Trapping of a Methanesulfonanilide by Closure of the HERG Potassium Channel Activation Gate

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abstract Deactivation of voltage-gated potassium (K^+) channels can slow or prevent the recovery from block by charged organic compounds, a phenomenon attributed to trapping of the compound within the inner vestibule by closure of the activation gate. Unbinding and exit from the channel vestibule of a positively charged organic compound should be favored by membrane hyperpolarization if not impeded by the closed gate. MK-499, a methanesulfonanilide compound, is a potent blocker (IC_{50} = 32 nM) of HERG K^+ channels. This bulky compound (7 × 20 Å) is positively charged at physiological pH. Recovery from block of HERG channels by MK-499 and other methanesulfonanilides is extremely slow (Carmeliet, 1992; Ficker et al., 1998), suggesting a trapping mechanism. We used a mutant HERG (D540K) channel expressed in *Xenopus* oocytes to test the trapping hypothesis. D540K HERG has the unusual property of opening in response to hyperpolarization, in addition to relatively normal gating and channel opening in response to depolarization (Sanguinetti and Xu, 1999). The hyperpolarization-activated state of HERG was characterized by long bursts of single channel reopening. Channel reopening allowed recovery from block by 2 μM MK-499 to occur with time constants of 10.5 and 52.7 s at −160 mV. In contrast, wild-type HERG channels opened only briefly after membrane hyperpolarization, and thus did not permit recovery from block by MK-499. These findings provide direct evidence that the mechanism of slow recovery from HERG channel block by methanesulfonanilides is due to trapping of the compound in the inner vestibule by closure of the activation gate. The ability of HERG channels to trap MK-499, despite its large size, suggests that the vestibule of this channel is larger than the well studied *Shaker* K^+ channel.

key words: MK-499 • voltage clamp • *Xenopus* oocyte

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INTRODUCTION

The voltage and channel state-dependent modulation of ionic currents by drugs has provided insights into the structure of voltage-gated ion channels. 30 yr ago, Armstrong (1969, 1971) showed that block of a voltage-gated K^+ channel in squid giant axon by a quaternary ammonium ion (QA)[1] required channel opening, that QA access to the binding site inside the pore occurred from the intracellular side, and that elevation of external [K^+] hastened dissociation of the QA. Moreover, channel block by small QA compounds (e.g., TEA) was not associated with a change in deactivation rate. These findings suggested that TEA could be trapped inside the channel pore by closure of the activation gate. Drug trapping has also been described for TEA or decyltriethylammonium by a mutant *Shaker* K^+ channel (Holmgren et al., 1997), and small local anesthetics by sodium channels (Strichartz, 1973). These later studies supported Armstrong's model that the inner pore of a channel was a relatively large water-filled vestibule, situated between the putative selectivity filter and activation gate and lined by hydrophobic residues (Armstrong, 1969, 1971). These predicted features of the vestibule were confirmed when the crystal structure of the KcsA channel was solved (Doyle et al., 1998). The inner pore of KcsA, and by inference other K^+ channels, is lined by the inner helices (homologous to S6 transmembrane domains) from each of four subunits, arranged to form an inverted teepee structure.

Most previous investigations of open channel-dependent block and trapping by drugs have used low affinity, charged molecules such as QA compounds for K^+ channels, or local anesthetic agents for Na^+ channels. High micromolar to millimolar concentrations of local anesthetics or QA compounds are required to block these channels. In contrast, methanesulfonanilide compounds such as dofetilide and MK-499, antiarrhythmic agents that also appear to block by a trapping mechanism (Carmeliet, 1992), reduce the rapid delayed rectifier K^+ current (I_{Kr}) of cardiac myocytes at nanomolar concentrations. I_{Kr} channels are formed by coassembly of HERG subunits (Sanguinetti et al., 1995; Trudeau et al., 1995). Recent studies have confirmed that methanesulfonanilides are potent blockers of heterologously expressed HERG channels (Kiehn et al., 1996;

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[1] *Abbreviations used in this paper:* I_{Kr}, rapid delayed rectifier K^+ current; QA, quaternary ammonium ion; WT, wild type.

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Snyders and Chaudhary, 1996; Spector et al., 1996a; Herzberg et al., 1998). Block by these compounds requires channel opening and occurs from the cytoplasmic side of the membrane (Zou et al., 1997). Although still controversial, it appears that methanesulfonanilide compounds preferentially bind to the inactivated state of the HERG channel (Wang et al., 1997). Recovery from HERG block by these compounds is extremely slow, even at negative holding potentials when most channels are in the low-affinity closed state. Because methanesulfonanilides are positively charged at physiological pH, it is likely that a negative transmembrane potential would favor unbinding and electrostatic repulsion of the drug from the channel pore, if such egress was not impeded by a closed activation gate. As noted above, drug trapping caused by closure of the activation gate of K^+ channels has been described previously for small QA compounds. However, methanesulfonanilides are large and bulky compounds. Based on x-ray analysis, MK-499 is 20×7 Å (personal communication from Dr. C. Culberson, Merck Research Laboratories, West Point, PA), much larger than TEA, which is ~6.9 Å in cross section (Stanfield, 1983).

The drug-trapping hypothesis predicts that, if channels could reopen from the closed state in response to hyperpolarization, then positively charged drugs would dissociate from their receptor site and exit the channel. Trapping of methanesulfonanilide compounds could be directly tested if gating of HERG channels could be altered and the activation gate made to open at very negative transmembrane potentials. We recently discovered that a single missense mutation in the S4–S5 linker (LS4S5) of HERG (D540K) altered channel gating in exactly this manner (Sanguinetti and Xu, 1999). D540K HERG channels have the unusual property of opening in response to strong hyperpolarization, in addition to normal gating associated with channel opening in response to depolarization. Hyperpolarization-dependent opening of D540K is half-maximal at −117 mV, whereas depolarization-dependent opening of these channels is half-maximal at −9 mV.

The aim of the present study was to exploit the unique gating properties of D540K HERG and determine whether the very slow recovery from block of HERG channels by methanesulfonanilide compounds results from a trapping mechanism. We first characterized the properties of hyperpolarization-activated channel opening of D540K HERG and showed that these openings were associated with long periods of single channel bursting activity. We then demonstrated that strong hyperpolarization of D540K HERG, but not wild-type (WT) HERG channels, promotes recovery from block by MK-499. These studies provide a direct demonstration that, if not for closure of the activation gate, membrane hyperpolarization favors unbinding of a large positively charged compound. More specifically, these studies provide a molecular mechanism for the slow recovery from HERG channel block by methanesulfonanilide compounds, a potent and clinically important class of antiarrhythmic agents.

