Molecular Determinants of Voltage-dependent Human Ether-a-Go-Go Related Gene (HERG) $K^+$ Channel Block*

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The structural determinants for the voltage-dependent block of ion channels are poorly understood. Here we investigate the voltage-dependent block of wild-type and mutant human ether-a-go-go related gene (HERG) $K^+$ channels by the antimalarial compound chloroquine. The block of wild-type HERG channels expressed in Xenopus oocytes was enhanced as the membrane potential was progressively depolarized. The IC$_{50}$ was 8.4 ± 0.9 μM when assessed during 4-s voltage clamp pulses to 0 mV. Chloroquine also slowed the apparent rate of HERG deactivation, reflecting the inability of drug-bound channels to close. Mutation to alanine of aromatic residues (Tyr-652 or Phe-656) located in the S6 domain of HERG greatly reduced the potency of channel block by chloroquine (IC$_{50}$ > 1 mM at 0 mV). However, mutation of Tyr-652 also altered the voltage dependence of the block. In contrast to wild-type HERG, block of Y652A HERG channels was diminished by progressive membrane depolarization, and complete relief from block was observed at +40 mV. HERG channel block was voltage-independent when the hydroxyl group of Tyr-652 was removed by mutating the residue to Phe. Together these findings indicate a critical role for Tyr-652 in voltage-dependent block of HERG channels. Molecular modeling was used to define energy-minimized dockings of chloroquine to the central cavity of HERG. Our experimental findings and modeling suggest that chloroquine preferentially blocks open HERG channels by cation-π and π-stacking interactions with Tyr-652 and Phe-656 of multiple subunits.

HERG1 encodes the pore-forming subunits of channels that conduct the rapid delayed rectifier $K^+$ current, $I_{Kr}$ (2, 3). Mutation of HERG is a common cause of inherited long QT syndrome, a disorder of cardiac repolarization that predisposes affected individuals to torsade de pointes arrhythmia and sudden death (4). Acquired long QT syndrome is far more common than inherited long QT syndrome and is most often caused by block of HERG channels as a side effect of treatment with commonly used medications including antiarrhythmic, antiemetic, antibiotic, and psychoactive agents (5, 6). Although rare, treatment with the antimalarial drug chloroquine has also been associated with acquired arrhythmias. Prolonged therapy with chloroquine can lead to electrocardiographic changes including T-wave depression or inversion and prolonged QRS and QT intervals (7, 8). Prolonged QT intervals caused by chloroquine can induce torsade de pointes (9, 10). At the cellular level, chloroquine decreases the maximum upstroke velocity due to block of sodium current and prolongs the duration of action potentials due to block of inward rectifier current (I$_{K1}$) and I$_{Kr}$ (11). Elucidating the molecular mechanisms of HERG channel block by chloroquine and other drugs may enable the rational design of new pharmaceuticals devoid of this unwanted side effect.

The structural basis of HERG channel block by several potent drugs was recently investigated using alanine-scanning mutagenesis (12). Mutation of several amino acid residues of the HERG channel reduced the block of HERG current by several chemically unrelated compounds. These key residues were located on the S6 domain or near the pore helix, and homology modeling predicted that they faced the central cavity of the channel. Binding to two residues in particular (Tyr-652 and Phe-656) was proposed to be the most important determinants of the binding site. A critical role for Phe-656 had been proposed previously (13) for binding of the antiarrhythmic agent dofetilide and quinidine. Most HERG channel blockers that have been studied in detail (e.g. MK-499, dofetilide, and cisapride) are high affinity ligands and exhibit little or no voltage-dependent block. In contrast, blockade of HERG channels by low affinity ligands (e.g. chloroquine, quinidine) is characterized by significant voltage-dependent kinetics and steady state effects (11).

