Molecular Determinants of Dofetilide Block of HERG K⁺ Channels

Eckhard Ficker, Wolfgang Jarolimek, Johann Kiehn, Arnd Baumann, Arthur M. Brown

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 (Ikr). HERG/Ikr 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
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). BEAG was subcloned into a modified pSP64 vector. HERG/pSP64 was a gift from M.T. Keating (University of Utah, Salt Lake City). cDNA for the point mutation HERG S631V (accession No. Y13430). BEAG was subcloned into a modified pSP64 cassette generated in pBluescript HERG ISS, and HERG MT and all point mutations were generated (HERG, HBS5, HBS6, HERG GGPS, HERG KNSV, HERG ISS, HERG MT, and all point mutations in HERG) or (HERG, HBS5, HBS6, HERG GGPS, HERG KNSV, HERG ISS, HERG MT, and all point mutations in BEAG).

Macrophage Recordings

Macrophage 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⁺ 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 HBS6 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.

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

$$I_{\text{dofetilide}} / I_{\text{control}} = 1 / [1 + (\text{IC}_{\text{50}} / \text{[D]}^{n})]$$

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). On return to negative membrane potentials, removal of C-type inactivation resulted in an initial
increase in tail currents with slow deactivation kinetics. BEAG gave rise to delayed rectifier–like outward currents with activation kinetics strongly dependent on the holding potential and an extremely fast current deactivation at more hyperpolarized membrane potentials (Fig 1C and D). Overall, the biophysical properties of BEAG K+ channels were identical to those described for mouse and rat eag channels (Fig 1C and 1D).16,17 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.18

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 > .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 washout. C, BEAG: 2-microvolt whole-cell macropatch recordings of BEAG. Dofetilide was perfused with extracellular bath solution.

Figure 1. Macroscopic current recordings from Xenopus oocytes injected with HERG or BEAG. A, HERG: holding potential, −80 mV; test pulses, from −100 to +80 mV with 10-mV increments; and return potential, −70 mV. B, I-V relationship measured at the end of the test pulse for same cell as in panel A. C, BEAG: holding potential, −80 mV; test pulses, from −100 to +80 mV with 10-mV increments; and return potential, −70 mV. Note that current deactivation is very fast at −70 mV (2 to 3 milliseconds) and cannot be resolved at this time scale. D, I-V relationship measured at the end of the test pulse for same cell as in panel C. All recordings were performed in 5 mmol/L [K+]o.

Figure 2. Differential sensitivity and time dependence of dofetilide block. A, HERG: two-microelectrode (2-micro) voltage-clamp recording; holding potential, −80 mV; test pulse, to 0 mV for 1600 milliseconds, preceded by 20 conditioning prepulses to 0 mV for 400 milliseconds applied at a frequency of 1 Hz; and return potential, −70 mV. Current traces were elicited in 0.1, 0.3, and 1 μmol/L dofetilide. B, Slow time-dependent development of dofetilide block. Note slow washout. C, BEAG: 2-microvolt voltage-clamp recording; holding potential, −80 mV; test pulse, to 0 mV for 1600 milliseconds, preceded by 20 conditioning pre- pulses to 0 mV for 400 milliseconds applied at a frequency of 1 Hz; and return potential, −70 mV. Current traces were elicited in 1, 10, and 100 μmol/L dofetilide. D, Time dependence of dofetilide block in BEAG. E, BEAG: cell-attached (cell-att) macropatch recording with pulse protocol analogous to 2-micro recordings described in panel C. F, Time dependence of dofetilide block in cell-att macropatch recordings of BEAG. Dofetilide was perfused with extracellular bath solution.
The IC₅₀ value of dofetilide block in whole oocytes expressing HERG K⁺ channels was approximately 10-fold higher than those measured in inside-out patches from oocytes expressing HERG (IC₅₀ = 0.035 μmol/L) or in whole-cell patch-clamp recordings performed in HEK293 cells transiently transfected with HERG (IC₅₀ = 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 IC₅₀ 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 (Figs 2E and 5). In contrast, the reverse mutation BHS56 CHM (with the HERG conduction pathway implanted into BEAG) showed an IC₅₀ (0.7 ± 0.14 μmol/L [n = 6]) 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 [Mg²⁺] (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.³⁴,¹⁹ Replacement of either S5 (HS5 CHM) or S6 (HS6 CHM) in HERG with the corresponding BEAG domain preserved C-type inactivation. Both HBS5 and HBS6 CHMs were very sensitive to dofetilide, with IC₅₀ 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.

