Molecular Determinants of Dofetilide Block of HERG K⁺ Channels

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Abstract—The human ether-a-go-go–related gene (HERG) encodes a K⁺ channel with biophysical properties nearly identical to the rapid component of the cardiac delayed rectifier K⁺ current (I_Kr). HERG/I_Kr channels are a prime target for the pharmacological management of arrhythmias and are selectively blocked by class III antiarrhythmic methanesulfonanilide drugs, such as dofetilide, E4031, and MK-499, at submicromolar concentrations. By contrast, the closely related bovine ether-a-go-go channel (BEAG) is 100-fold less sensitive to dofetilide. To identify the molecular determinants for dofetilide block, we first engineered chimeras between HERG and BEAG and then used site-directed mutagenesis to localize single amino acid residues responsible for block. Using constructs heterologously expressed in Xenopus oocytes, we found that transplantation of the S5-S6 linker from BEAG into HERG removed high-affinity block by dofetilide. A point mutation in the S5-S6 linker region, HERG S620T, abolished high-affinity block and interfered with C-type inactivation. Thus, our results indicate that important determinants of dofetilide binding are localized to the pore region of HERG. Since the loss of high-affinity drug binding was always correlated with a loss of C-type inactivation, it is possible that the changes observed in drug binding are due to indirect allosteric modifications in the structure of the channel protein and not to the direct interaction of dofetilide with the respective mutated site chains. However, the chimeric approach was not able to identify domains outside the S5-S6 linker region of the HERG channel as putative candidates involved in drug binding. Moreover, the reverse mutation BEAG T432S increased the affinity of BEAG K⁺ channels for dofetilide, whereas C-type inactivation could not be recovered. Thus, the serine in position HERG 620 may participate directly in dofetilide binding; however, an intact C-type inactivation process seems to be crucial for high-affinity drug binding. (Circ Res. 1998;82:386-395.)

Key Words: human ether-a-go-go–related gene bovine ether-a-go-go channel K⁺ channel antiarrhythmic drug dofetilide

The human ether-a-go-go–related gene, HERG, encodes a K⁺ channel with biophysical properties nearly identical to the rapid component of I_Kr.¹ The electrophysiological fingerprint of both I_Kr and HERG currents is dominated by slow current activation at negative membrane potentials, large long-lasting tail currents on repolarization, modulation of current amplitudes by external K⁺ ions, and a limited amount of outward current. This strong inward rectification is unusual for voltage-activated K⁺ currents and has been attributed to a fast C-type inactivation mechanism.²⁻⁵ In humans, I_Kr plays a central role in repolarization of the myocardium and termination of individual heartbeats. Of all class III antiarrhythmic agents, I_Kr is blocked with highest affinity and selectivity by methanesulfonanilides, such as E-4031, ibutilide, and dofetilide.⁶⁻⁹ Heterologously expressed HERG currents share these pharmacological properties with I_Kr,¹⁰ and open-channel block by nanomolar concentrations of methanesulfonanilides (dofetilide and MK-499) has been demonstrated.¹¹⁻¹³ I_Kr/HERG represents a unique target for the pharmacological management of arrhythmias. The pharmacological potential of class III antiarrhythmic drugs, however, is limited by their tendency to produce an excessive prolongation of action potentials, which can cause acquired long QT syndrome and sudden cardiac death. Interestingly, hereditary long QT syndrome has been linked to mutations in HERG that produce loss of function or dominant-negative suppression of I_Kr.¹⁴

In the present study, we used dofetilide to probe the structural requirements governing highly selective methanesulfonanilide block of HERG. The closely related BEAG channel is 100-fold less sensitive to dofetilide and was used to engineer CHMs with HERG. We analyzed HERG/BEAG CHMs to determine which protein domain is responsible for high-affinity block by dofetilide. We found that with transplantation of the S5-S6 linker from BEAG into HERG, the high-affinity block of dofetilide was lost. To further refine our structural analysis, we constructed a series of smaller CHMs and point
mutations in the S5-S6 linker region of HERG. Point mutations of HERG amino acid 620 abolished high-affinity drug binding and revealed a strong interaction between C-type inactivation and high-affinity dofetilide binding.

Materials and Methods

The cDNA clone for BEAG was isolated by A. Baumann (GenBank accession No. Y13430). cDNA for the point mutation HERG S631V was engineered in a position equivalent to the one introduced in positions equivalent to naturally occurring MluI and KpnI restriction sites in HERG. All changes in the coding regions were verified by sequencing. A NarI-KpnI fragment was excised from HERG BstEI-Xhol and swapped with the corresponding BEAG fragment in the BEAG-pBluescript construct, resulting in the chimeric pore region of BH516. In a final step, the BstEI-Xhol fragment was excised and subcloned into full-length HERG-pSP64 from which the wild-type fragment had been removed. For construction of BH556 and BH555, MluI-KpnI fragments were excised and subcloned into the opposite pBluescript plasmids. In a second step, BstEI-Xhol and BstEI-KpnI fragments were excised and subcloned into full-length HERG-pSP64 and BEAG-pSP64, respectively.