Two models have been proposed to explain voltage-dependent block of ion channels by drugs (14). The modulated receptor model proposes that the binding affinity ($K_d$) of a drug varies as a function of channel state. For example, many local anesthetic agents appear to preferentially block sodium channels in the open and/or inactivated state but have little affinity for the closed states that predominate at the resting membrane potential. However, the molecular basis for the apparent state-dependent change in binding affinity is poorly understood. An alternative hypothesis is the guarded receptor model that assumes a constant and state-independent $K_d$ but proposes that access to the binding site by charged drugs is prevented (“guarded”) by the activation gate (15–17).
FIG. 1. Effect of chloroquine on HERG current in *Xenopus laevis* oocytes. A and B, representative HERG currents recorded from an oocyte before (A) and after (B) incubation with 15 μM chloroquine. Currents were recorded at test potentials between −70 and +40 mV. Tail currents were recorded after repolarization to −70 mV. C, I-V relationships for currents measured at the end of the 4-s test pulse before and after application of 5, 15, and 30 μM chloroquine (*n* = 5). Currents were normalized to control current at −20 mV for each oocyte. D, I-V relationships for peak tail currents before and after application of 5, 15, and 30 μM chloroquine. Currents were normalized to the peak current measured in control conditions for each oocyte.

Here we characterize the block by chloroquine of WT and mutant HERG channels expressed in *Xenopus* oocytes. Our results suggest a molecular explanation for voltage-dependent block of HERG channels by chloroquine that further implicates the importance of the aromatic residues located on the S6 domains. We propose that block of HERG by chloroquine requires channel opening and sequential interaction with two aromatic residues (Phe-656 and Tyr-652) that face the central cavity of the HERG channel. This model incorporates features of both the guarded receptor and the modulated receptor hypotheses to describe the voltage-dependent block of HERG channels by chloroquine.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology**—Several HERG channel mutants (V625A, Y652A, and F656A) were chosen for study based on our previous finding (12) that these mutations decreased the potency of channel block by MK-499. These and additional (Y652F, Y652T, and Y652E) missense mutations were introduced into WT HERG cDNA by the megaprimer method as described previously (12) and subcloned into the pSP64 plasmid vector (Promega, Madison, WI). Before use in experiments, each construct was confirmed with restriction mapping and DNA sequencing of the PCR-amplified segment. G628C/S631C HERG was kindly provided by G.-Y. Tseng. Complementary RNAs for injection into oocytes were prepared with SP6 Cap-Scribe (Roche Molecular Biochemicals) following linearization of the expression construct with EcoRI.

**Voltage Clamp of Oocytes**—Isolation and maintenance of *Xenopus* oocytes and cRNA injections were performed as described (19). A GeneClamp 500 amplifier (Axon Instruments, Burlingame, CA) and standard two microelectrode voltage clamp techniques (20) were used to record currents. Currents were recorded at room temperature (22–24 °C) 2–4 days after cRNA injection. Glass microelectrodes were filled with 3 M KCl, and their tips were broken to obtain resistances of 0.5 to 1 megohms. The external solution contained 96 mM NaMes, 2 mM KMes, 2 mM CaMes, 5 mM HEPES, 1 mM MgCl2, adjusted to pH 7.6 with methanesulfonic acid. Voltage commands were generated using pCLAMP software (version 6; Axon Instruments, Burlingame, CA). Currents were not corrected for leak or endogenous currents, and capacitance transients were not nullled. To generate current-voltage (I-V) relationships, pulses were applied in 10-mV increments at a frequency of 0.05 Hz. Test potentials ranged from −70 to +40 mV and were applied from a holding potential of −80 mV. Deactivating (tail) currents were measured at −70 mV.

The time-dependent block of current was determined by dividing current recorded during a 4-s pulse in the presence of drug (I<sub>drug</sub>) by the current recorded before application of drug (I<sub>control</sub>). The resulting ratio, I<sub>drug</sub>/I<sub>control</sub>, was fit with a single exponential function to obtain the time constant for the onset of HERG current blockade. Currents measured at the end of 4-s test pulses were used to measure steady state fractional block ("fraction blocked"), defined as the amplitude of current reduced by drug divided by control current amplitude.

Chloroquine (Sigma) was dissolved in the external solution to obtain the desired drug concentrations. Oocytes were exposed to chloroquine solutions until steady state effects were achieved, usually in about 15 min. To determine the concentration-effect relationships, a single oocyte was exposed to cumulative concentrations of chloroquine.

