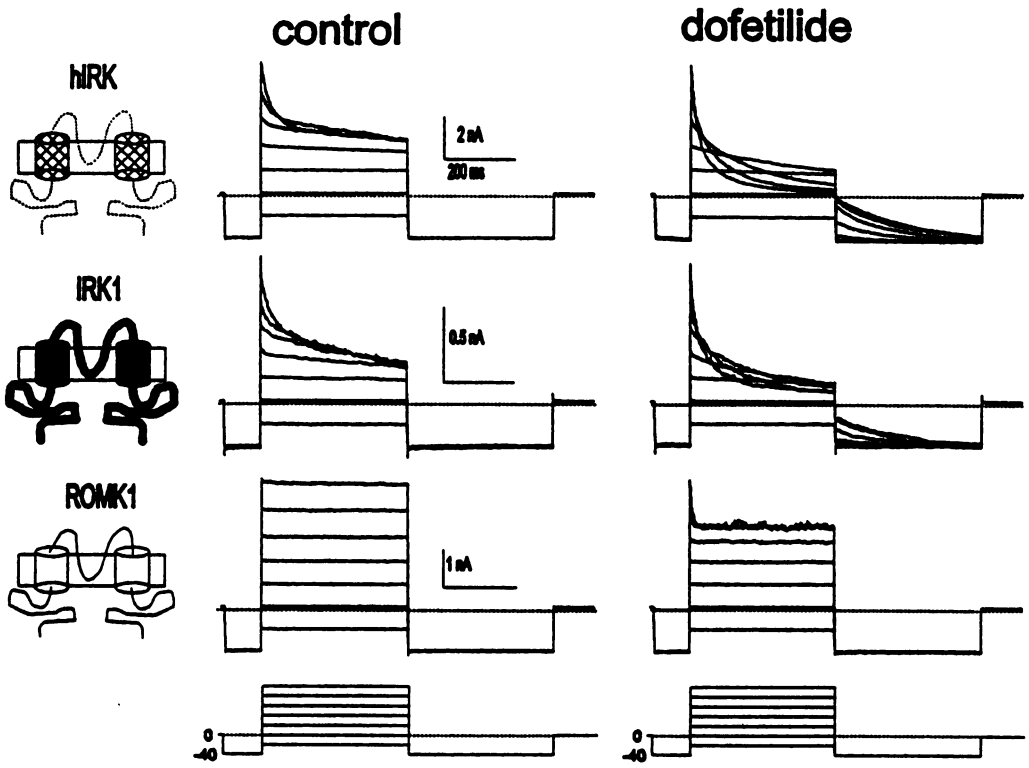

Fig. 2. Control currents (*top left*) and the effect of dofetilide on hIRK (*top right*), IRK1 (*middle*), and ROMK1 (*bottom*) in excised macropatches in a 0 Mg^{2+} and 0 polyamine solution. hIRK and IRK1 are blocked by dofetilide, and the unblocking during the step back to −40 mV is very slow. In contrast, ROMK1 is only weakly blocked at 80 and 100 mV, and the unblocking is virtually instantaneous. The concentration of dofetilide was 10 μM. Holding potential, 0 mV; prepulse, −40 mV/100 msec; test pulse, −20–100 mV in 20-mV steps (400 msec); return pulse, −40 mV/400 msec. Bath, EDTA-K^+ solution; pipette, high-K^+ solution (100 mM K^+).

IRK1 by dofetilide has approximately the same time dependence and characteristics; i.e., the unblocking at hyperpolarized potentials is also very slow: τ = 197 ± 32 msec (three experiments). In contrast, block of ROMK1 is inconsequential. ROMK1 control current kinetics display no time dependence, and the currents are not affected by dofetilide at ≤60 mV. At 100 mV, ROMK1 is only weakly (33 ± 5.8%) blocked by dofetilide, and the unblocking during the step back to −40 mV is virtually instantaneous (Fig. 2).

Identification of M2 as the domain determining dofetilide block. Given that IRK1 and hIRK are blocked by dofetilide, whereas ROMK1 is practically unaffected, we used chimeras between IRK1 and ROMK1 and constructed new chimeras between hIRK and ROMK1 to localize the structural domain responsible for block. The most dramatic manifestation of block is unblocking at hyperpolarized potentials, and we used this to measure the effects of genetic engineering.

R. CHM. 1 consists of the amino and carboxyl termini of IRK1 with the transmembrane domains M1 and M2 as well as H5 of ROMK1 (Fig. 3, *top*). Dofetilide (10 μM) showed only a weak block of R. CHM. 1 [36 ± 13% block at 100 mV (four experiments)] with a very fast unblocking [τ = 10.1 ± 5.9 msec (four experiments)]. Therefore, the sequence determining the slow unblocking must be within the domains of M1, M2, or H5.

We then tested a more limited chimera. R. CHM. 2 is primarily IRK1 with only H5 replaced by ROMK1 (Fig. 3). This chimera was strongly blocked by dofetilide and showed the same slow unblocking as IRK1 and hIRK [83 ± 7.7% block at 100 mV, τ = 379 ± 207 msec (four experiments)]. Based on this measurement, we concluded that the sequence determining the slow unblocking was within M1 and/or M2.