Expression in Xenopus Oocytes

In brief, Xenopus oocytes were surgically removed and enzymatically defolliculated by treatment with collagenase (2 mg/mL, 1.5 hours) in calcium-free OR2 solution (mmol/L): NaCl 82.5, KCl 2.5, MgCl2 1, and HEPES 5 (pH 7.6). Stage V-VI oocytes were injected with 46 nL of 1 to 1000 ng/µL cRNA solution and incubated at 19°C in SOS solution (mmol/L): NaCl 100, KCl 2, CaCl2 1.8, MgCl2 1, HEPES 5, and pyruvic acid 2.5 (+100 µg/mL gentamicin), pH 7.6.

Electrophysiology

Two-Microelectrode Voltage-Clamp Recordings

Two to 7 days after injection, oocytes were voltage-clamped using a Dagan 8500 two-electrode voltage-clamp amplifier. Current and voltage electrodes were filled with 3 mol/L KCl and had resistances of ~1 MΩ. Bath solutions in experiments using whole oocytes were as follows (mmol/L): NaCl 96, KCl 5, CaCl2 1.8, MgCl2 1.0, HEPES 5 (K+ Ringer’s solution 5, pH 7.4), CaCl2 1.8, and HEPES 10 with either KCl 115 (K+ Ringer’s solution 115, pH 7.4) or CsCl 115 (Cs+ Ringer’s solution 115, pH 7.4). In some recordings 1.0 mmol/L MgCl2 was added to the 115 mmol/L K+ Ringer’s solution. The pClamp software (Axon Instruments) was used for the generation of voltage-clamp protocols and for data acquisition. No leak subtraction was performed. For IC50 measurements, dofetilide (N-[4-(N-[4-(methanesulfonamido)-phenoxyl]-N-methyl)phenyl]methanesulfonamide, provided by Pfizer Central Research, Groton) was perfused in increasing concentrations with 5 mmol/L K+ Ringer’s solution. All IC50 measurements were performed in Xenopus oocytes held at ~80 mV. During measurements, oocytes were continuously stimulated at a frequency of 1 Hz with conditioning prepulses of 0 mV and 400-millisecond duration to accelerate the onset of block. Every 30 seconds, currents were recorded with a depolarizing 1600-ms test pulse to 0 mV, followed by a 1600-ms pulse to ~70 mV. In general, current amplitudes were measured at the end of the depolarizing test pulse to 0 mV. Concentration-response measurements in BH56 CHMs, however, were made by analysis of current amplitudes on return to ~120 mV and not at the end of the depolarizing voltage command to 0 mV.

Macrophot Records

Macrophot recordings were performed as previously described.35 Patch pipettes had resistances of 0.2 to 0.6 MΩ and were filled with 5 mmol/L K+ Ringer’s solution (mmol/L): NaCl 96, KCl 5, CaCl2 1.8, MgCl2 1.0, and HEPES 5 (pH 7.4). The depolarizing bath solution had the following composition (mmol/L): KCl 100, EDTA 5, EGTA 5, and HEPES 10 (iso-K+, pH 7.4). The pClamp software was used for data acquisition. No leak subtraction was used. For IC50 measurements in the cell-attached configuration, whole oocytes were perfused with increasing concentrations of dofetilide in iso-K+ bath solution. Concentration-response measurements in the cell-attached mode were performed using the same voltage protocol as described for two-microelectrode recordings in whole oocytes.

Concentration-response relationships for dofetilide block were fit to a Hill equation of the following form:

\[ I_{\text{infitilide}} / I_{\text{control}} = 1 / \left[ 1 + (D / IC_{50})^{n} \right] \]

where I indicates current, [D] is the dofetilide concentration, n is the Hill coefficient, and IC50 is the concentration necessary for 50% block. All data are expressed as mean±SEM. All measurements were performed at room temperature (20°C).

Results

Fig 1A shows a typical HERG K+ current family elicited by depolarizing voltage steps ranging between −100 and +80 mV with a return potential of −70 mV measured in 5 mmol/L [K+]o. HERG K+ channels activated slowly on moderate membrane depolarizations and exhibited limited outward current at more positive membrane depolarizations. The characteristic bell-shaped I-V relationship of HERG was attributed to a C-type inactivation mechanism that dominates at depolarized potentials (Fig 1B).7 On return to negative membrane potentials, removal of C-type inactivation resulted in an initial
At the protein level, BEAG is 96% identical to both mouse and rat eag channels (Fig 1C and D). Overall, the biophysical properties of BEAG K⁺ channels are identical to those described for mouse and rat eag channels (Fig 1C and 1D). At the protein level, BEAG is 96% identical to both rat and mouse eag. When compared with HERG, BEAG diverges considerably and shows only ~50% identity with HERG in the hydrophobic core region S1-S6 of the channel proteins.