**Data Analyses**—Data are presented as mean ± S.E. Clampfit software (Axon Instruments) was used to perform nonlinear least squares kinetic analyses of time-dependent currents. The fractional block of current (*γ*) was plotted as a function of drug concentration ([D]) and the data were fit with Hill Equation 1 to determine the concentration (IC<sub>50</sub>) required for 50% block of current magnitude and the Hill coefficient, *h*.

\[
y = \frac{[D]^h}{([D]^h + IC_{50}^h)}
\]

(Eq. 1)

Statistical comparisons between experimental groups were performed using analysis of variance and Dunnett’s method. Differences were considered significant at *p* < 0.05.

**Molecular Modeling**—The 1BL8 KcsA structure (21) was retrieved from the Protein Data Bank and used as the template for an HERG homology model. Based on a BLAST search of protein crystal structures, the HERG channel turret (amino acids 572–609) was modeled after 3PRC (residues Met-10 to Met-45). The SS domain was modeled using an alignment between the outer helix of KcsA and the putative SS of HERG. The MOE program (Chemical Computing Group) was used to produce a homology model for the HERG monomer. Energy minimization was performed to eliminate close contacts but not to allow helix unfolding. The tetramer was constructed by copying the derived monomer conformation onto the KcsA tetramer. Low energy conformations of chloroquine were generated and docked using the Flexible Ligands Oriented on Grid procedure (22).

**RESULTS**

**Block of WT HERG Current by Chloroquine**—Currents were elicited by 4-s depolarizing pulses to potentials ranging from −70 to +40 mV. The step currents measured at the end of 4-s pulses and the tail currents measured on return to −70 mV were reduced in a concentration-dependent manner by chloroquine (Fig. 1, A and B). The I-V relationship for step currents peaked at −20 mV in control and at −30 mV in the presence of
chloroquine. Block of step currents was pronounced at more depolarized test potentials (Fig. 1C), indicating voltage-dependent block. The decrease in tail currents by chloroquine was also voltage-dependent (Fig. 1D), with greater block apparent at more depolarized potentials. For example, 5 μM chloroquine reduced peak tail currents on average by 1% at −50 mV and 36% at +40 mV.

The time- and voltage-dependent block of WT HERG current by chloroquine was studied in greater detail using a concentration of 15 μM. Superimposed traces of currents recorded during a 4-s pulse to −50 mV or +10 mV, before and after addition of 15 μM chloroquine, are shown in Fig. 2, A and B. Time-dependent block of current during the pulse made the rate of current activation appear faster, whereas delayed recovery from block initiated by repolarization slowed the rate of deactivation (Fig. 2, A and B). The slower apparent rate of deactivation has been described for other compounds as a “foot in the door” effect, meaning that drug must first exit the central cavity of the channel before the activation gates (“door”) can close (23).

The ratio Idrug/Icontrol as a function of time during the pulse was used to estimate initial block and the onset rate of block (Fig. 2, C and D). The current ratio had an initial value of 1.0, indicating that channels completely recovered from block during the 16-s interval between test depolarizations. The time constants (τ) for the onset rate of block were voltage-dependent (17 ± 3 mV for e-fold change in τ, where e = 2.71828), decreasing with membrane depolarization from 420 ms at −50 mV to 140 ms at +40 mV (Fig. 2E). Because the onset of block was relatively fast, steady state reduction of current by 15 μM chloroquine was adequately estimated using a 4-s pulse. Steady state fractional block of current varied as a function of test potential, decreasing from 0.18 at −50 mV to >0.7 at potentials positive to 0 mV (Fig. 2F). These findings demonstrate that 15 μM chloroquine blocks open, but not closed, WT HERG channels and that steady state block was increased as a positive function of membrane potential.

**Removal of Inactivation Reduces Sensitivity but Not Voltage Dependence of HERG Block**—The hypothesis that chloroquine might preferentially bind to inactivated channels was tested with G628C/S631C HERG, a mutant channel that does not inactivate (24). G628C/S631C current was reduced by chloroquine in a concentration-dependent manner (Fig. 3, A and B). Similar to WT HERG, the block of current conducted by mutant channels was increased as a positive function of voltage (Fig. 3C). The IC50 for current block at +20 mV was 27.2 ± 2.7 μM (Fig. 3D), 4-fold less sensitive than WT channels (p < 0.05). These findings indicate that although removal of inactivation reduced the steady state block of HERG, it did not affect the voltage dependence of block.