The next step was to determine whether dofetilide block could be localized to either M1 or M2. We tested the IRK1/ROMK1 chimeras CHM 11 and CHM 12 in which the ROMK1 M1 or M2 segments, respectively, plus some flanking H5 sequence had been transplanted into IRK1 (Fig. 3). Because we had already shown that H5 substitution did not alter dofetilide block, these chimeras could be used to analyze the contributions of M1 and M2. CHM 12 was blocked by dofetilide, but the unblocking was very fast [87 ± 5.0% block at 100 mV, τ = 11 ± 1.1 msec (three experiments)], indicating that M2 was the essential structural domain responsible for the slow unblocking in IRK1. In CHM 11, the unblocking was virtually as slow as wild-type IRK1 [82.2 ± 3.7% block at 100 mV, τ = 143 ± 5.0 msec (four experiments)], suggesting that sequences in M1 do not contribute to the slow unblocking of dofetilide.

We extended this approach to hIRK and constructed the chimera REVCHM M2, which is basically hIRK with a ROMK1 M2 substitution (Fig. 3). In this chimera, only the 22 residues of M2 were substituted by ROMK1, without additional residues of H5. Although the expression level of this chimera was very low, we were able to analyze the dofetilide block. REVCHM M2 was blocked by dofetilide, and the unblocking was very fast [51 ± 2.8% block at 100 mV, τ = 7.5 ± 1.4 msec (two experiments)](Fig. 3). We also constructed the opposite chimera in ROMK1, but this did not express. In summary, the putative transmembrane domain M2 was the main macroscopic structural domain determining the slow unblocking of dofetilide in hIRK and IRK1.

Pinpointing the residues in M2 determining block. An alignment of the M2 sequences in hIRK, IRK1, and ROMK1 demonstrates that there are eight identical positions in hIRK and IRK1 that differ from ROMK1 (Fig. 4, *row 1*). Among the eight amino acids, we classified four as minor and four as major differences. The amino acids at positions 159, 162, 163, and 168 differed only in one side-chain carbon