MATERIALS AND METHODS

Isolation and maintenance of *Xenopus* oocytes, site-directed mutagenesis, and cRNA injection were performed as described previously (Goldin, 1991; Goldin and Sumikawa, 1992; Sanguinetti and Xu, 1999). Whole cell currents were recorded 2–4 d after cRNA injection using a GeneClamp 500 amplifier, a Pentium computer with a Digidata 1200 computer interface (Axon Instruments), and standard two-electrode voltage clamp techniques (Stühmer, 1992). To attenuate endogenous chloride currents, Cl^- was replaced with Mes in the external solution that contained (mM): 96 NaMES, 2 KMES, 2 $CaMES_2$, 5 HEPES, 1 $MgCl_2$, adjusted to pH 7.6 with methane sulphonic acid. Some oocytes had large endogenous inward currents when the membrane was pulsed to very negative potentials. The presence of these currents varied between batches of oocytes and was easily discernible from the D540K HERG inward currents based on their negative activation threshold and very slow activation kinetics. Batches of oocytes with large endogenous currents were discarded.

Bath solutions were applied with a switching device that directed flow through a 1-ml Gilson pipette tip and could be placed within 0.5 mm of the oocyte. Flow rates of 3–4 ml min^{-1} were thus restricted to a small volume around the cell, ensuring complete and rapid exchange of solutions and preventing external K^+ accumulation/depletion when current amplitudes were limited to ±4 μA. Whole-cell current records were on-line filtered and digitized at 1 kHz, unless otherwise stated. Currents were not leak subtracted.

Single channel recording from *Xenopus* oocytes was performed as described previously (Zou et al., 1997) using an Axopatch 200 patch clamp amplifier (Axon Instruments). The extracellular solution contained (mM): 120 KCl, 10 HEPES, 5 EGTA; adjusted to pH 7.2 with KOH. Current signals were on-line filtered at 1 kHz with a low-pass Bessel filter and digitized at 2.5 kHz with a Pentium computer and Digidata 1200 interface. Single channel data were analyzed using Fetchan and Pstat software (Pclamp 6; Axon Instruments). Single channel amplitudes were generated from all point histograms of binned single channel data and fit with Gaussian distributions using a Levenberg-Marquardt least-squares fit routine. Amplitude threshold analysis was used to generate idealized single channel traces for calculating dwell time and time-dependent open probability histograms.

MK-499 (Claremon et al., 1993), supplied by Merck and Co., Inc., was prepared daily by dilution to the required concentration from a 5-mM stock solution that was dissolved in dimethylsulphonic acid and kept at −20°C.

Data are expressed as mean ± SEM. Statistical analysis was performed using paired and unpaired *t* tests (Excel; Microsoft Corp.). Curve fitting was performed with Origin software (Microcal).

RESULTS

Properties of Whole Cell D540K HERG Channel Current Activated by Hyperpolarization

We previously described the biophysical properties of D540K HERG (Sanguinetti and Xu, 1999). Before studying the block of D540K HERG by MK-499, we fur-

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ther characterized the unique gating properties of this mutant channel using whole-cell and single-channel recording techniques. D540K HERG current elicited by depolarization from a holding potential of −90 mV appeared to activate instantaneously, followed by rapid inactivation. In the example shown in Fig. 1 A, currents were elicited by 2-s depolarizations to potentials ranging from −60 to +20 mV. Deactivation elicited by repolarization to −70 mV was relatively fast. In the same cell, hyperpolarization from the holding potential of −90 mV activated a small instantaneous current followed by a much slower inward current (Fig. 1 B). The instantaneous component represents channels that were open at the holding potential of −90 mV. In contrast to deactivation of depolarization-activated current, the deactivation of hyperpolarization-activated channels was quite slow at −70 mV (compare tail currents in Fig. 1, A and B). Thus, channels opened by depolarization activate and deactivate rapidly, whereas channels opened by hyperpolarization activate and deactivate slowly. The time course of current activation in response to depolarizing pulses is obscured by the rapid onset of inactivation. However, when other mutations (G628C, S631C) that remove inactivation (Smith et al., 1996) were introduced into the D540K HERG background, the rate of activation was easily discernible (Fig. 1 C), with time constants of 15 ± 1 and 92 ± 8 ms at 0 mV (n = 9). Inactivation-removed D540K HERG channels also reopened upon hyperpolarization (Fig. 1 C, bottom). The I-V relationship for steady state D540K HERG channel currents activated by either depolarizing or hyperpolarizing pulses showed inward rectification, with a peak outward current near −20 mV (Fig. 1 D). The small slope conductance between −60 and −90 mV indicates the voltage range where D540K HERG channels are primarily in a closed state.

The contrast between the properties of depolarization and hyperpolarization-activated open states of D540K HERG channels are most obvious using a two-pulse protocol. Channels were opened and/or inactivated by a 1-s prepulse to +40 mV, followed by a pulse to −160 mV (Fig. 2 A). Hyperpolarization elicited a large transient inward current followed by a second, slowly activating inward current (Fig. 2 B). The fast component resulted from rapid recovery of depolarization-activated channels from inactivation and their subsequent rapid deactivation (Schonherr and Heinemann, 1996; Smith et al., 1996; Spector et al., 1996b). The slow component of inward current resembles the hyperpolarization-activated current (Fig. 1 B), and presumably is caused by channels reopening from the deactivated, closed state. After extrapolating back to time zero to correct for deactivation (Fig. 2 C), the amplitude of the depolarization-activated current component was clearly larger than the second component, even though the driving force was the same. The disparity in amplitude could result from either a difference in single channel current amplitude or open probability between the two open states. To investigate this further, we compared the single channel properties of the depolarization-activated