**Characterization of the Chloroquine-binding Site**—Several residues located on the S6 and pore helix domains of HERG compose the putative binding site for methanesulfonanilide drugs such as MK-499 (IC50 = 34 nM). Mutation of Val-625 of the pore helix or Tyr-652 or Phe-656 of the S6 domain to Ala caused the most profound reductions in potency for block of HERG current by MK-499 (12). Chloroquine is a weak blocker of HERG, so it was possible that the putative binding sites for this drug would differ from those determined previously for the high affinity ligand MK-499 (12). Therefore, we determined the concentration-effect relationship for chloroquine on V625A,
Fig. 3. Chloroquine blocks inactivation-removed HERG channels. A and B, G628C/S631C HERG currents recorded before and after exposure of oocyte to 50 μM chloroquine. C, normalized I-V relationships for G628C/S631C HERG currents (n = 5). D, concentration-dependent block by chloroquine of G628C/S631C HERG current recorded at a test pulse of +20 mV. The IC_{50} was 27.2 ± 2.7 μM; h = 0.84 (n = 5).

Y652A, and F656A HERG channels and compared the potency for block to WT channel current. For this measurement, current was measured at the end of a 4-s pulse to 0 mV for WT, V625A, and Y625A HERG channels. The reduction of current caused by exposure to 50 μM chloroquine was nearly identical for WT and Y625A HERG channels (Fig. 4, A and B). Because the V625A mutation reduces K^+ selectivity of the HERG channel, the tail currents were inward at −70 mV. Unlike MK-499, where the V625A mutation reduced potency by 54-fold (12), this mutation did not alter the potency of channel block by chloroquine. The IC_{50} values were 8.4 ± 0.9 μM for WT and 7.2 ± 0.8 μM for Y625A HERG (Fig. 4C).

Similar to our previous findings with MK-499, mutation of aromatic residues located in the S6 domain greatly reduced chloroquine potency. The IC_{50} for block of Y652A HERG was increased ~500-fold relative to WT HERG (Fig. 4C). To increase the amplitude of poorly expressing F656A mutant channels, tail currents were recorded at −140 mV instead of −70 mV (Fig. 4, D and E). By using this protocol, the IC_{50} for WT current was increased to 19.7 ± 1.7 μM. Block of F656A HERG was minimal at 0.5 mM, indicating a decrease in potency of nearly 3 orders of magnitude compared with WT HERG (Fig. 4F; p < 0.01). These findings suggest that chloroquine blocks WT channels by interaction with Tyr-652 and Phe-656 residues located in the S6 domain, but it does not interact with Val-625 located at the base of the pore helix.

Time- and Voltage-dependent Block of Tyr-652 Mutant HERG Channels—The block of Y652A HERG channels was more prominent for weak than for strong membrane depolarization, a pattern opposite to that observed for WT current. Voltage-dependent block is obvious in the superimposed current traces of Fig. 5, A and B, where 150 μM drug reduced steady state current by about 35% at a test potential of −30 mV but reduced current by only 5% at +20 mV. Furthermore, the apparent rate of Y652A HERG tail current deactivation was much faster in the presence of drug (Fig. 5, A and B), as opposed to the slowed deactivation associated with block of WT current (Fig. 2).

The ratio I_{app}/I_{control} during 4-s test pulses to −30 or +20 mV is plotted in Fig. 5, C and D. The current ratio had an initial value of 1, indicating that block occurred only after channels had opened. The time course of the current ratio was biphasic, with an initial rapid phase of block followed by a slower partial recovery from block. The time constants for the onset and recovery from block were strongly voltage-dependent and decreased at more depolarized potentials (Fig. 5F). The voltage dependence for block onset (16 ± 3 mV/e-fold change in τ) was almost the same as measured for WT HERG. Steady state block of Y652A HERG, expressed as fractional block, was also voltage-dependent and varied from 0.7 at −50 mV to 0 at +40 mV (Fig. 5F). These findings demonstrate that chloroquine blocked Y652A channels only after opening of the activation gate and that block decreases with increasing membrane depolarization. The increased rate of deactivation (Fig. 5, A and B) was likely caused by rapid re-block of mutant channels by drug in response to repolarization to −70 mV. In support of this interpretation, the rate of Y652A HERG deactivation was strongly dependent on chloroquine concentration (Fig. 5, G and H).