To analyze the high-affinity binding site of dofetilide in HERG K⁺ channels, we took advantage of the fact that BEAG is ~100-fold less sensitive to dofetilide than HERG. IC₅₀ values were measured in two-microelectrode whole-cell recordings with a prepulse protocol to accelerate the onset of block (see "Materials and Methods"). Fig 2A shows HERG currents recorded at 0 mV during perfusion of different dofetilide concentrations. Current amplitudes became more and more reduced with increasing drug concentrations. Current activation and deactivation kinetics, however, were not modified. To test drug effects on current inactivation, we isolated the inactivation process with the following voltage-clamp protocol: cells were held at -40 mV, hyperpolarized for 20 milliseconds to -100 mV to remove any inactivation accumulated at holding potential, and finally depolarized to 0 mV. The voltage step to 0 mV elicited a rapidly decaying outward current, which reflects the highly synchronized inactivation of HERG channels available in a particular cell (inset, Fig 2A). Time constants characterizing the inactivation process were analyzed by fitting monoexponential functions to the decaying outward currents. At membrane potentials of -40, -20, 0, and +20 mV, time constants were 13.5±0.4, 12.1±0.5, 9.1±0.6, and 5.7±0.4 milliseconds (n=5). With 1 μmol/L dofetilide in the extracellular perfusate, time constants were 13.1±0.5, 11.6±0.6, 8.3±0.6, and 5.0±0.5 milliseconds (n=5) and proved not to be different from control values by means of Student’s t test (P>0.05). Although this negative result does not exclude inactivated-state block, it further supports the conclusion of two more recent reports that the inactivated state is not a major target for dofetilide binding.11,13 Fig 2B shows that steady-state block was slowly approached within 10 to 20 minutes after starting perfusion with dofetilide. Steady-state inhibition was determined by monoexponential fits to the time course of amplitude reductions at each drug concentration applied. The IC₅₀ of dofetilide block was 0.32±0.04 μmol/L (n=24) in HERG K⁺ channels.
The IC_{50} value of dofetilide block in whole oocytes expressing HERG K^+ channels was ≈10-fold higher than those measured in inside-out patches from oocytes expressing HERG (IC_{50}, 0.035 μmol/L) or in whole-cell patch-clamp recordings performed in HEK293 cells transiently transfected with HERG (IC_{50}, 0.012 μmol/L). The lower affinity measured in whole oocytes does not affect our study since we focus on relative differences in sensitivity to dofetilide between wild-type channels and chimeric constructs/point mutations.

Much higher dofetilide concentrations had to be used to block BEAG channels (Fig 2C). The IC_{50} was 31.8±7.5 μmol/L (n=6). The onset of block in BEAG K^+ channels was much faster. Steady state was reached within 3 to 5 minutes. In marked contrast to HERG, during washout, 80% to 100% of initial control values were reached within 3 to 10 minutes even at high dofetilide concentrations (Fig 2D). The fast onset and offset of block in BEAG K^+ channels prompted the question of whether dofetilide might block BEAG K^+ channels via an extracellular interaction site. To resolve this, we added dofetilide in increasing amounts to the extracellular bath solution while BEAG currents were recorded from cell-attached macropatches. When the above-described whole-cell pulse protocol was used, steady-state inhibition was reached within 1 to 2 minutes in cell-attached macropatch recordings. Even more important, block by dofetilide could be reversed within 3 to 4 minutes to 60% to 80% of control levels (Fig 2F). The time course of the developing block as well as the washout are comparable to the time course seen in whole-cell recordings. The IC_{50} in cell-attached macropatch recordings was 3.4±0.3 μmol/L (fit with a Hill coefficient of 1, n=7). Thus, dofetilide blocks BEAG from the cytoplasmic side of the membrane.

HERG and BEAG K^+ channels were also differentially blocked by E4031, another important methanesulfonanilide drug. In HERG K^+ channels, we measured an IC_{50} of 3.5 μmol/L for E4031 block (n=4). In BEAG K^+ channels, the IC_{50} was 180 μmol/L (n=4). As described for dofetilide, the onset/washout of block by E4031 was slow in HERG K^+ channels and fast in BEAG channels (data not shown).

Transplantation of BEAG S5-S6 Linker Into HERG Removes High-Affinity Block

Dofetilide has been described as a slow-onset/slow-offset open-channel blocker of HERG, suggesting a binding site in the conduction pathway.11,17 Therefore, we constructed "large scale" CHMs between HERG and BEAG channels with a main focus on those domains of the ion channel protein thought to line the conduction pathway for K^+; (1) transmembrane domain S5, (2) S5-S6 linker, or "pore" region, and (3) transmembrane domain S6.