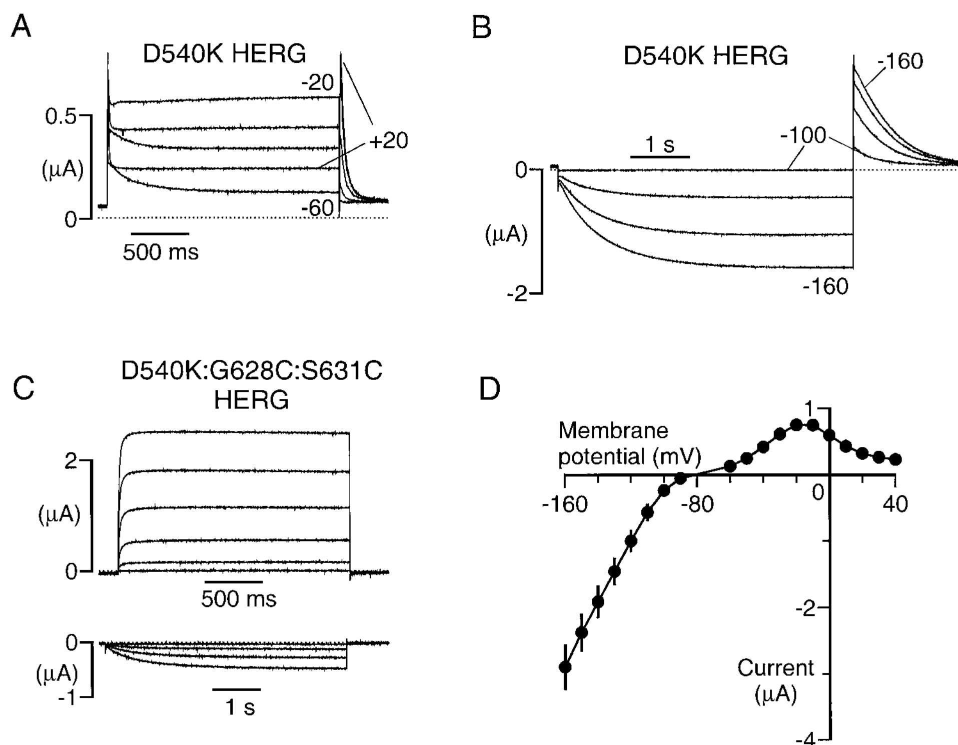

Figure 1. Electrophysiological properties of D540K HERG channels expressed in *Xenopus* oocytes. (A) Representative whole cell recordings of D540K HERG currents elicited by 2-s depolarizing test pulses from −60 to +20 mV in 20-mV increments at a stimulation frequency of 0.1 Hz. (B) D540K HERG currents from the same cell as A, elicited by 5-s hyperpolarizing test pulses from −100 to −160 mV in 20-mV increments at a stimulation frequency of 0.09 Hz. The holding potential was −90 mV, tail currents were recorded at −70 mV, and dotted lines indicate zero current level. (C) Inactivation-removed mutant HERG (D540K:G628C:S631C) currents activated by pulses ranging from −50 to +50 mV (top) and −100 to −160 mV (bottom). (D) Steady state I-V relationship for D540K HERG (n = 17 cells).

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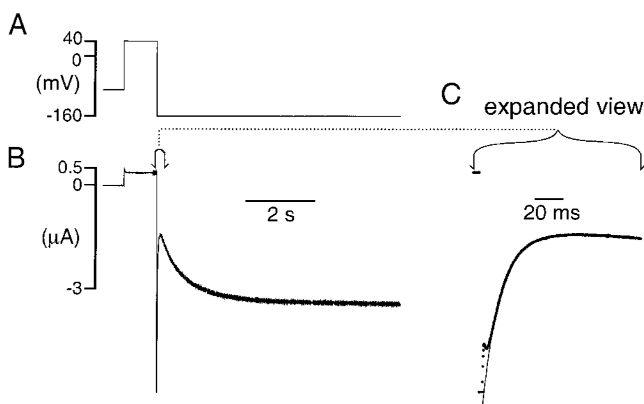

Figure 2. Two components of inward current elicited by hyperpolarization. (A) Membrane potential was stepped from a holding potential of −90 to +40 mV for 500 ms to maximally activate depolarization-dependent current, and then stepped to −160 mV for 5 s. (B) Currents elicited by voltage protocol in A. Sampling frequency was changed from 1 to 5 kHz just before hyperpolarization. The transient current component represents rapid recovery from inactivation followed by deactivation, whereas the slow current represents channel reactivation. (C) Expanded view of current after hyperpolarization to −160 mV. Current deactivation was fit with an exponential function (solid line) and extrapolated back to time zero to correct for deactivation during recovery from inactivation.

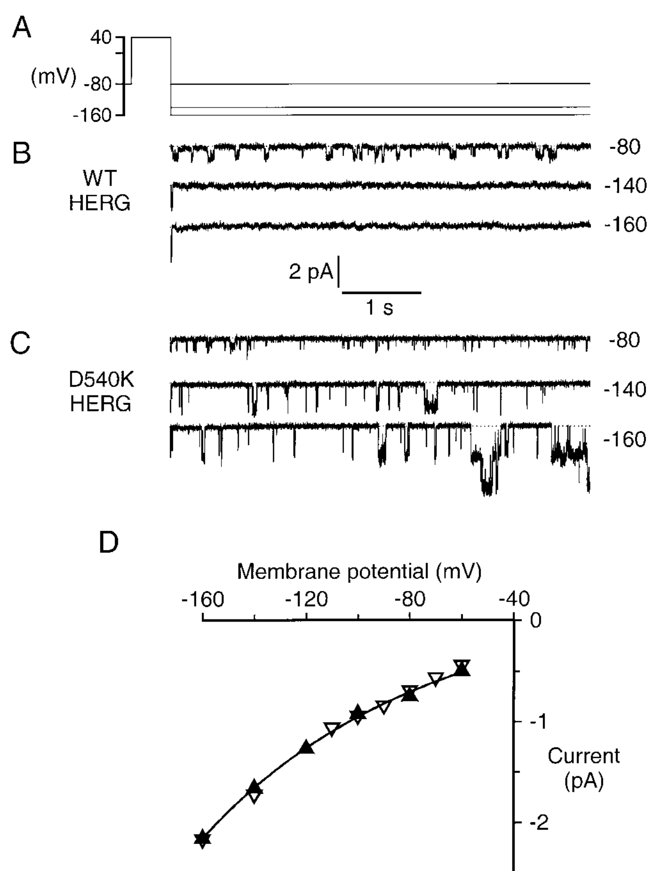

Figure 3. Comparison of WT and D540K HERG single channel currents. Currents were recorded in cell-attached patches of *Xenopus* oocyte membrane. (A) Voltage protocol used to elicit single channel currents. From a holding potential of −80 mV, membrane potential was stepped to +40 mV for 500 ms, followed by a 5-s step to either −80, −140, or −160 mV. Representative WT HERG (B) and D540K HERG (C) single channel currents were recorded at the indicated membrane potentials. Dotted lines indicate zero current level. (D) Mean single channel I-V relationships for WT (▽, $n = 5$) and D540K (▲, $n = 12$) HERG. Standard error bars were smaller than the symbols. WT HERG data between −110 and −60 mV were taken from Zou et al. (1997).

current of WT HERG with the hyperpolarization-activated current of D540K HERG.