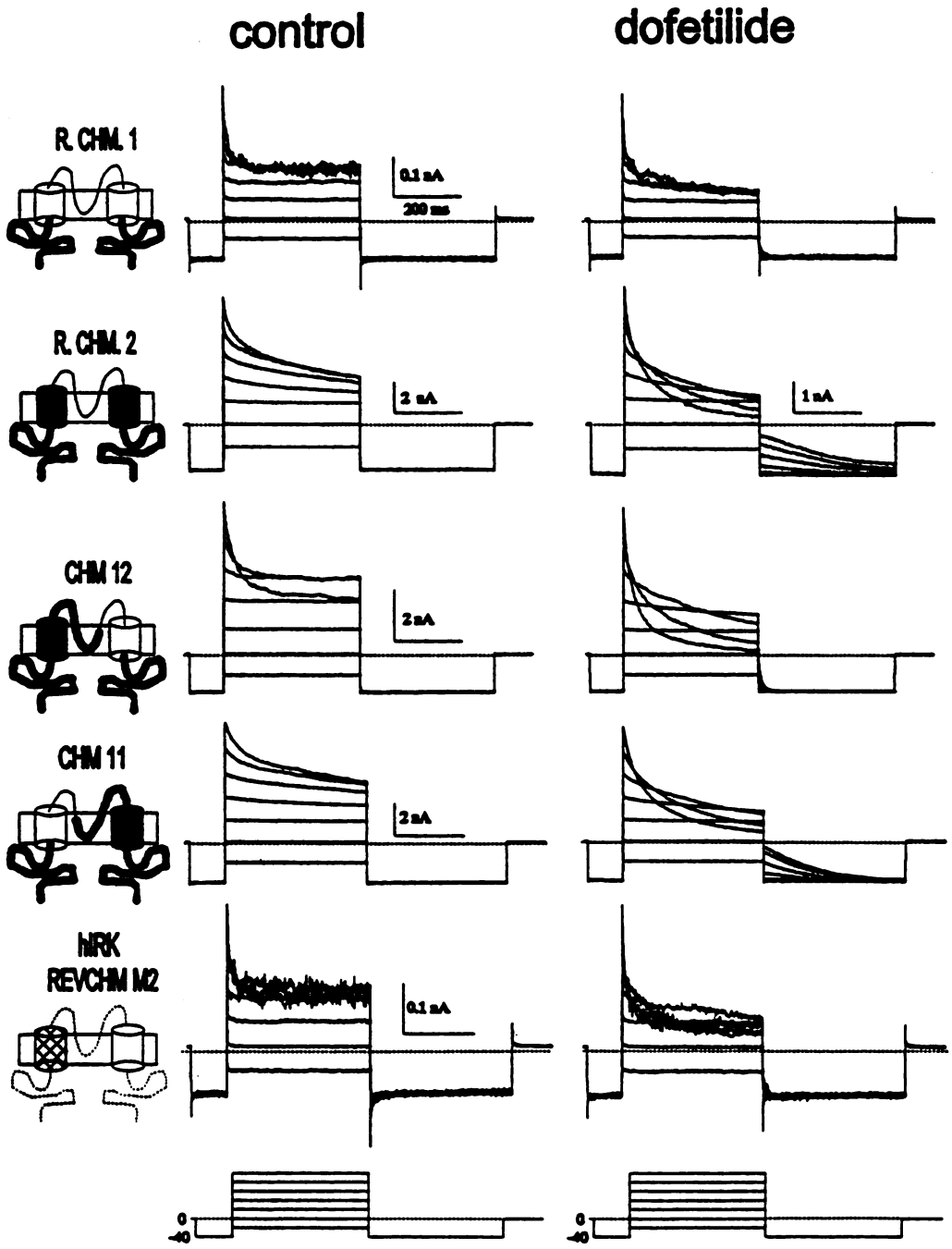

Fig. 3. Control currents and the effect of dofetilide (10 μM) on four chimeras between IRK1 and ROMK1 (R. CHM. 1, R. CHM. 2, CHM 12, and CHM 11) and one chimera between hIRK and ROMK1 (REVCHM M2) in excised macropatches. The slow unblocking of dofetilide correlates strongly with M2; i.e., only the chimeras that have the transmembrane spanning domain M2 from IRK1 or hIRK (R. CHM. 2 and CHM 11) have a slow unblocking during the step back to −40 mV. The chimeras with the M2 domain of ROMK1 (R. CHM. 1, CHM 12, REVCHM M2) have a fast unblocking. Holding potential, −80 mV; prepulse, −40 mV/100 msec; test pulse, −20–100 mV in 20-mV steps/400 msec; return pulse, −40 mV/400 msec. Bath, EDTA-K^+ solution; pipette, high-K^+ solution (100 mM K^+).

atom. Therefore, we focused on the major differences at hIRK-M161L, hIRK-C170V, hIRK-D173N, and hIRK-I177C (Fig. 4, *row 2*).

hIRK-M161L, hIRK-C170V, and hIRK-D173N were all blocked by dofetilide and showed slow unblocking (Fig. 5) [hIRK-M161L: 64 ± 23% block at 100 mV, τ = 206 ± 52 msec (four experiments); hIRK-C170V: 56 ± 7.2% block at 100 mV, τ = 209 ± 40 msec (three experiments); hIRK-D173N: 45.9 ± 8.6% block at 100 mV, τ = 126 ± 29 msec (four experiments)]. In contrast, hIRK-I177C was also blocked by dofetilide but showed very fast unblocking [78.1 ± 13.4% block at 100 mV, τ = 17.4 ± 1.7 msec (eight experiments)]. Therefore, I177 is the primary determinant for the slow unblocking of dofetilide in hIRK.

Recovery from block at physiological extracellular K^+ concentrations. The unblocking of dofetilide is also reflected in the recovery from block. We measured the recovery from block at −80 mV with 5 mM extracellular K^+. Wild-type hIRK had a recovery time constant of 1168 ± 553 msec (three experiments) at −80 mV recovery potential. The point mutation hIRK-I177C recovered 10-fold faster from drug block, with a time constant of 112 ± 38 msec (three experiments) at −80 mV (Fig. 6).

We also determined the IC_{50} values for dofetilide block in hIRK and hIRK-I177C with extracellular 5 mM K^+. We calculated an IC_{50} for hIRK of 530 ± 254 nM (three experiments) and 2090 ± 282 nM (four experiments) for hIRK-I177C at the end of a 400-msec/40-mV test pulse.

	161												170		173				177			
hIRK	A	V	F	M	V	V	A	Q	S	I	V	G	C	I	I	D	S	F	M	I	G	A
IRK1							F										A		I			
ROMK	I			L	L	I	F				L		V			N				C		
Row 1	V			M	V	V					V		C			D				I		
Row 2				M									C			D				I		

Fig. 4. Alignment of the amino acids in M2 of hIRK, IRK1, and ROMK1. *Blanks*, identical amino acids. *Row 1*, amino acids that are identical in hIRK as well as IRK1 and differ from the corresponding position in ROMK1. *Row 2*, amino acids from row 1 that differ by more than one carbon atom from the corresponding ROMK1 position.

Voltage dependence of unblocking. The unblocking of dofetilide is voltage dependent and occurs faster at high [K°] than at 5 mM [K°], in agreement with open channel block (9). We compared the unblocking of dofetilide in hIRK and the mutant hIRK-I177C over the voltage range of −20 to −100 mV at high [K°] (Fig. 7). After a test pulse to 40 mV, the unblocking time constant of dofetilide in hIRK at −20 mV was 422 ± 99 msec (six experiments), whereas in hIRK-I177C, it was 19-fold faster [22.1 ± 8.4 msec (five experiments)]. At −100 mV, hIRK was unblocked with a time constant of 16.4 ± 2.3 msec (six experiments), and hIRK-I177C was unblocked 24-fold faster [0.66 + 0.1 msec (five experiments)]. Unblocking was monoexponential at all potentials. The values over the entire voltage range are displayed in Fig. 8.

Estimating the on and off rates of dofetilide block. Consider the simplest reversible drug/receptor reaction:

$$R + D \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} RD \qquad (1)$$

where R is the open unblocked channel, D is the drug, and RD is the drug-bound blocked channel. k_{on} is the rate constant of the blocking reaction (on rate), and k_{off} is the rate constant of unblocking (off rate). To estimate rate constants, we performed the calculations at voltages at which only one of both reactions was the dominant reaction; this was −40