Chloroquine had qualitatively similar effects on Y652T and Y652E HERG channels (Fig. 6). The IC_{50} measured at 0 mV was increased 500-fold for both mutant channels compared with WT channels. Fractional block by 150 μM chloroquine varied as a function of test potential, diminishing from 0.68 ± 0.04 at −30 mV to 0.01 ± 0.01 at +40 mV for Y652T HERG (n = 4), and from 0.72 ± 0.03 at −30 mV to 0.17 ± 0.03 to +40 mV for Y652E HERG (n = 5). Unblock of current observed with increasing depolarization suggests that the drug dissociates from a receptor site and either enters the cytosol or moves into a position within the central cavity that does not block K^+ conduction. The results presented below suggest the second explanation is more likely.

We next determined the effect of a more conserved amino acid substitution of Tyr-652. Phenylalanine only differs from tyrosine by the absence of an −OH. We had shown previously (12) that mutation of Tyr-652 to Phe had no significant effect on channel block by MK-499. In contrast to MK-499, the Y652F mutation reduced the potency of chloroquine by more than 10-fold. More surprising, however, was the finding that block of Y652F HERG current was nearly voltage-independent. Block was nearly the same when currents were elicited with a pulse to −50 or +10 mV, and the apparent rate of deactivation was only slightly faster in the presence of drug (Fig. 7, A and B). Similar to WT and Y652A HERG channels, block of Y652F channels only occurred after the channels were opened (as
fig. 4. concentration-dependent block of wt and mutant herg channels by chloroquine. a and b, superimposed traces of wt herg (a) and v625a (b) currents elicited by application of depolarizing pulses to -10 mV, before and after exposure to 15 μM chloroquine. c, concentration-effect relationship for block of herg current by chloroquine. the IC50 was 8.4 ± 0.9 μM (h = 0.81) for WT, 7.2 ± 0.8 μM (h = 0.76) for v625a (n = 5), and >3 mM for Y652A (n = 6). d and e, superimposed traces of WT herg (D) and Phe-656 HERG (E) currents elicited by application of depolarizing pulses to 0 mV and upon repolarization to -140 mV, before and after exposure of chloroquine at 50 μM (D) and 500 μM (E). F, concentration-effect relationship for current inhibition by chloroquine. the IC50 determined by using a Hill equation was 19.7 ± 1.7 μM (h = 0.9) for WT HERG, and >10 mM for Phe-656 HERG (p < 0.01). n = 4-6 oocytes for each channel type.

Discussion
Chloroquine Interacts with Phe-656 and Tyr-652 of the S6 Domain to Cause Open Channel Block of HERG—Block of HERG K+ channels by chloroquine exhibits several features typical of an open channel blocker. First, there was no block of initial current in response to a depolarizing pulse, consistent with a lack of interaction with channels in the closed state. Second, the extent and rate of onset of block was voltage-dependent, increasing at more depolarized potentials. Third, slowed deactivation and tail current crossover in the presence of chloroquine suggested that when drug was bound to the channel it prevented closure of the activation gate. This is similar to the so-called foot in the door mechanism first hypothesized by Armstrong (23) to describe the kinetics of unblock of squid axon K+ channels by tetraethylammonium derivatives (25). the inactivation-deficient mutant channel, G628C/S631C HERG, was about 4 times less sensitive to block by chloroquine. It is likely that the charged form of chloroquine is responsible for channel block because the alkylammoniums (N1 and N2 in fig. 8C, inset) have pK values of 8.4 and 10.8, respectively. Thus, the positively charged form of chloroquine preferentially blocks open HERG channels by a foot in the door mechanism, and block is enhanced by, but not dependent on, inactivation.

Chloroquine blocked HERG current with a potency similar to that of quinidine (13, 26, 27), meloquine (28), sparfloxacin (29), and vesnarinone (30, 31). All these compounds exhibit increased block with increasing membrane depolarization, and channels fully recover from block in less than a minute at a holding potential of -80 mV. in contrast, methanesulfonylilide drugs such as MK-499 and dofetilide are high affinity ligands with IC50 values in the 10-100 nM range and do not exhibit such obvious voltage-dependent block of current (32, 33). Moreover, the recovery from block of HERG channels by methanesulfonylilides is extremely slow and incomplete (33, 34). Despite these kinetic differences, the amino acids that compose the binding site for low and high affinity blockers appear to be similar. The most important residue appears to be Phe-656 that is located in the S6 domain and faces the central cavity of the HERG channel pore. Mutation of Phe-656 to Val increased the IC50 for block by chloroquine of HERG by 120-fold for dofetilide drugs such as MK-499 and dofetilide are high affinity ligands. This could occur by interaction of a positively charged amine (N2 or N3) of chloroquine with the negatively charged amine (N2 or N3) of chloroquine with the negative electrostatic potential provided by the face of a Phe-656 residue. Such cation-π interactions (35, 36) have been shown to be important in other ligand-binding sites such as the acetylcholine receptor (37).