Single Channel Properties of Hyperpolarization-activated D540K HERG

WT and D540K HERG single channel currents were recorded using the cell-attached configuration of the patch-clamp technique. Channel openings were elicited by stepping to −80, −140, or −160 mV for 5 s, after a 500-ms prepulse to +40 mV. At −80 mV, inward single channel events were observed throughout the 5-s step for both WT and D540K HERG currents. However, at −140 and −160 mV, single channel WT HERG currents were only observed at the beginning of the pulse (Fig. 3 B). In contrast, D540K HERG single channels opened repetitively throughout the pulse (Fig. 3 C). There was no significant difference between WT and D540K HERG single channel amplitudes at any potential ($P > 0.05$, Fig. 3 D). The I-V relationship for both channels displayed weak inward rectification, as previously reported for WT HERG (Zou et al., 1997) and cardiac I_{Kr} (Ito and Ono, 1995) channels. This may result from voltage-dependent transitions to a substate or flicker block by an unidentified intracellular molecule (Zou et al., 1997). The single channel conductance, determined by linear regression analysis of mean data was 22.0 pS between −160 and −120 mV and 12.4 pS between −120 and −60 mV. The ensemble-averaged current at −160 mV for a macropatch containing at least six channels is shown in Fig. 4. The number of open channels increased progressively during the 5-s pulse and the ensemble average closely resembled the whole-cell current at this test potential (Fig. 1 B).

The single channel behavior of D540K HERG at −80 and −160 mV was most clearly observed during long gap-free recordings. At −80 mV, the open probability was low and open channel bursting was rare and of short duration (Fig. 5 A). In contrast, the open probability at −160 mV was much greater and was associated with long bursts of channel openings (Fig. 5 B). Fig. 5, C and D, shows plots of open probability during 4–5 min of continuous recording. At −80 mV, single channel openings were rare and appeared random. In contrast, single channel open probability appeared modal at −160 mV, with long periods of bursting activity inter-

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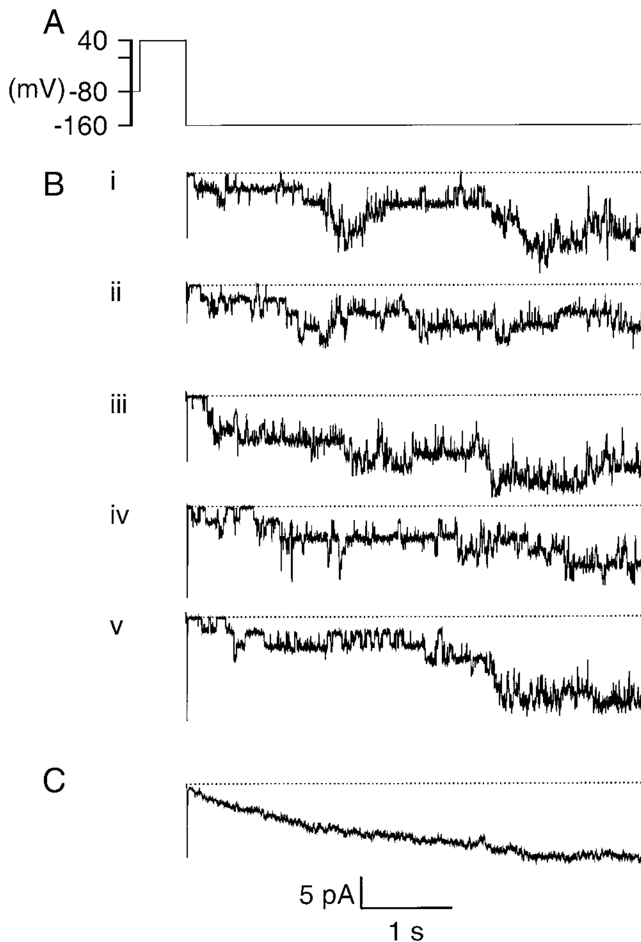

Figure 4. Ensemble average of D540K HERG single channel currents. (A) Voltage protocol used to elicit single channel currents. (B) Representative currents recorded during five different pulses, showing that channel activity increased throughout the 5-s pulse to −160 mV. This patch contained at least six single channels, with an average amplitude of 2.15 pA corresponding to a conductance of 22 pS. (C) Ensemble average of 17 current traces, including those shown in B.

rupted by prolonged pauses. These data suggest that destabilization of the closed state of D540K is voltage dependent. This was confirmed by estimating the open probability of channels as a function of membrane potential. Single channel open probability (P_o) was strongly voltage dependent (Fig. 5 E), increasing from 0.01 ± 0.003 (n = 6) at −80 mV to 0.37 ± 0.027 (n = 3) at −180 mV. The voltage dependence of P_o determined from single channel analysis was shifted in the negative direction compared with whole cell recordings, where P_o was half-maximal at −117 mV (Sanguinetti and Xu, 1999). This shift was largely due to the different ionic conditions used for whole-cell and single-channel recordings. When we recorded whole-cell currents in ionic conditions more similar to the single-channel recording conditions (96 mM K^+, nominally Ca^{2+}-free solution), the voltage dependence of D540K HERG activation was shifted by at least −40 mV (not shown).

In summary, the single channel conductance of D540K HERG activated by hyperpolarization was identical to WT HERG activated by depolarization. Progressive hyperpolarization increased single channel burst duration and open probability. These results suggest that the D540K mutation destabilizes the closed state of HERG, but does not affect the conductance or selectivity properties of the channel.

Hyperpolarization-induced Channel Opening of D540K HERG Facilitates Recovery from Block by MK-499

The properties of HERG channel block by the methanesulfonanilide MK-499 has many of the hallmarks typical of drug trapping. Block occurs from the intracellular side of the channel (Zou et al., 1997) and requires prior channel opening (Spector et al., 1996a). Moreover, block is essentially irreversible and the rate of HERG channel deactivation is not altered by MK-499 (Spector et al., 1996a). These findings suggest that block results from trapping, and not a "foot in the door" mechanism. MK-499 is a large molecule (Fig. 6 A) that is nearly all charged at physiological pH. At the pH 7.6 of the extracellular solution, ∼99% of the drug has a single positive charge and ∼65% of the drug has two positive charges (pK_a = 7.86 and 9.52). The drug trapping hypothesis predicts that if HERG channels could reopen from the closed state in response to hyperpolarization, then the channels should recover from block by a positively charged drug like MK-499. We exploited the unique gating properties of D540K HERG to determine whether block of HERG channels by MK-499 results from a trapping mechanism.