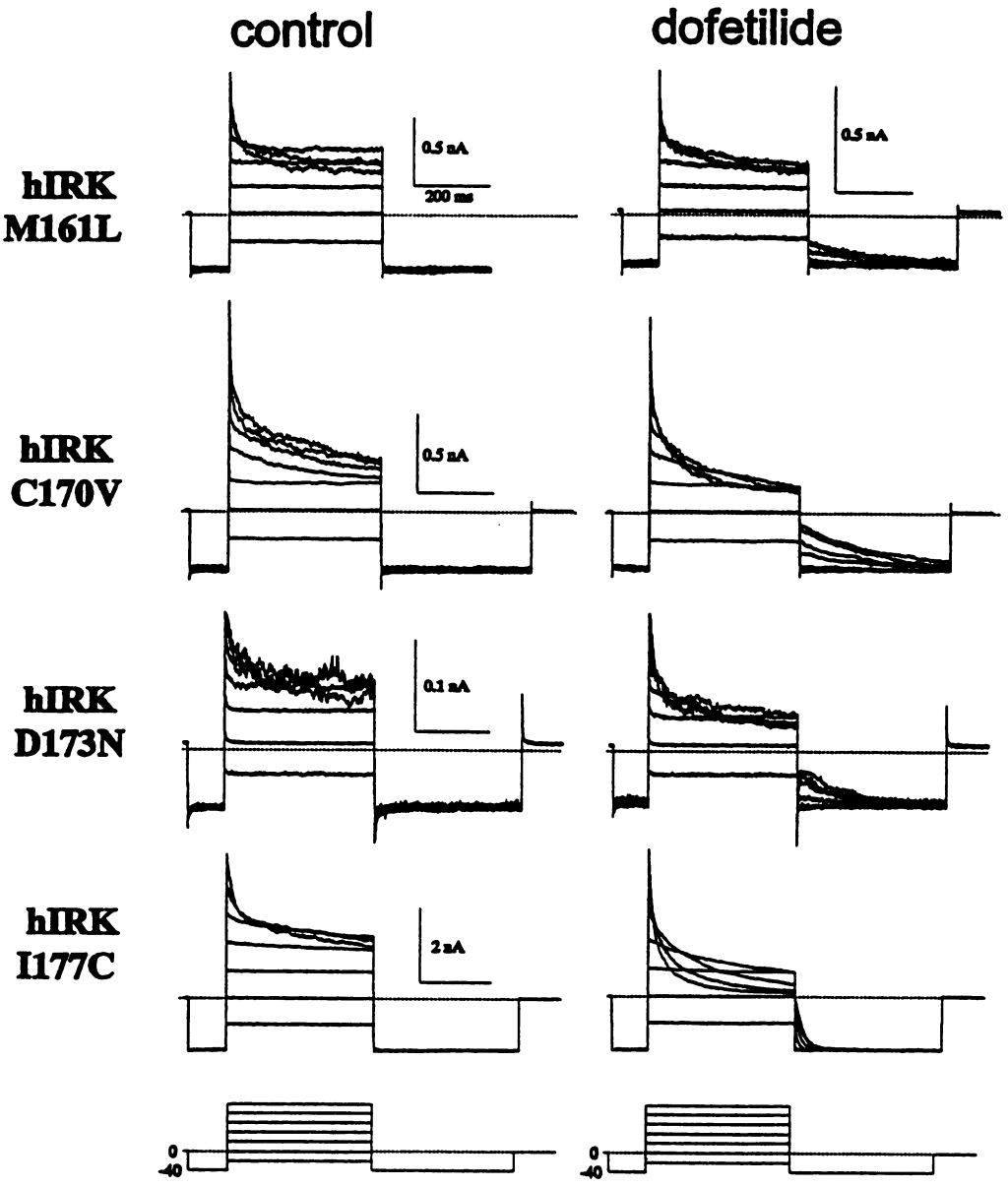

Fig. 5. Control macropatch measurements and the effect of dofetilide (10 μM) on four point mutations in the putative transmembrane domain M2 in hIRK. Only the point mutation I177C produces a fast unblocking of dofetilide. Holding potential, −80 mV; prepulse, −40 mV/100 msec; test pulse, −20–100 mV in 20-mV steps/400 msec; return pulse, −40 mV/400 msec. Bath, EDTA-K^+ solution; pipette, high-K^+ solution (100 mM K^+).

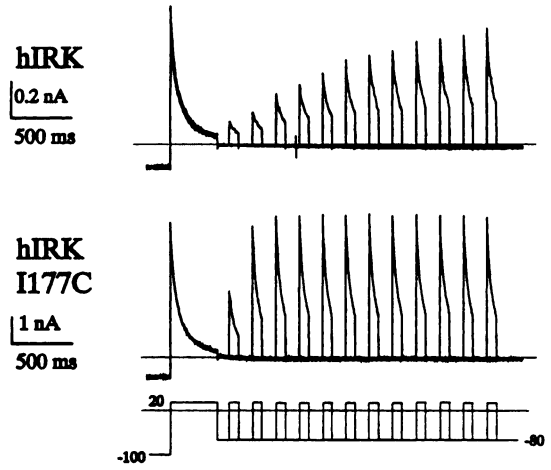

Fig. 6. Macropatch measurements of the recovery from drug block at the recovery potential −80 mV in hIRK and the mutant hIRK-I177C. hIRK shows a slow recovery time constant of 1206 msec (*top*). In contrast, the single point mutation hIRK-I177C accelerates the time constant of recovery from block to 124 msec. Holding potential, −100 mV; test pulse 1, 20 mV/400 msec; delays at the recovery potential of −80 mV to the test pulse 2 were 50–950 msec in 100-msec increments; test pulse 2, 20 mV/50 msec. Bath, EDTA-K^+ solution; pipette, low-K^+ solution (5 mM K^+).