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.¹¹,¹³ 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 IC₅₀ 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.³⁴ Transplantation of the complete conduction pathway S5–S6 of BEAG into HERG (HBS56 CHM) resulted in a noninactivating BEAG-like channel with an IC₅₀ of dofetilide block close to the one measured for BEAG WT (17.2 ± 2.4 μmol/L [n = 6] versus 31.8 ± 2.7 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 IC₅₀ (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 [Mg²⁺] (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.³⁴,¹⁹ Replacement of either S5 (HS5 CHM) or S6 (HS6 CHM) in HERG with the corresponding BEAG domain preserved C-type inactivation. Both HBS5 and HBS6 CHMs were very sensitive to dofetilide, with IC₅₀ 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⁺] 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 BHS56 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⁺]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⁺]o was 5 mmol/L. On the right is the corresponding I-V relationship C, HBSS (transplantation of BEAG S5 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⁺]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⁺]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⁺]o was 5 mmol/L. Panel E2 shows the corresponding I-V relationship. For panel E3, extracellular Mg²⁺ regulates activation of BHS56. The holding potential was −90 mV, test pulse was to +40 mV, return potential was −70 mV, and K⁺ was 5 mmol/L. Panel E3 shows the corresponding I-V relationship. For panel E4, HBPore (transplantation of HERG S5 into HERG). For panel F1, the holding potential was −80 mV, test pulses were from −140 to +40 mV, and [K⁺]o was 5 mmol/L. For panel F2, the pulse protocol was as in panel F1 but 115 mmol/L K⁺. Ringer’s solution with 1 mmol/L Mg²⁺ 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. It indicates 5 mmol/L [K⁺]o; ■, 115 mmol/L K⁺. Ringer’s solution with Mg²⁺. For panel F4, C-type inactivation is preserved in HBSS6. 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, HBSS6 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. Furthermore, current inactivation in HBSS6 CHM was slowed by increasing the [K⁺]o, and by extracellular applied tetraethylammonium (data not shown). These results were consistent with the idea that C-type inactivation is not impaired in HBSS6 CHMs. Since switching neither the S5 nor the S6 domain resulted in major changes of IC₅₀ values, we transplanted the BEAG S5-S6 linker region into HERG (HBPor CHM, Fig 6A). In HBPor 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 HBPor CHM, an IC₅₀ 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 GGPSS) changed neither C-type inactivation nor high-affinity dofetilide block (IC₅₀ HERG GGPSS, 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⁺ 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 (IC₅₀ 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 IC₅₀ was 123±27 µmol/L (n=5), which was nearly identical to the one measured for the large-scale CHM HBPor (Fig 5). Thus, major determin-
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 ± 0.06 μmol/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 inactivated state (Fig 8C, right).

Figure 6. A. Sequences of HERG, BEAG, and CHMs engineered in the S5-S6 linker region. The BEAG-derived sequence is shadowed. HERG MT corresponds to the double mutation HERG F619M & S620T. HERG 620 and HERG 631 indicate amino acids in HERG central for C-type inactivation and dofetilide binding and their counterparts BEAG 432 and BEAG 443. Symbols in panel A correspond to those in panel B. n.e. indicates CHM not expressing. B, Concentration-dependent dofetilide block of CHMs in S5-S6 linker region. Whole-cell measurements were performed at a holding potential of −80 mV. The prepulse protocol was as follows: prepulse of 0 mV for 400 milliseconds applied at 1 Hz; test pulse of 0 mV for 1600 milliseconds, recorded every minute; and return potential, −70 mV for 1600 milliseconds. [K+]o was 5 mmol/L. Concentration-response relationships were fit with Hill equations (n = 1).

Figure 7. Macroscopic current recordings from oocytes injected with the following CHMs. A, HERG GGPS. B, HERG ISS. C, HERG MT (HERG F619M & HERG S620T). Holding potentials were −80 mV, test pulses were from −100 to +80 mV in 10-mV increments, return potentials were −70 mV, and [K+]o was 5 mmol/L. The graphs on the right show the corresponding I-V relationships.
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.\(^20\) It is completely removed in inside/out recordings, since 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 phenomenological 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 a complete loss of C-type inactivation. Substitution although current inactivation was abolished. The experiments performed in HERG S620T were consistent with the idea that this amino acid position contributes to the lining of the inner pore region of HERG K\(^{\text{+}}\) channels. In HERG S620C, half-maximal block by dofetilide was reached with a concentration of 5.7±0.9 \(\mu\text{mol/L}\) (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...
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 ~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 results obtained with mutations in HERG S620, however, have to be contrasted with mutations of an amino acid residue in the extracellular mouth of the HERG K\(^+\) channel pore, HERG S631A and HERG S631V. Although similar to HERG S620T in terms of removal of C-type inactivation and lowered drug affinity, HERG S631 faces the opposite site of the membrane from HERG S620. The tight coupling between 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.\(^11\)\(^13\) 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.\(^11\)\(^13\) Also, the IC\(_{50}\) 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\(_1\) tumor cells block of native I\(_K\) 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 ~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
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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 antiarrhythmic 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/IKr 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 S631, shifted the IC₅₀ 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 IC₅₀ 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).24 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.25

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/IKr 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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Eckhard Ficker, Wolfgang Jarolimek, Johann Kiehn, Arnd Baumann and Arthur M. Brown

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