We first determined the concentration-response relationship for MK-499 block of HERG channels. Since HERG activation is slow, we used long test pulses with a short inter-pulse duration so that channels spent the majority of time in an activated or inactivated state. From a holding potential of −90 mV, 5-s pulses to 0 mV were followed by repolarization to −70 mV for 400 ms. The membrane was held at −90 mV for only 600-ms between successive pulses. Oocytes that had a steadily incrementing current magnitude during the control period, indicative of a shift in the voltage dependence of inactivation due to extracellular accumulation of K^+, were discarded. WT and D540K HERG channels were blocked by MK-499 in a concentration-dependent manner (Fig. 6). Concentration-response curves were constructed by measuring steady state current amplitudes at each MK-499 concentration and normalizing to control, and then fitting with a Hill equation. The concentration at which 50% inhibition occurred (IC_{50}) was 32 ± 4 nM (n = 4) and 104 + 8 nM (n = 5–7) for WT and D540K HERG channel currents, respectively. Steady

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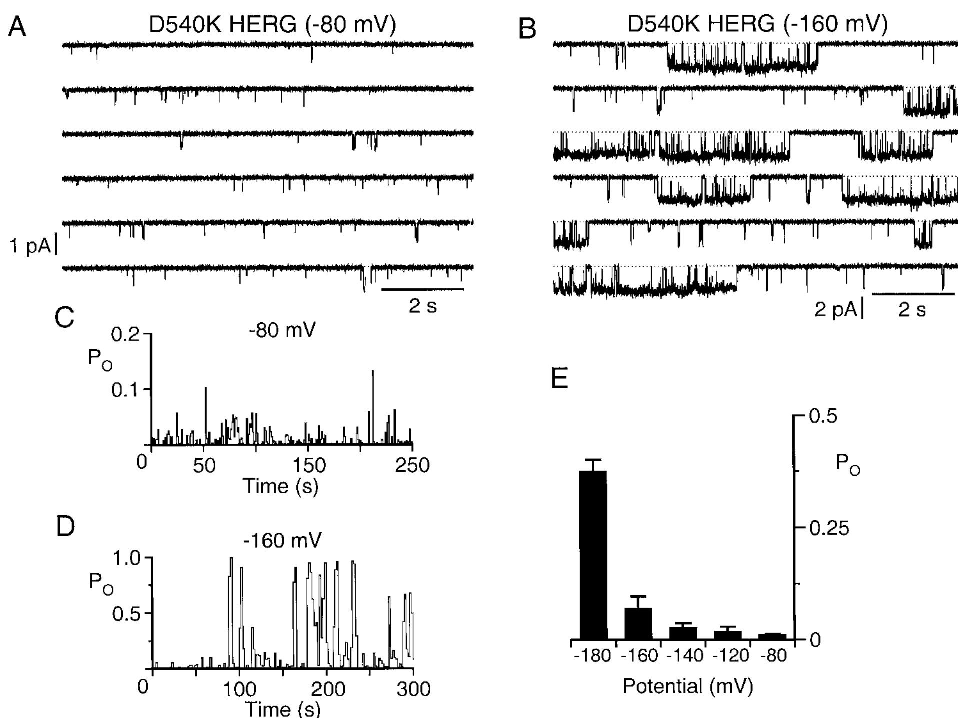

Figure 5. Open probability of D540K HERG channels is voltage dependent. Representative gap-free recordings of single channel activity from a single patch recorded at −80 mV (A) and −160 mV (B). Open probability was calculated at 1-s intervals and plotted against time for −80 mV (C) and −160 mV (D). (E) Bar graph of P_o-voltage relationship for D540K HERG single channel currents (n = 3–6), determined from idealized traces of gap-free current recordings with a minimum duration of 2 min.

state (end pulse) current reflects off and on rates for MK-499 binding at −90 mV (holding potential) and 0 mV (test potential). At −90 mV, a small fraction of D540K, but not WT HERG channels, are in the open state. The reduced potency of MK-499 for block of D540K HERG channels could be caused by unblock of open channels that occurred at the holding potential of −90 mV. For the remaining experiments, we used 2 μM MK-499 to investigate the mechanisms of recovery from HERG channel block by this drug. Under steady state conditions, this concentration completely blocked D540K HERG channels.

To investigate WT HERG recovery from MK-499 block, we used the voltage protocol illustrated in Fig. 7 A (top). Voltage pulses to 0 mV were applied repetitively at 10-s intervals. Once current amplitudes had stabilized in control conditions (Fig. 7 A, a), 2 μM MK-499 was applied. When block reached >85% (Fig. 7 A, b), 5-s hyperpolarizing pulses to −160 mV were applied in the continued presence of MK-499 (Fig. 7 A, c). These repetitive hyperpolarizing pulses were applied at 20-s intervals for 15 min. To assess recovery from block, a depolarizing pulse to 0 mV was applied (Fig. 7 A, e). The amplitude of peak current as a function of time for the whole experiment is illustrated in Fig. 7 B. The percent recovery from block was calculated from the difference in peak current amplitude between e and b, divided by peak current in a. After 40–50 hyperpolarizing pulses (∼13–17 min), the mean recovery from block of WT HERG was 5.0 ± 0.9% (n = 5).

The same protocol described for WT HERG was used to examine the kinetics of recovery from block by MK-499 of D540K HERG current. Whole cell "control" current (Fig. 8 A, a) was blocked >85% by repetitive pulsing to 0 mV in the presence of 2 μM MK-499 (b). The first (Fig. 8 B, c) and last (d) of 38 hyperpolarizing pulses to −160 mV are shown. In response to the first hyperpolarizing pulse (c), the current was initially near 0, but became progressively larger during the 5-s pulse. Currents at the end of the pulse and peak tail currents were substantially larger for the last (d) compared with the first (c) hyperpolarizing pulse, indicating recovery from block. Recovery was assessed with a depolarizing pulse to 0 mV (e). Comparing a and e, it is apparent that repetitive pulsing to −160 mV resulted in considerable recovery (in this example, 85%) from MK-499 block. The time course of MK-499 block and unblock is illustrated in Fig. 8 B. Both onset and recovery from block had an exponential time course. D540K HERG recovery from MK-499 block was calculated in the same way as described above for WT HERG. The mean recovery with 35–50 hyperpolarizing pulses was 95.3 ± 2.8% (n = 6).