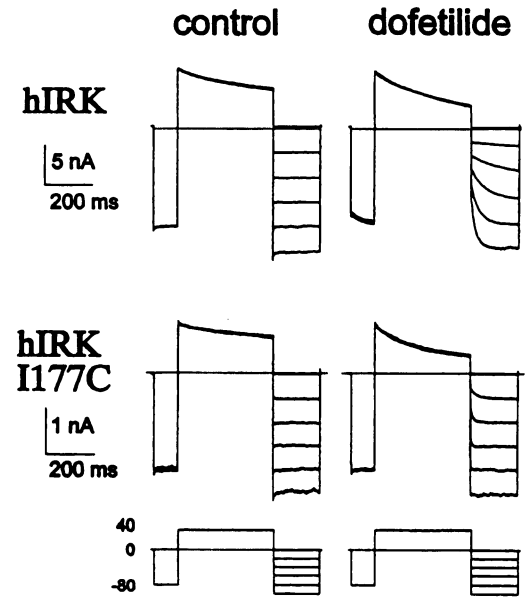

Fig. 7. Control macropatch currents and the effect of dofetilide (10 μM) on hIRK and the mutant hIRK-I177C. *Left*, control measurements. *Right*, block of dofetilide during depolarization and unblocking during various hyperpolarizing potentials. The unblocking of hIRK is 15–25-fold slower than hIRK-I177C over the entire voltage range. Holding potential, 0 mV; prepulse, −80 mV/100 msec; test pulse, 40 mV/400 msec; hyperpolarizing pulse 0 to −100 mV in 20-mV steps. Bath, EDTA-K^+ solution; pipette, high-K^+ solution (100 mM K^+).

mV for the unblocking reaction and +100 mV for the blocking reaction.

The time constant of the exponential approach to equilibrium is (22)

$$\tau = 1/(k_{on}[D] + k_{off}) \quad (2)$$

where $[D]$is the drug concentration. At −40 mV, k_{off} is much larger than k_{on}; i.e., the on rate can be neglected. Eq. 2 can be simplified to the following:

$$\tau_{off} = 1/k_{off} \quad (3)$$

We measured unblocking time constants of τ = 232 msec in hIRK wild-type and τ = 17.4 msec in hIRK-I177C. By using eq. 3, we calculated the off rate k_{off} as 4.31 sec^{-1} at −40 mV in the wild-type and as 57.4 sec^{-1} at −40 mV in hIRK-I177C.

To calculate the on rate k_{on} at +100 mV, we used the following equations (22):

$$K_D = k_{off}/k_{on} \quad (4)$$

where we substituted k_{off} by eq. 2. This resulted in the following:

$$k_{on} = 1/\{\tau_{on}([D] + K_D)\} \quad (5)$$

where τ_{on} is the time course of the blocking reaction during the activation (100 mV/400 msec) test pulse (Fig. 9), and the IC_{50} value was calculated at the end of the 400-msec test pulse and used as K_D.

To estimate an apparent τ_{on}, we used the "dofetilide-sensitive current" by digital subtraction of the blocking trace from the control trace (Fig. 9). The time course of the dofetilide-sensitive current could be fitted by a single exponential equation, consistent with the kinetics in equation (1). The fitted graph is also displayed in Fig. 9. The values for hIRK wild-type were τ_{on} = 29.3 ± 11.2 msec (six experiments) and

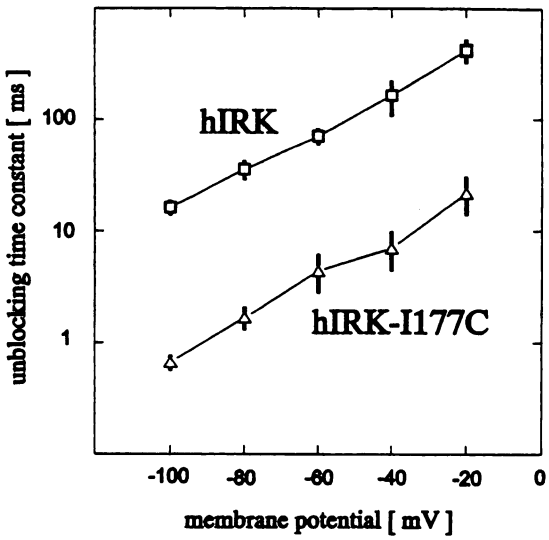

Fig. 8. The time constants of unblocking in hIRK and hIRK-I177C as a function of unblocking membrane potential. Values are as mean ± standard deviation.

τ_{on} = 27.6 ± 7.8 msec for hIRK-I177C (five experiments). We calculated the IC_{50} values at the end of the 100-mV/400-msec test pulse and used them as K_D values. hIRK wild-type was blocked with an IC_{50} = 0.96 ± 0.41 μM (three experiments) and hIRK-I177C with an IC_{50} = 2.6 ± 1.4 μM (six experiments). Using eq. 5, we calculated the on rates k_{on} = 3.11 × 10^6 M^{-1} sec^{-1} for hIRK wild-type and k_{on} = 2.87 × 10^6 M^{-1} sec^{-1} for hIRK-I177C.