Overall, six "large-scale" CHMs were constructed (Fig 3). Transplantation of the complete S1 to S6 transmembrane region of HERG into BEAG (BHS16 CHM) resulted in an inactivating HERG-like current with an IC_{50} of dofetilide block close to HERG WT (0.6±0.22 μmol/L [n=5] versus 0.32±0.04 μmol/L [n=24] for HERG WT) and accelerated current activation and deactivation kinetics (Figs 4A and 5). Overall, the phenotype of BHS16 CHM was reminiscent of a HERG construct with a deletion in the N-terminus, HERG Δ2-373, which similarly did not eliminate inactivation but accelerated deactivation by ≈10-fold.3,4 Transplantation of the complete conduction pathway S5-S6 of BEAG into HERG (HBS56 CHM) resulted in a noninactivating BEAG-like channel with an IC_{50} of dofetilide block close to the one measured for BEAG WT (17.2±2.4 μmol/L [n=6] versus 31.8±7.5 μmol/L [n=6] for BEAG WT, Figs 4B and 5). In contrast, the reverse mutation BHS56 CHM (with the HERG conduction pathway implanted into BEAG) showed an IC_{50} (0.7±0.14 μmol/L [n=3]) close to the one measured in HERG WT (Fig 5). Current activation in BHS56 was exceedingly slow and showed a characteristic biphasic behavior (Fig 4E1). Beside the effects on kinetics, the voltage dependence of activation was shifted to more positive values (Fig 4E2). Both phenomena were controlled by [Mg^{2+}], (Fig 4E3). Although the current activation in BHS56 CHM was dominated by the transplanted HERG domains, the current deactivation was fast, like BEAG. This provides further evidence that major determinants for current deactivation reside in N-terminal domains, whereas current inactivation is largely controlled by the S1-S6 regions of these channel proteins.3,4,19 Replacement of either S5 (HBS5 CHM) or S6 (HBS6 CHM) in HERG with the corresponding BEAG domain preserved C-type inactivation. Both HBS5 and HBS6 CHMs were very sensitive to dofetilide, with IC_{50} values of 0.48±0.09 μmol/L (n=4) and 0.15±0.02 μmol/L (n=7), respectively (Fig 5). HBS5 CHM expressed HERG-like currents with respect to activation, deactivation, and inactivation (Fig 4C). Currents expressed by HBS6, however, resembled inward rectifier currents (Fig 4F1 and 4F2). This conversion from an outward rectifier to an inward rectifier resulted from a disrupted activation gate in combination with a C-type inactivation process left intact in HBS6 CHM. In fact, we were not able to close HBS6 CHM channels with hyperpolarizations up to −180 mV. The putative K^+ dependence suggested by the I-V relationships shown in Fig 4F3 can be explained as the simple reflection of changes in reversal potential and driving force while [K^+], increased.

Figure 3. Concentration-dependent dofetilide block in HERG/BEAG CHMs. Whole-cell measurements in Xenopus oocytes were performed at −80 mV. Oocytes were continuously stimulated at a frequency of 1 Hz with conditioning prepulses of 0 mV for 400 milliseconds to accelerate the onset of block. Every minute, currents were recorded with a depolarizing 1600-millisecond test pulse to 0 mV followed by a 1600-millisecond pulse to −70 mV. [K^+]o was 5 mmol/L. Concentration-response measurements in HBS56 CHM were made by analysis of current amplitudes on return to −120 mV. Concentration-response relationships in HERG WT, BHS16, HBS5, HBS6, and HBPore were fit with Hill equations according to a one-to-one binding scheme (Hill coefficient n=1). Fits to concentration-response curves measured in BEAG WT and HBS56 are shown, assuming the same binding scheme. For these two constructs, however, better fits could be obtained with Hill coefficients of ±0.6.
Figure 4. Macroscopic current recordings from oocytes injected with the following CHMs. A, BHS16 (transplantation of HERG S1 to S6 into BEAG). On the left, the holding potential was −80 mV, test pulses were from −100 to +80 mV in 10-mV increments, return potential was −70 mV, and [K\(^{\text{o}}\)] was 5 mmol/L. On the right is the corresponding I-V relationship. B, HBS56 (transplantation of BEAG S5 to S6 into HERG). On the left, the holding potential was −90 mV, test pulses were from −100 to +90 mV in 10-mV increments, return potential was −70 mV, and [K\(^{\text{o}}\)] was 5 mmol/L. On the right is the corresponding I-V relationship C, HBSS5 (transplantation of BEAG S5 into HERG). On the left, the holding potential was −90 mV, test pulses were from −100 to +60 mV in 10-mV increments, return potential was −70 mV, and [K\(^{\text{o}}\)] was 5 mmol/L. On the right is the corresponding I-V relationship D, HBPore (transplantation of BEAG S5-S6 linker region into HERG). On the left, the holding potential was −85 mV, test pulses were from −100 to +60 mV in 10-mV increments, return potential was −70 mV, and [K\(^{\text{o}}\)] was 5 mmol/L. On the right is the corresponding I-V relationship, E1 to E3, BHS56 (transplantation of HERG S5 to S6 into BEAG). For panel E1, the holding potential was −80 mV, test pulses were from −80 to +80 mV in 10-mV increments, return potential was −70 mV, and [K\(^{\text{o}}\)] was 5 mmol/L. Panel E2 shows the corresponding I-V relationship. For panel E3, extracellular Mg\(^{2+}\) regulates activation of BHS56. The holding potential was −90 mV, test pulse was to −40 mV, return potential was −70 mV, and K\(^{\text{r}}\) Ringer’s solution was 115 mmol/L with and without [Mg\(^{2+}\)]\. F1 to F4, HBS6 (transplantation of HERG S5-S6 into HERG). For panel F1, the holding potential was −80 mV, test pulses were from −140 to +40 mV, and [K\(^{\text{r}}\)] was 5 mmol/L. For panel F2, the pulse protocol was as in panel F1 but 115 mmol/L K\(^{\text{r}}\) Ringer’s solution with 1 mmol/L Mg\(^{2+}\) was used (same cell as in panel F1). For panel F3, the I-V relationship corresponds to current recordings shown in panels F1 and F2, indicates 5 mmol/L [K\(^{\text{r}}\)]\. F4, 115 K\(^{\text{r}}\) Ringer’s solution with Mg\(^{2+}\). For panel F4, C-type inactivation is preserved in HBS6. Holding potential was −80 mV, hyperpolarizing prepulse was 350 milliseconds at −140 mV, and test pulses were from −130 to +40 mV. Experiment was performed in 115 mmol/L Cs\(^{+}\) Ringer’s solution.