It could be argued that the greater recovery from block of D540K HERG than WT HERG channels was caused by an allosteric affect on the drug binding site caused by mutation of D540 and not by untrapping when channels reopened in response to hyperpolarization. To test for this possibility, we performed the same recovery from block experiment on D540A HERG channels. These channels activate and deactivate rapidly and have identical voltage dependence for depolarization-activated current as D540K HERG, but do

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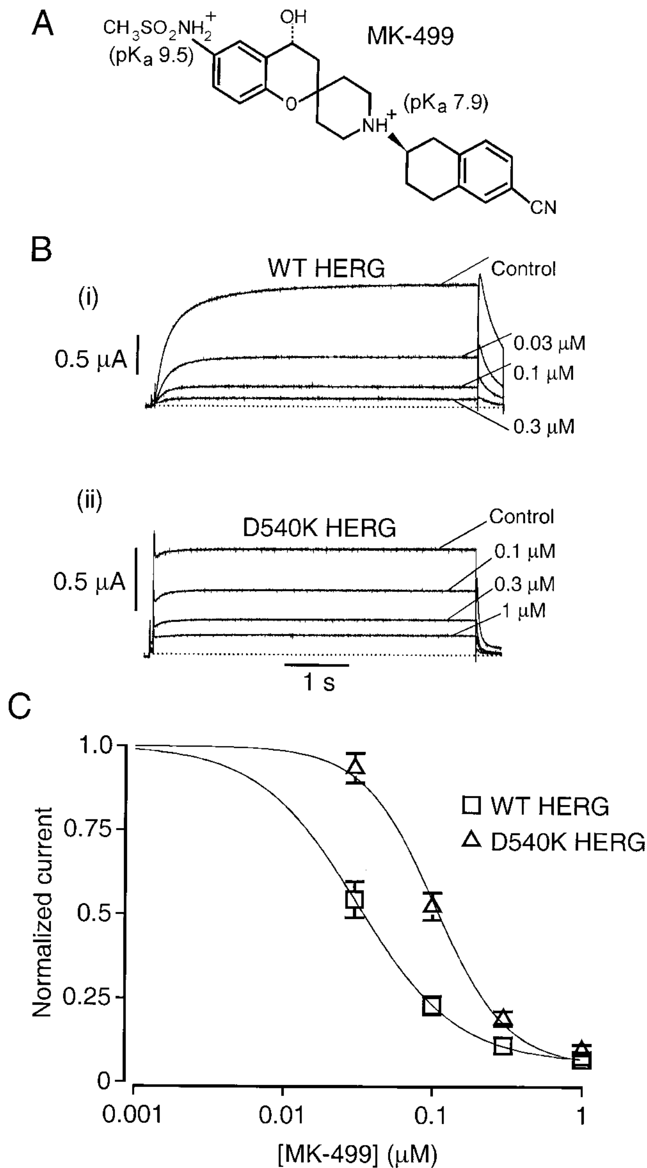

Figure 6. Concentration-dependent block of HERG by MK-499. (A) Structure of MK-499 showing pK_a values for nitrogen groups. (B) Examples of WT (i) and D540K HERG (ii) currents before and after exposure of oocytes to solutions containing various concentrations of MK-499. From a holding potential of −90 mV, currents were recorded during 5-s pulses to 0 mV; tail currents were recorded at −70 mV. (C) Concentration-response relationships for block of WT and D540K HERG current in oocytes. Steady state peak current amplitudes at 0 mV for each [MK-499] were normalized to control. Mean values were plotted against [MK-499] and fitted with the Hill equation. Mean IC_{50}s were 32 ± 4 nM (Hill coefficient = 1; n = 4) for WT HERG and 104 ± 8 nM (Hill coefficient = 1.8; n = 5–7) for D540K HERG.

not open with hyperpolarization (Sanguinetti and Xu, 1999). Representative D540A HERG current traces are shown in Fig. 9 A and current amplitudes are plotted against time in B. Greater than 90% block was obtained with 2 μM MK-499 (b), but little recovery (e) was observed after 50 pulses to −160 mV (c and d). The mean

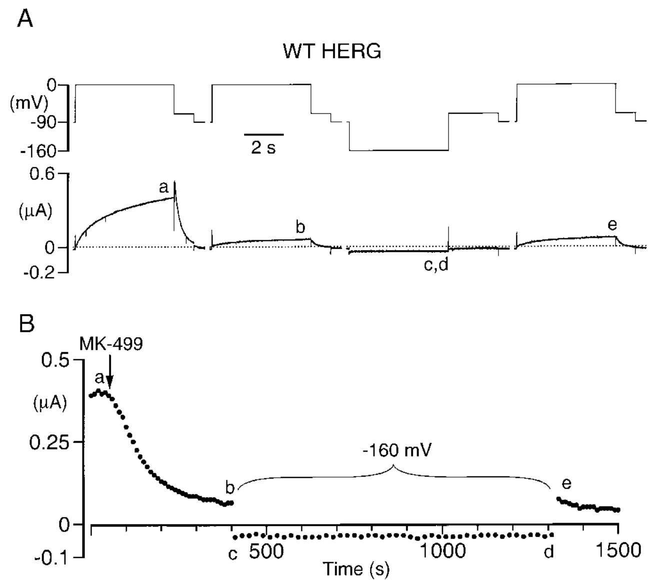

Figure 7. WT HERG channels do not significantly recover from MK-499 block. (A, top) Voltage pulse protocol, (bottom) WT HERG currents. *Xenopus* oocyte expressing WT HERG channels was continuously stimulated at a frequency of 0.1 Hz with 5-s voltage steps to 0 mV from a holding potential of −90 mV. The oocyte was pulsed until currents reached steady state (a) before switching to a solution containing 2 μM MK-499. When block of WT HERG currents was more than 85% (b), repetitive 5-s hyperpolarizing voltage steps to −160 mV were applied (45 total) at a frequency of 0.05 Hz, in the continuous presence of MK-499. c and d are the first and last hyperpolarizing pulses, respectively. Tail currents were induced by steps to −70 mV. The extent of recovery was determined with a 5-s depolarizing pulse to 0 mV (e). (B) Peak current magnitudes for experiment in A. Each point represents current elicited by a single voltage pulse. Arrow designates time at which MK-499 was administered.

recovery from block was 12.4 ± 1.7% (n = 6). Thus, recovery from block was minimal for WT and D540A HERG channels that do not reopen with hyperpolarization. The small, but significant recovery from block of these channels was likely due to rare channel openings at the holding potential of −90 mV. Overall, our data suggest that unblock of the channel by MK-499 can only occur when the activation gate is open.