Tyr-652 is located one helical turn above Phe-656 in the S6 domain and also faces the central cavity of the channel pore. We found that mutation of Tyr-652 to Ala, Thr, or Glu increased the IC50 for block by chloroquine of HERG by >500-
fold. In a previous study (12) we also found that mutation of Val-625 to Ala, located at the base of the pore helix and adjacent to the K\(^{\text{11001}}\) selectivity filter, reduced the potency of MK-499 but not cisapride or terfenadine. Chloroquine is more like these latter two drugs with respect to lack of interaction with Val-625. Thus, our findings with chloroquine provide further evidence that HERG channel blockers, despite significant structural diversity, interact principally with one or more aromatic residues of the S6 domain.

Substitution of Tyr-652 with Ala or Phe only slightly altered the gating properties of HERG channels but markedly influenced voltage-dependent block. Block of WT HERG current by chloroquine was enhanced by progressive depolarization. In contrast, block of Y652A HERG current was diminished by increased depolarization, whereas block of Y652F current was relatively insensitive to voltage. Thus, substitution of a phenyl with a benzyl moiety (Y652F) eliminated the voltage dependence of HERG block, whereas removal of the aromatic group reversed the voltage dependence of block. Considering the evident importance of the \(-\text{OH}\), we determined the effect of chloroquine on Y652T and Y652E HERG channels. Both mutations reduced the potency and reversed the voltage dependence of block by chloroquine similar to Y652A HERG. Thus, the voltage-dependent block of WT HERG cannot be explained by H-bonding of the drug with a \(-\text{OH}\) or \(-\text{COOH}\) group of non-aromatic amino acids. A possible explanation for the critical role of Tyr-652, but not simply an \(-\text{OH}\) group in voltage-dependent block of HERG, might be the cation-\(\pi\) interaction discussed above for Phe-656. Cation-\(\pi\) interactions are stronger for Tyr than Phe residues (35), perhaps because the phenolic \(-\text{OH}\) assists in positioning the face of the aromatic ring in a preferred orientation for interaction with the cation (N\(_2\) or N\(_3\) of chloroquine).

**A Model to Explain Voltage-dependent Block of WT and Mutant HERG Channels by Chloroquine**—Molecular modeling was used to define two energy-minimized dockings for chloroquine inside the central cavity of a homology model of the HERG channel (Fig. 8). It is important to note that 1) other ligand dockings are plausible, 2) the homology model is based on a static KcsA crystal structure, and 3) that the most important S6 residues comprising the putative binding sites (Tyr-652 and Phe-656) likely change orientation in response to channel

![Fig. 5. Voltage-dependent block of Y652A HERG currents by chloroquine.](image)
gating. Despite these obvious limitations, the dockings illustrated in Fig. 8 provide a framework for the discussion of a model that we base primarily on experimental findings.