from 5 to 115 mmol/L. The activation of this current no longer depended on movement of the voltage sensor; instead, HBS6 channels were gated by removal of an inactivation gate, which prevents outward current flow at more depolarized potentials. The kinetics and voltage dependence of this inactivation process could be clearly resolved in recordings with 115 mmol/L [Cs\(^{+}\)], (Fig 4F4). Cs\(^{+}\) is known for slowing C-type inactivation in HERG WT channels.\(^{3}\) Furthermore, current inactivation in HBS6 CHM was slowed by increasing the [K\(^{\text{r}}\)]\(^{\text{r}}\), and by extracellular applied tetraethylammonium (data not shown). These results were consistent with the idea that C-type inactivation is not impaired in HBS6 CHMs. Since switching neither the S5 nor the S6 domain resulted in major changes of I\(_{\text{C0}}\) values, we transplanted the BEAG S5-S6 linker region into HERG (HBPore CHM, Fig 6A). In HBPore CHM, C-type inactivation and high-affinity dofetilide binding were abolished, whereas the slow current activation and deactivation characteristics for the parent channel HERG were preserved (Fig 4D). For HBPore CHM, an I\(_{\text{C0}}\) of 148±34 μmol/L (n=8) was measured (Fig 5). Thus, the S5-S6 linker seems to contain the determinants for both dofetilide binding and C-type inactivation.

**Chimeric Constructs in the HERG S5-S6 Linker Region**

The S5-S6 linker with the putative binding site for dofetilide was divided into two regions: (1) 40 amino acids preceding H5 and (2) the H5 domain (Fig 6A). Transplantation of the first 40 amino acids of the S5-S6 linker of BEAG into HERG (HERG GGPS) changed neither C-type inactivation nor high-affinity dofetilide block (I\(_{\text{C0}}\) HERG GGPS, 0.21±0.03 μmol/L [n=6]; Figs 6B and 7A). In the second region, the conserved H5 domain of the S5-S6 linker region, which is thought to harbor the selectivity filter in K\(^{\text{r}}\) channels, the amino acid sequence is largely preserved between HERG and BEAG. We analyzed the sequence differences between these two channels with three separate constructs in this region (Fig 6A). HERG KNSV did not express currents. HERG ISS showed wild-type behavior with respect to kinetics, C-type inactivation, and dofetilide binding (I\(_{\text{C0}}\) HERG ISS, 0.32±0.06 μmol/L [n=8]; Figs 6B and 7B). In contrast, analysis of the double mutation HERG MT (HERG F619M and S620T) exhibited major changes. C-type inactivation was completely removed (Fig 7C), and the affinity for dofetilide was reduced (Fig 6B). The I\(_{\text{C0}}\) was 123±27 μmol/L (n=5), which was nearly identical to the one measured for the large-scale CHM HBPore (Fig 5). Thus, major determi-
nants of the HBPore phenotype could be located to amino acids in position HERG 619 and/or HERG 620.

A Serine in Position 620 Is Critical for High-Affinity Dofetilide Block and C-Type Inactivation

Since the characteristics of HBpore could be mimicked by the double mutation HERG MT, we constructed two single point mutations, HERG F619M and HERG S620T, to evaluate contributions from both positions separately. HERG F619M preserved C-type inactivation (Fig 8A). In addition, this mutation was blocked by very low dofetilide concentrations (IC₅₀, 0.43±0.06μmol/L [n=7]; Fig 9). In contrast, the conservative substitution of serine with threonine in position 620 resulted in a complete loss of C-type inactivation (Fig 8C). This was further confirmed with inside/out macropatch recordings. First, channels were maximally activated with depolarizations to +20 mV; after 650 milliseconds, the membrane was stepped to potentials between −100 and +90 mV (Fig 8C, right). Even at +90 mV, no residual inactivation was detectable. Correspondingly, the onset of current deactivation was instantaneous and no longer delayed by channels coming out of the open state.
of inactivation. The rectification process, which dominates the I-V relationship in whole-cell recordings at very positive potentials (see Fig 8C, middle), is due to a fast block by Na$^+$ ions. It is completely removed in inside/out recordings, since macropatches were excised in a nominally Na$^+$-free solution (iso-K$^+$). The IC$_{50}$ for dofetilide block increased dramatically to 248±29 μmol/L (n=8, Fig 9). Of all the constructs engineered in the present study, HERG S620T was the least sensitive to dofetilide, even less sensitive than HBPore CHM or the double mutation HERG MT (Fig 5). We conclude that HERG position 620 constitutes a hot spot in the pore region for conformational changes tightly associated with C-type inactivation and high-affinity dofetilide binding. A binding site in the inner pore region as proposed for HERG S620 would fit inactivation and high-affinity dofetilide binding. A binding site for conformational changes tightly associated with C-type inactivation and high-affinity dofetilide binding could not be reached with constructs: K$_{0}$ permeability was negligible for HERG WT and HERG S620T channels, whereas the Na$^+$ permeability might be consistent with the idea that this amino acid position contributes to the lining of the inner pore region of HERG K$^+$ channels. In HERG S620C, half-maximal block by dofetilide was reached with a concentration of 5.7±0.9 μmol/L (n=8, Fig 9). This value is intermediate to HERG WT and HERG S620T, as if part of the dofetilide binding site could be preserved with this substitution although current inactivation was abolished.