Assuming an equivalence between block and binding, we estimated the difference in binding energy ΔΔG of dofetilide

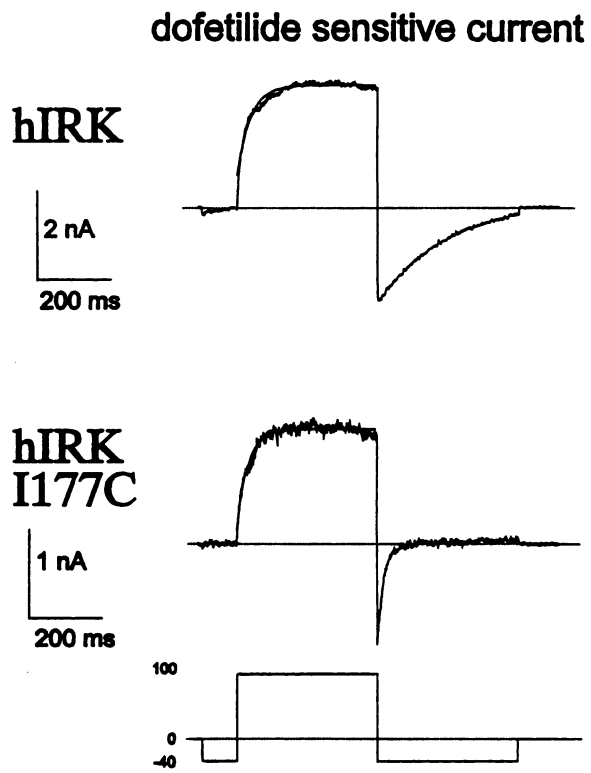

Fig. 9. Digitally subtracted "dofetilide sensitive current" in hIRK and hIRK-I177C. The time constants of blocking are virtually identical. The time constant of unblocking is 13 times faster in hIRK-I177C. Holding potential, 0 mV; prepulse, −40 mV/100 msec; test pulse, 100 mV/400 msec; hyperpolarizing pulse, −40 mV. Bath, EDTA-K^+ solution; pipette, high-K^+ solution (100 mM K^+).

to hIRK and hIRKI177C using equations from enzyme kinetics (23–25). $\Delta\Delta G$ was calculated as the following:

$$\Delta\Delta G = -RT \log_e[k_{onhIRK}\, k_{offI177c}/k_{offhIRK}\, k_{onI177c}] \quad (6)$$

The voltage dependence of the off rates was not altered by I177C (Fig. 8). The time constants of unblocking and the k_{off} between hIRK and hIRKI177C differed by a factor of 19–24 (mean, 21.5) over the entire voltage range at which unblocking was measured (Fig. 8). The on rates of hIRK and hIRKI177C at 100 mV were similar (k_{on} = 3.11 × 10^6 M^{-1} sec^{-1} for hIRK, k_{on} = 2.87 × 10^6 M^{-1} sec^{-1} for hIRK-I177C). We assumed a similar voltage dependence for the on rates as for the off rates. The time constants for blocking at 100 mV had a ratio of $k_{onhIRK}/k_{onhIRK\text{-}I177C}$ = 1.08. For the calculation of $\Delta\Delta G$ we used $k_{onhIRK}/k_{onhIRK\text{-}I177C}$ = 1.08 and $k_{offI177c}/k_{offhIRK}$ = 1/21.5. The calculated difference in binding energy between hIRK and hIRK-I177C was $\Delta\Delta G$ = 1.78 kcal/mol.

Discussion

Our results demonstrate that the putative transmembrane domain M2 controls unblocking and recovery from dofetilide block in hIRK. Furthermore, the major determinant is I177. In models of drug/receptor interaction using membrane current measurements, unblocking is assumed to be due to the off rate and equilibrium binding to on and off rates. The dramatic change in unblocking for hIRK-I177C indicates that I177 is a major determinant of block.

Isoleucine has a hydrophobicity index of 2.5 kcal/mol (26) and cysteine has a hydrophobicity index of −2.8 kcal/mol, indicating that hydrophobicity at 177 was greatly reduced by the I/C substitution. Because dofetilide has strongly hydrophobic aromatic functions, the substitution should greatly reduce the hydrophobic interaction and result in much faster unblocking. Considering that hIRK is probably an homotetramer (27, 28), as many as four hydrophobic interaction sites may have been altered. Thus, the maximum difference in hydrophobicity clearly accommodates our calculated difference in binding energy of 1.78 kcal/mol. Point mutations in the nucleotide-gated channel of bovine photoreceptors (24) and human plasma factor VIIa (25) have produced similar changes in binding energy of cyclic nucleotides and human tissue factor, respectively.

At physiological pH, dofetilide is 28.5% protonated and positively charged (5). It is likely that the positive N^+ charged form of the compound is involved in voltage dependence of block (9). We previously calculated that the blocking site of dofetilide in hIRK is 85% of the electrical distance across the membrane from the cytoplasmic surface (9). To test whether I177C produced a change, we investigated the unblocking at different hyperpolarized potentials (Figs. 7 and 8). The time constants of unblocking were 15–25-fold faster in hIRK-I177C than in hIRK wild-type over the entire voltage range. The steepness of the voltage dependence of unblocking, however, was not significantly changed, resulting in two parallel curves for hIRK and hIRK-I177C (Fig. 8). We conclude that the position of dofetilide in the electrical field was unaffected by the mutation.

Our interpretation of the mutational analysis is that the large acceleration of unblocking and the weak change in the IC_{50} for hIRK-I177C result from a loss of hydrophobicity at the binding site that normally traps dofetilide and is responsible for its slow unblocking. This interpretation is similar to that for block of K^+ channels by quaternary ammonium ions in internally perfused squid giant axon, where it was concluded that quaternary ammonium ions with hydrophobic tails dissociated more slowly from the receptor (29, 30). Our estimated rate constants are of the same order as the rate constants estimated for tetrapentylammonium block of K^+ currents in squid axons (k_{on} = 1.1 × 10^6 M^{-1} sec^{-1}, k_{off} = 10 sec^{-1}) (30). The quaternary ammonium receptor was thought to have a hydrophobic pocket, large enough to interact with the hydrophobic tails of the compounds. We suggest that the dofetilide receptor in hIRK contains a similar hydrophobic pocket. However, we cannot exclude the possibility that I177C produced its effects in an allosteric manner.