To determine whether recovery of D540K HERG channels from MK-499 block was sensitive to membrane potential, we determined rates for recovery at two different potentials. A single 2-min test pulse was applied to −120 mV (Fig. 10 A) or −160 mV (B) before (Control) and after repetitive pulsing to 0 mV to achieve steady state block with 2 μM MK-499. At either test potential, control currents activated within 4 s to a steady state level that was maintained throughout the 2-min pulse. After block and in the continued presence of MK-499, the holding and initial inward currents were attenuated. However, with continued hyperpolarization, the inward current slowly increased as channels

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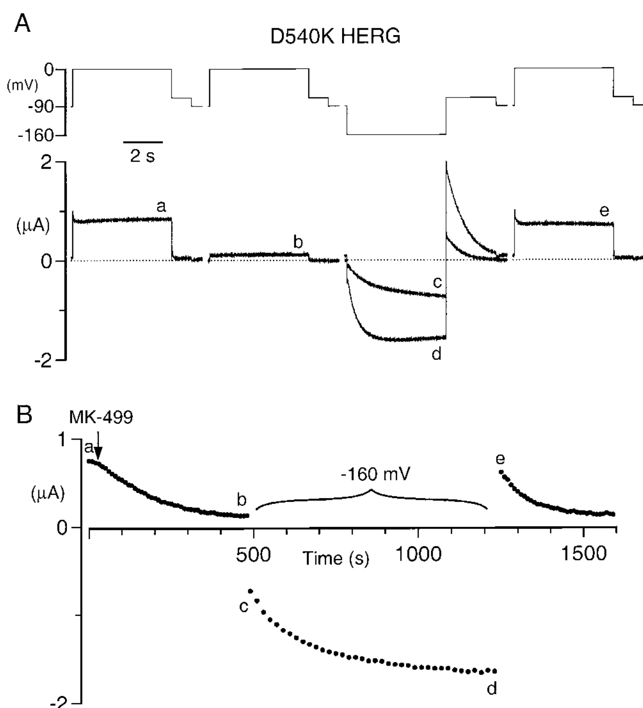

Figure 8. Untrapping of MK-499 from D540K HERG channels. Recovery from MK-499 block of D540K HERG was investigated using the same protocol described in Fig. 7. (A) Voltage protocol and representative current traces. a and b are currents elicited before and after block by MK-499, respectively. c and d are currents elicited by the first and last of 38 hyperpolarizing pulses. e is current in response to the first depolarizing voltage step after the hyperpolarizations. (B) Peak current magnitudes for experiment in A. Each point represents current elicited by a single voltage pulse. Arrow designates time at which MK-499 was administered.

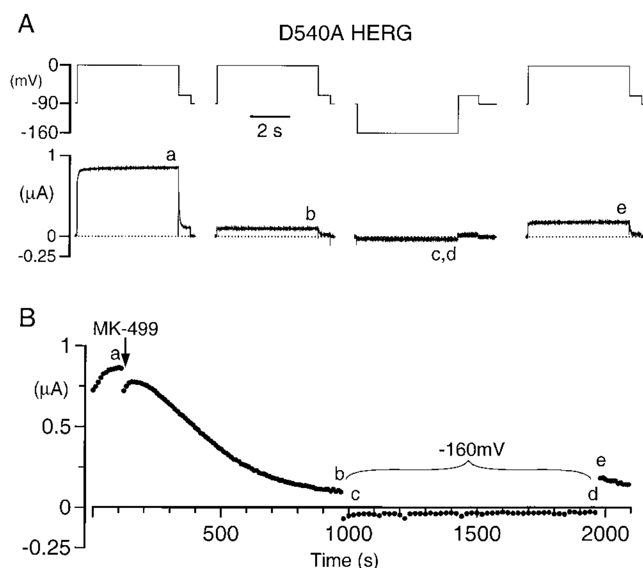

Figure 9. D540A HERG channels do not significantly recover from MK-499 block. The same protocol as described in Fig. 7 was used. (A, top) Voltage pulses, (bottom) representative D540A HERG current traces. Note that D540A HERG channels do not open with hyperpolarization. a and b are currents elicited before and after block by MK-499, respectively. c and d are currents elicited by the first and last of 50 hyperpolarizing pulses to −160 mV. e is current in response to a depolarizing voltage step applied immediately after the final hyperpolarizing pulse. (B) Peak current magnitudes for the experiment in A. Each point represents current elicited by a single voltage pulse. Arrow designates time at which MK-499 was administered.

became unblocked. The rate and extent of recovery from block (MK-499 current as a proportion of control current) was greater at −160 than at −120 mV. Time constants for recovery from block were determined by curve-fitting the ratio of drug-sensitive current to control current ($I_{d\text{-}s}/I_c$) as a function of time (Fig. 10 C). Recovery was best described with a biexponential function at −160 mV and a single exponential function at −120 mV. In five cells, the τ_f and τ_s for recovery at −160 mV was, respectively, 5.1 ± 1.6 and 52.6 ± 9.3 s. At −120 mV, τ was 116.5 ± 30.9 s ($n = 5$; significantly different from τ_s at −160 mV, $P < 0.05$). Thus, as expected for a positively charged compound, the recovery from block of D540K HERG channels was accelerated by greater hyperpolarization.

DISCUSSION

Mechanism of Hyperpolarization-dependent Channel Opening of D540K HERG Channels

As we previously reported, replacement of Asp with Lys at position 540 in LS4-S5 of HERG destabilized the closed state, permitting the channel to reopen in response to membrane hyperpolarization (Sanguinetti and Xu, 1999). This destabilization results in a biphasic voltage dependence of channel opening, a unique behavior for a voltage-gated ion channel. Hyperpolarization-activated current was associated with an increased burst duration and probability of channel opening, but no change in single channel conductance. The P_o-V relationship for D540K HERG current is shaped like an inverted bell. Depolarization-dependent opening of D540K HERG channels is half-maximal at −9 mV, whereas hyperpolarization-dependent opening is half-maximal at −117 mV (Sanguinetti and Xu, 1999). The P_o-V relationship is steeper for hyperpolarizing than depolarizing pulses, but both types of channel openings are likely to occur in response to movement of the S4 domain. However, it is unclear whether the D540K mutation alters the movement of the S4 domain in response to hyperpolarization, the interaction of LS4–S5 with another portion of the channel (S6 domain?), or in some other way destabilizes the closed state at negative transmembrane potentials. Mutation of residues other than D540 located in the LS4-S5 also affect activation and deactivation of HERG (Wang et al., 1998; Sanguinetti and Xu, 1999). The structure of the activation gate is not known, but it has been suggested that twist-