To gain further insight in the tight coupling between C-type inactivation and dofetilide binding, we tested two point mutations in position HERG 619/620 and BEAG 432/443. Concentration-response relationships were fitted with Hill equations (Hill coefficient n=1).

A clear separation between effects on C-type inactivation and dofetilide binding could not be reached with constructs analyzed so far. Mutations supposedly located on opposite sites of the membrane gave similar phenotypes: loss of drug binding was always correlated with a loss of C-type inactivation. Three additional point mutations in HERG S620 were constructed in an attempt to further separate drug binding and current inactivation. Substitution of an alanine (HERG S620A) or of the bulkier valine (HERG S620V) in position 620 resulted in constructs that did not express current levels suitable for analysis. The introduction of a cysteine in position 620 (HERG S620C) gave functional channels with C-type inactivation removed. Fig 8B shows currents recorded at membrane potentials between −100 and +80 mV. In these recordings, it was striking that the rectification observed in HERG S620T, which could be attributed to a fast block by Na$^+$ ions, was not present. Therefore, we tested whether the permeability of HERG S620C was altered. Permeability ratios for HERG WT, HERG S620T, and HERG S620C channels were obtained from reversal potentials measured in two-microelectrode voltage-clamp experiments. The overall sequence of cation permeabilities was preserved between these three constructs: K$^+\geq$Rb$^+\geq$Cs$^+\geq$Na$^+$. However, whereas the Na$^+$ permeability was negligible for HERG WT and HERG S620T (<0.01, n=3), it was increased in HERG S620C. We measured a Na$^+\cdot$K$^+$ permeability of 0.07±0.02 (n=5). This change in Na$^+$ permeability might be consistent with the idea that this amino acid position contributes to the lining of the inner pore region of HERG K$^+$ channels. In HERG S620C, half-maximal block by dofetilide was reached with a concentration of 5.7±0.9 μmol/L (n=8, Fig 9). This value is intermediate to HERG WT and HERG S620T, as if part of the dofetilide binding site could be preserved with this substitution although current inactivation was abolished.

The experiments performed in HERG S620C were consistent with the idea that dofetilide binding could be at least partially conserved in a noninactivating channel. Therefore, we asked...
whether it is possible to increase dofetilide binding in noninactivating BEAG channels by engineering a serine in the position equivalent to HERG S620. The $I_{\text{C}0}$ for dofetilide block was shifted in BEAG T432S from 31.8 $\mu$mol/L measured in BEAG WT channels to 7.8±1.2 $\mu$mol/L (n=5, Fig 9). BEAG T432S channels expressed noninactivating delayed rectifier currents, as shown with two-microelectrode and high resolution inside/out macropatch recordings (Fig 8D). Neither current activation nor current deactivation was modified compared with the parent channel BEAG WT. As a control, we mutated amino acid position 443 in BEAG. Position 443 is equivalent to HERG S631, the above-described position in the extracellular mouth of the HERG channel pore interfering with C-type inactivation and dofetilide binding. The alanine in the BEAG WT channel was replaced by a serine as expressed in HERG WT in the corresponding position (see Fig 6A). BEAG A443S channels expressed noninactivating outward currents. The I-V relationship for these currents showed that the voltage dependence was not significantly different from that for BEAG T432S and BEAG WT (Fig 8E). The $I_{\text{C}0}$ value of dofetilide block, however, was not shifted to lower values as described for BEAG T432S ($I_{\text{C}0}$, 7.8 $\mu$mol/L). Instead, it was increased from 31.8 $\mu$mol/L (BEAG WT) to 41.8±5.0 $\mu$mol/L (n=5, Figs 5 and 9).