Although I177 is critical for block by dofetilide, other regions may also be involved in binding because the chimeras R. CHM. 1, CHM 12, REVCHM M2, and the mutant hIRK-I177C made unblocking much faster but not instantaneous. Interestingly, the negatively charged D173 is the main determinant for block by polyamines in IRKs (15–17, 31) but does not strongly affect the slow unblocking of dofetilide. We observed only a 1.8-fold acceleration of unblocking in hIRK-D173N relative to hIRK. hIRK has approximately the same sensitivity to polyamines as IRK1 (9) and in hIRKD173 is also a major determinant of block by polyamines.[2]

In this study, we used an artificial cytoplasmic solution

[2] J. Kiehn, B. Wible, A. E. Lacerda, and A. M. Brown, unpublished observations.

without natural blocking particles, Mg^{2+} and polyamines. However, in a previous study, we demonstrated that dofetilide can significantly block outward current of hIRK in the presence of Mg^{2+} and polyamines (9). We also used a high concentration relative to the therapeutic dose (32). It seems that hIRK is 10–30-fold less sensitive to dofetilide than I_{Kr} in cardiomyocytes. Nevertheless, dofetilide block of hIRK might be relevant to toxic effects of class III drugs because high concentrations may inhibit final repolarization, thereby increasing cardiac excitability.

A similar hydrophobic position determining dofetilide block in hIRK may exist in other molecular targets for this class of drugs. Such might be the case for the HERG channel, which probably underlies I_{Kr} (33, 34) and I_{KACh}, which is a heteromultimer of two different inward rectifier potassium channels, CIR and GIRK1 (28). The HERG channel is sensitive to dofetilide with an IC_{50} of 34 mM (35). An alignment of the putative pore-forming sequences of HERG (S5≤S6) and hIRK (M1–M2) showed 51% similarity and 21% identity of the corresponding amino acids. We speculate that dofetilide may block at S6 in HERG, especially because HERG has many properties of an inward rectifier potassium channel (34). The same may be the case for I_{KACh}, which can be blocked at higher concentrations with the very similar methanesulfonanilides D-sotalol and E4031, an MS-551 in guinea pig atrial cells (8). The M1–M2 region of GIRK1, which is a component of I_{KACh}, is very homologous to hIRK (49% identity). GIRK1 also has an isoleucine at the corresponding position hIRK-I177, which may be part of the binding site for methanesulfonanilides in I_{KACh}.

Further studies are needed to demonstrate which effects on different channel types result in beneficial or disadvantageous effects for drug-treated patients. By resolving the structural determinants of drug action, a model of each ion channel drug receptor site can be made for use in the modification and creation of more specific cardiac drugs lacking deleterious side effects.