As shown in Fig. 2F, chloroquine blocks with relatively low potency to WT HERG channels in response to a weak depolarization from a holding potential of ca. 80 mV. Low potency could result from an initial docking involving a cation–π interaction, followed by π-stacking of the quinoline of chloroquine between Phe-656 residues from multiple subunits as depicted in docking 1 (Fig. 8B). The requirement for initial drug docking with Phe-656 could explain why the voltage dependence for the onset of channel block was nearly the same (∼17 mV/e-fold change in τ) regardless if the fractional block was a positive (WT), negative (Y652A, Y652E, Y652T), or independent (Y652F) function of transmembrane voltage. If docking to Phe-656 was prevented, for example by mutation of the residue to Ala, then channel block would be expected to be drastically reduced, as was observed (Fig. 4F). Increased block of WT HERG in response to greater membrane depolarization could be explained by an enhanced occupancy of drug with a distinct higher affinity site. Based on our experimental findings with Tyr-652 mutant HERG channels, we propose that interaction with Tyr-652 mediates higher affinity binding, perhaps as shown for docking 2 depicted in Fig. 8D. Although not explicitly predicted by this docking, our experimental findings further suggest that N₃ may bind with Tyr-652 by a cation–π interaction. Decreased interaction of the drug molecule with multiple Phe-656 residues (docking 1) and enhanced interaction with Tyr-652 residues could be facilitated by an electrostatic effect on the positively charged drug as the membrane potential is progressively depolarized. Mutation of Tyr-652 to Ala would be expected to eliminate the higher affinity site, but depolarization might still provide an electrostatic effect that could cause movement of the charged drug further into the central cavity, away from the Phe-656 residues. Consistent with this interpretation, complete relief of Y652A and Y652T HERG channel block was observed in response to strong membrane depolarization (Figs. 5F and 6F).

Blockade of Y652F HERG channels was relatively insensitive to transmembrane voltage. Perhaps mutation of Tyr-652 to Phe reduced the affinity of the depolarization-favored drug-binding site to a value similar to the affinity defined by interaction with multiple Phe-656 residues. In the absence of the Tyr –OH group, the interaction between drug and Phe-652 might be similar to the interaction that stabilizes binding of drug to Phe-656. Two sites with similar binding affinities could
account for the lack of voltage-dependent block.

Chloroquine slowed the apparent rate of WT HERG current deactivation but increased the rate of Tyr-652 mutant HERG current deactivation. Slowed deactivation and crossover of the tail currents of WT HERG channels are consistent with a foot in the door mechanism, where it is assumed that channels...
cannot close until the drug unbinds from the channel. The increase in the apparent rate of deactivation of Tyr-652 mutant HERG channels in the presence of drug is consistent with re-binding of drug to Phe-656 residues located lower in the cavity that would be favored upon membrane repolarization. In the case of Y652A channels, re-block presumably occurs by a drug molecule that is present in the central cavity but not bound to the S6 domain.

Non-aromatic Substitutions of Tyr-652 Residues—The block of Y652A HERG channels by chloroquine was completely reversed by strong depolarization. This was a surprising result because an increase in membrane depolarization should favor movement of a positively charged drug further into the central cavity and either result in no change in block or perhaps an increased block as was observed for WT channels. Moreover, a single hydrated K+ ion located in the middle of the central cavity and coordinated by the pore helices is evidently vital for ion conduction in KcsA and presumably voltage-gated K+ channels (21, 38), and binding of the N-terminal inactivation peptide to S6 residues that line the channel pore induces inactivation (39). It may seem unexpected that Y652A HERG channels would conduct K+ ions normally if chloroquine was present within the central cavity. However, there is a precedent for such an effect in the L-type Ca2+ channel. Dihydropyridine antagonists block current conducted by α1, Ca2+ channels by binding to several S6 residues that face the central cavity (40). Dihydropyridine agonists interact with some of the same residues, yet cause an increase in Ca2+ channel conductance (41).

There are at least two alternative explanations for the reverse voltage dependence of Y652A HERG channel block. For example, chloroquine might bind to a low affinity site located on the extracellular side of the channel pore and be increasingly knocked off by outward flux of K+ as the membrane potential was progressively depolarized. Alternatively, a strong depolarization could cause rotation of S6 and a reduction in binding affinity, allowing drug to diffuse back into the cytoplasm. This seems unlikely because the positive transmembrane potential would not favor the diffusion of a positively charged drug into the cytoplasm. Moreover, neither of these alternative mechanisms is compatible with the finding that block of Y652P by chloroquine was voltage-independent.

In summary, we propose that depolarization-dependent HERG channel block by chloroquine is a multistep process that involves sequential binding of a drug molecule to low and high affinity sites that are accessible only when the channel is in the open state. Repolarization of the membrane promotes recovery from block, but channels can only close after drug has unbound from Tyr-652. This model incorporates features of both the guarded receptor hypothesis (requirement for channel opening) and the modulated receptor hypothesis (binding affinity that varies with voltage) to describe the voltage-dependent block of HERG channels by chloroquine.

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