**Discussion**

Several chimeric and mutant channels were engineered to identify amino acid residues critical for block by dofetilide. A single point mutation, HERG S620T, which introduced an additional methyl group in the side chain of the native channel, has been found to reduce the affinity for dofetilide by $\approx$800-fold. The reduction in dofetilide block correlated with a complete loss of channel inactivation. Substitution of a cysteine for the native serine in this position resulted in a channel with an altered permeability for Na$^+$ ions. This might indicate that the residue in position HERG S620 (by analogy to topological models proposed for K$^+$ channels in the Kv gene family$^{22}$) is directed to the cytoplasmic part of the ion conduction pathway and would be consistent with an intracellular binding site for dofetilide as proposed more recently. The $I_{\text{C}0}$ for dofetilide block, however, was not shifted to lower values as described for BEAG T432S ($I_{\text{C}0}$, 7.8 $\mu$mol/L). Instead, it was increased from 31.8 $\mu$mol/L (BEAG WT) to 41.8±5.0 $\mu$mol/L (n=5, Figs 5 and 9).

Since mutations and CHMs in the pore region that showed high-affinity block by dofetilide and C-type inactivation obtained by independent mutations at two residues might be most easily explained by a model in which dofetilide binds preferentially to the inactivated state of HERG. In such a model, abolishing the inactivated state would necessarily result in a loss of high-affinity drug binding. On the other hand, our data do not support dofetilide binding to the inactivated state of this channel since (1) dofetilide does not accelerate the inactivation time course of HERG channels and (2) strong depolarizations of long duration delay the development of block rather than enhance it.$^{11,12}$ Thus, the loss of high-affinity drug binding that correlates with a loss of C-type inactivation might be better explained by the disruption of an earlier obligatory conformational step, which is essential for both drug binding and C-type inactivation. More recently, the kinetic properties of HERG currents were successfully simulated by a linear model consisting of three closed states, one open state, and one inactivated state.$^{23}$ Since HERG channels were not blocked when kept closed, high-affinity block of closed states could be eliminated. On the contrary, the requirement for channel activation suggested open-channel block. However, it has been reported that dofetilide does not produce significant changes in single-channel open time.$^{13}$ Also, the $I_{\text{C}0}$ measured for dofetilide and MK–499 in heterologously expressed HERG channels seemed not to depend on [K$^+$]o, as one would expect for open-channel block.$^{12,13}$ The influence of [K$^+$]o, however, remains controversial, since in AT, tumor cells block of native I$_{\text{Ks}}$ channels by dofetilide was dependent on [K$^+$]o.$^{24}$ Furthermore, open-channel block is hard to reconcile with mutations such as HERG S620T, in which removal of the inactivated state would be linked to a dramatic increase in the availability of a binding site associated with the open state. At this point, it may be important to reconsider that HERG channels showed bursting behavior. Such bursting behavior can result from a frequently revisited brief closed state, which exists as the final step in the activation pathway. Alternatively, these short closures might represent dwells in a preinactivated state. Binding of dofetilide to such a short-lived closed state would help to understand (1) why there is no competition of drug binding with external K$^+$ and (2) why the on rates for block are exceedingly slow, although dofetilide concentrations reached steady-state levels in Xenopus oocytes after equilibration times of only 2 to 4 minutes, as shown in experiments with BEAG. Such short equilibration times are expected for a drug that is only $\approx$30% protonated at physiological pH.$^{25}$ In contrast, it took 10 to 20 minutes to reach steady-state block in measurements with HERG expressed in oocytes. On the other hand, it is difficult to envisage a kinetic model in which simple removal of inactivation (as shown for HERG S620T) would decrease the availability of a short-lived closed state in the activation pathway. At the same time, our experiments do not provide enough information for an interpretation of these short closures as dwells into a preinactivated state. According to such an interpretation, however, the prediction would be that bursting behavior should be strongly reduced in mutations such as HERG S620T, i.e., mutations with low affinity for dofetilide binding and no C-type inactivation. A clear answer to the question of which state may be the target for dofetilide is currently not possible.

Since mutations and CHMs in the pore region that showed a loss of drug binding always exhibited a loss of C-type inactivation, it is possible that the change observed in drug binding is due to indirect nonspecific allosteric modifications in the structure of the channel protein and not to the direct interaction of dofetilide with the respective mutated site chains. If we assume that the pore region might not participate directly in dofetilide binding, the chimeric approach used in the present study should still have identified alternative protein domains as candidates for drug binding, assuming that residues conserved between both parent channels play no crucial role in drug binding. CHMs such as BHS16, HBS56, and BHS56 reveal no contribution from the N- and C-terminus or
transmembrane domains S1 to S4 to a binding site. This result is in line with the low homology between HERG and BEAG on the protein level, especially in the N- and C-terminal regions. The dofetilide affinity of all chimeric constructs studied seemed to be entirely controlled by the ion conduction pathway composed of S3, S5–S6 linker, and S6. If this part of the protein originated from HERG, we observed high-affinity drug binding; if it originated from BEAG, we observed low-affinity binding. Revisiting the CHMs engineered in the conduction pathway shows clearly that transplantation of the BEAG S5 domain into HERG resulted in a channel with HERG-like properties (ie, C-type inactivation, high-affinity dofetilide binding site, and slow current activation and deactivation) and gave no hint for an association of drug binding with S5. At this point, it is important to mention that 16 of 24 amino acids are conserved between BEAG and HERG S5 domains. On the other hand, transplantation of the BEAG S6 domain into HERG disrupted the gating properties of the recipient channel completely; the activation gate was largely destroyed, whereas C-type inactivation was entirely conserved. Although this transplantation caused major structural changes, HBs6 CHM was very sensitive to dofetilide. In fact, it was the only CHM engineered with an affinity clearly greater than HERG WT. In this case, 18 of 29 amino acids are conserved. It is not clear whether the enhanced affinity measured in HBs6 CHM is due to allosteric effects or due to a contribution of specific amino acid residues in BEAG S6. The effects caused by transplantation of BEAG S6, however, deserve special attention, since in voltage-gated Kv1.5 channels, it has been shown that hydrophobic residues in the S6 transmembrane domain play a central role in block by the antiaarrhythmic drug quinidine.26 A comparison to quinidine binding in voltage-gated Na$^+$ channels identifies common structural motifs in both voltage-gated K$^+$ and Na$^+$ channels, which define this drug-channel interaction.27 Surprisingly, this domain seems to be less important for methanesulfonanilide binding in HERG/I$_{Kr}$ as judged from our chimeric approach. A more systematic analysis of all amino acid residues in S6 (eg, by alanine scanning mutagenesis) might be necessary to evaluate the role played by S6 residues in dofetilide binding.