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References

1. Carmeliet, E. Mechanisms and control of repolarization. *Eur. Heart J.* **14** (Suppl. H):3–13 (1993).
2. Sanguinetti, M. C., and N. K. Jurkiewicz. Two components of cardiac delayed rectifier K^+ current. *J. Gen. Physiol.* **96**:195–215 (1990).
3. Baumgarten, C. M., and H. A. Fozzard. The resting and pacemaker potentials, in *The Heart and Cardiovascular System* (H. A. Fozzard, eds.). Raven Press, New York, 601–636 (1986).
4. Vaughan-Williams, E. M. A classification of antiarrhythmic actions reassessed after a decade of new drugs. *J. Clin. Pharmacol.* **24**:129–147 (1984).
5. Cross, P. E., J. E. Arrowsmith, G. N. Thomas, M. Gwilt, A. Burges, and A. J. Higgins. Selective class III antiarrhythmic agents. 1. Bis(arylalkyl)-amines. *J. Med. Chem.* **33**:1151–1155 (1990).
6. Jurkiewicz, N. K., and M. C. Sanguinetti. Rate-dependent prolongation of cardiac action potential by a methansulfonanilide class III antiarrhythmic agent, specific block of rapid activating delayed rectifier K^+ current by Dofetilide. *Circ. Res.* **72**:75–83 (1993).
7. Kiehn, J., P. Villena, T. Beyer, and J. Brachmann. Differential effects of the new class III agent Dofetilide on potassium currents in guinea pig cardiomyocytes. *J. Cardiovasc. Pharmacol.* **24**:566–572 (1994).
8. Mori, K., Y. Hara, T. Saito, Y. Masuda, and H. Nakaya. Anticholinergic effects of class III antiarrhythmic drugs in guinea pig atrial cells: different molecular mechanisms. *Circulation* **91**:2834–2843 (1995).
9. Kiehn, J., B. Wible, E. Ficker, M. Taglialatela, and A. M. Brown. Cloned human inward rectifier potassium channel as a target for class III methansulfonanilides. *Circ. Res.* **77**:1151–1155 (1995).
10. Duff, H. J., Z.-P. Feng., and R. S. Sheldon. High- and low-affinity sites for [^{3}H] dofetilide binding to guinea pig. *Circ. Res.* **77**:718–725 (1995).
11. Wible, B. A., M. DeBiasi, K. Majumder, M. Taglialatela, and A. M. Brown. Cloning and functional expression of an inwardly rectifying K^+ channel from human atrium. *Circ. Res.* **76**:343–350 (1995).
12. Kubo, Y., T. Baldwin., Y. N. Jan., and L. Y. Jan. Primary structure and functional expression of a mouse inward rectifier potassium channel. *Nature (Lond.)* **362**:127–133 (1993).
13. Ho, K., C. N. Nichols, W. J. Lederer, J. Lytton, P. M. Vassilew, M. V. Kanazirska, and S. C. Hebert. Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. *Nature (Lond.)* **362**:31–38 (1993).
14. Hilgemann, D. W., D. A. Nicoll, and K. D. Philipson. Charge movement during Na^+ translocation by native and cloned cardiac $Na^+\backslash a^{2+}$ exchanger. *Nature (Lond.)* **352**:715–718 (1991).
15. Ficker, E., M. Taglialatela, B. A. Wible, C. M. Henley, and A. M. Brown. Spermine and spermidine as gating molecules for inward rectifier K^+ channels. *Science (Washington D. C.)* **266**:1068–1072 (1994).
16. Lopatin, A. N., E. N. Makhina, and C. G. Nichols. Potassium channel block by cytoplasmatic polyamines as the mechanism of intrinsic rectification. *Nature (Lond.)* **372**:366–369 (1994).
17. Fakler, B., U. Brandle, C. Bond, E. Glowatzki, C. Koenig, J. P. Adelmann, H.-P. Zenner, and J. P. Ruppersberg. A structural determinant of differential sensitivity of cloned inward rectifier K^+ channels to intracellular spermine. *FEBS Lett.* **356**:199–203 (1994).
18. Taglialatela, M., B. A. Wible, R. Caporaso, and A. M. Brown. Specification of pore properties by the carboxyl terminus of inward rectifying K^+ channels. *Science (Washington D. C.)* **264**:844–847 (1994).
19. Taglialatela, M., E. Ficker, B. A. Wible, and A. M. Brown. C-terminus determinants for Mg^{2+} and polyamine block of the inward rectifier K^+ channel IRK1. *EMBO J.* **14**:5532–5541 (1995).
20. Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**:61–68 (1989).
21. Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. Site-directed mutagenesis by overlap extension using polymerase chain reaction. *Gene* **77**:51–59 (1989).
22. Hille, B. *Ionic Channels in Excitable Membranes.* 2nd ed. Sinauer, Sunderland, MA (1992).
23. Stryer. *Biochemistry.* 4th ed. WH Freeman and Company, New York (1995).
24. Varnum, M. D., K. D. Black, and W. N. Zagotta. Molecular mechanism for ligand discrimination of cyclic nucleotide-gated channels. *Neuron* **15**:619–625 (1995).
25. Kelley, R. F., E. C. Kimberly, M. P. O'Connell, and R. A. Lazarus. Analysis of the factor VIIa binding site on human tissue factor: effects of tissue factor mutations on the kinetics and thermodynamics of binding. *Biochemistry* **34**:10383–10392 (1995).
26. Von Heijne, G., and C. Blomberg. Transmembrane location of proteins: the direct transfer model. *Eur. J. Biochem.* **97**:175–181 (1979).
27. Glowatzki, E., G. Fakler, U. Brandle, U. Rexhausen, H.-P Zenner, J. P. Ruppersberg, and B. Fakler. Subunit-dependent assembly of inward-rectifier K^+ channels. *Proc. R. Soc. Lond. Ser. B Biol. Sci.* **261**:251–261 (1995).
28. Krapivinsky, G., E. A. Gordon, K. Wickman, B. Velimirovic, L. Krapivinsky, and D. E. Clapham. The G-protein-gated atrial K^+ channel I_{KACh} is a heteromultimer of two inwardly rectifying K^+ proteins. *Nature (Lond.)* **374**:135–141 (1995).
29. Armstrong, C. M. Inactivation of the potassium conductance and related phenomena caused by quaternary ammonium ion injected in squid axons. *J. Gen. Physiol.* **54**:553–575 (1969).
30. French, R. J., and J. J. Shoukimas. Blockage of squid axon potassium conductance by internal tetra-N-alkylammonium ions of various sizes. *Biophys. J.* **34**:271–291 (1981).
31. Wible, B. A., M. Taglialatela, E. Ficker, and A. M. Brown. Gating of inwardly rectifying K^+ channels localized to a single negatively charged residue. *Nature (Lond.)* **371**:246–249 (1994).
32. Yuan, S., B. Wohlfart, H. S. Rasmussen, S. Olsson, and C. Blomstroem-Lundqvist. Effect of dofetilide on cardiac repolarization in patients with ventricular tachycardia: a study using simultaneous monophasic action potential recordings from two sites in the right ventricle. *Eur. Heart. J.* **15**:514–522 (1994).
33. Sanguinetti, M. C., C. Jiang, M. E. Curran, and M. T. Keating. A mechanistic link between an inherited and an acquired cardiac arrhythmia: *HERG* encodes the I_{Kr} potassium channel. *Cell* **81**:1–8 (1995).
34. Trudeau, M. C., J. W. Warmke, B. Ganetzky, and G. A. Robertson. HERG, a human inward rectifier in the voltage gated potassium channel family. *Science (Washington D. C.)* **269**:92–95 (1995).
35. Kiehn, J., A. E. Lacerda, B. Wible, M. C. Sanguinetti, M. Keating, A. M. Brown. Single-channel properties of HERG, the channel encoding I_{Kr}. *Biophys. J.* **70-2**:A361 (1996).

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