Since the CHM approach was not able to identify domains outside the S5–S6 linker region of the HERG channel as putative candidates involved in drug binding, we have to ask whether the pore region itself participates in dofetilide binding. The main analytical problem is the tight coupling between drug binding and C-type inactivation. We were able to reconstruct a dofetilide binding site in BEAG by introducing a serine in BEAG 432, a position equivalent to HERG S620. BEAG T432S resulted in a channel 4-fold more sensitive to dofetilide than BEAG WT but did not introduce changes in activation or inactivation. On the contrary, mutations in the outer mouth of the BEAG channel at position 443, which is equivalent to HERG S631, shifted the IC$_{50}$ from 32 μmol/L in WT channels to 42 μmol/L in BEAG A443S. Again, the activation and inactivation properties were not affected by this mutation. Similarly, a comparison of HERG S620T with HERG S620C revealed that the introduction of a cysteine residue in position 620 shifted the IC$_{50}$ from 248 to 5.7 μmol/L, resulting in a channel ~40-fold more sensitive than HERG S620T. These results support the conclusion that in noninactivating channels, mutations of amino acid residues in HERG 620 or BEAG 432 alter the sensitivity of these channels to dofetilide. In both cases, the observed changes have to result from alterations in interactions of side chains with the bound drug. We could not observe any effects of these mutations on activation or inactivation when BEAG T432S and BEAG A443S were compared with BEAG WT or when HERG S620T was compared with HERG S620C. In summary, these observations point to a direct interaction of dofetilide with residue 432 in BEAG K$^+$ channels. A similar interaction site could be identified for noninactivating channels constructed in HERG, such as HERG S620T and HERG S620C. Similarly, mutations in the outer mouth of the pore at position HERG631 were ~8-fold more sensitive to dofetilide than was HERG S620T, with C-type inactivation largely abolished. The explanation might be that mutations in the outer mouth of the channel pore do not alter the amino acid sequence of an internal binding site but simply change the conformation of that site somewhat. Whether the same principles govern high-affinity block in HERG WT must be explored in greater detail in future experiments. The fact is that minor side-chain modifications at position HERG620 alter the function of the channel extensively and significantly. This argues strongly that the wild-type side chain interacts with some other part of the channel protein that confers the conformation needed to obtain inactivation. One explanation for our failure to reconstruct a binding site with nanomolar affinity might simply be that BEAG T432S does not reproduce C-type inactivation as indicated by the outwardly rectifying I-V relationship.

Although the role played by amino acid position 620 in HERG WT is not completely understood with respect to dofetilide binding, we find that this residue is crucial for C-type inactivation. It has been shown that the equivalent residues in Shaker (Shaker V438) and Kv2.1 channels (I369) are located within the ion conduction pathway.22,28 Interestingly, mutations in Kv2.1 I369 can introduce C-type inactivation (or P-type inactivation, as it was originally named in Kv2.1 or in chimeras closely related to Kv2.1).29 None of these Kv2.1 mutations, however, was blocked by dofetilide up to 100 μmol/L (data not shown). Although HERG K$^+$ channels belong to a different gene family, the importance of HERG S620 with respect to C-type inactivation has been preserved. All point mutations in HERG S620 interfered with C-type inactivation. These results indicate that C-type inactivation is not entirely localized to residues in the outer mouth of the pore region. C-type inactivation might also involve structural changes in deeper parts of the pore, with conformational changes imposed on a more extended group of amino acids than previously proposed.29

The structural requirements described in the present study for high-affinity binding of dofetilide to its receptor site might shed some light on the molecular basis underlying the exquisite specificity of methanesulfonanilide drugs for HERG/I$_{Kr}$ channel proteins. Our data should prove useful for a first understanding of class III antiarrhythmic drug action and might help in the future to circumvent some of the side effects that still restrict the therapeutic potential of those drugs.
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