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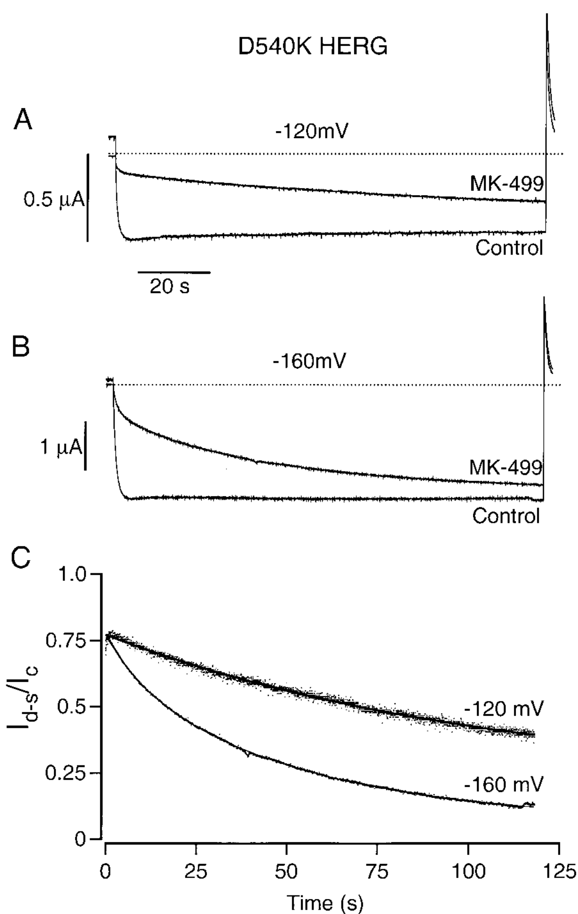

Figure 10. Rate of D540K HERG recovery from block is voltage dependent. Representative current traces elicited by 2-min hyperpolarizing voltage steps to −120 mV (A) or −160 mV (B). Control currents were elicited once outward currents stimulated with 5-s pulses to 0 mV had reached steady state. After the addition of 2 μM MK-499, the oocyte was pulsed repetitively to 0 mV to achieve >85% block. Then the test pulse to −120 was repeated. Block was reestablished before recording current at −160 mV. (C) Drug sensitive current, $I_{d\text{-}s}$ was calculated by subtracting the currents in the presence of MK-499 from the control currents, I_c, at each potential. $I_{d\text{-}s}$ was normalized to control current ($I_{d\text{-}s}/I_c$, dot plot) for each potential and fitted with exponential functions (solid lines) to obtain time constants for recovery from MK-499 block. $I_{d\text{-}s}/I_c$ at −120 mV was fitted with a single exponential function (τ = 109 s). $I_{d\text{-}s}/I_c$ at −160 mV was fitted with a biexponential function (τ_f = 10.5 s, τ_s = 52.7 s).

ing of the S6 domains in response to a change in transmembrane voltage could narrow or widen the access to the pore, analogous to opening and shutting of a "trap door." Based on mutational analysis (McCormack et al., 1993; Sanguinetti and Xu, 1999; Shieh et al., 1997), it is conceivable that LS4-S5 is an important component of the K^+ channel activation gate, perhaps by acting as a transducer between movement of the S4 voltage sensor and the narrow region of the S6 domains. Further studies are needed to define the molecular basis of the altered channel gating induced by the D540K mutation. Regardless of the exact gating mechanism, D540K HERG channel behavior is useful for testing the drug-trapping hypothesis that predicts that if channels could reopen from the closed state in response to hyperpolarization, then positively charged compounds would dissociate from their receptor site and exit the channel.

Block of HERG Channels by MK-499: A Clear Example of Drug Trapping

According to the modulated receptor model for channel block (Hille, 1977; Hondeghem and Katzung, 1977), a charged compound gains access to its binding site from the cytoplasm only after the channel has opened in response to membrane depolarization. Once bound to its receptor site within the inner vestibule of the pore (behind the activation gate), a charged compound can become "trapped" when the channel deactivates in response to membrane repolarization. A positively charged compound would not be capable of exiting the inner vestibule through the highly hydrophobic environment that typifies the wall of the pore or the surrounding membrane. Trapping can only occur if the drug is small enough to fit into the restricted space of the inner vestibule of the pore region (Hille, 1977; Hondeghem and Katzung, 1977). If the drug is charged and appropriately sized, then block is nearly irreversible as long as the channels are not reopened. High affinity block of HERG channels by methanesulfonanilide compounds is characterized by most of these properties. HERG channels are not blocked when oocytes are exposed to a high concentration (1 μM) of MK-499 if the membrane is held at −80 mV without pulsing. However, applying a train of repetitive depolarizing pulses causes block that is essentially irreversible. Thus, HERG block only occurs after the channels have opened. The pK_as of MK-499 are 7.86 for the piperidine group and 9.52 for the methanesulfonamide group. Thus, the drug is 99% charged in the bathing solution (pH 7.6) used in our experiments. At a concentration of 2 μM, ~20 nM of the drug would be in the uncharged form. Neutral MK-499 might not require channel opening to access the vestibule of the pore, but would then instantly reequilibrate in the aqueous environment of the vestibule, such that only 0.2 nM would be stable in the charged state. Because the IC_{50} of MK-499 is 32 nM, it is likely that only the charged form of the drug binds with high affinity to the HERG channel.

One property of MK-499 is not consistent with it being trapped by channel deactivation. MK-499 is a large molecule (20 × 7 Å) that would probably not fit into the inner vestibule of many K^+ channels, including *Shaker* and KcsA. Although there is little sequence homology between the inner helices of KcsA and S6 of *Shaker* or HERG, the basic pore structure is likely to be similar (Doyle et al., 1998; MacKinnon et al., 1998; reviewed in

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Yellen, 1998). The length of the inner vestibule of KcsA is ~18 Å, measured as the distance between the narrowest region between the crossing of the inner helices and the selectivity filter (Doyle et al., 1998). The inner vestibule of *Shaker* channels is likely to be even smaller because these channels are blocked by TEA (6.9 Å diameter), but it cannot trap this compound when applied from the intracellular side of the membrane. However, mutation of a single residue (I470 to Cys) alters the vestibule size of *Shaker* such that it can trap TEA as well as a much larger QA compound, decyltriethylammonium (Holmgren et al., 1997), that is comparable in length to MK-499 (Stanfield, 1983). Large QA compounds usually block K^+ channels by a foot in the door mechanism (Armstrong, 1969, 1971). These compounds require membrane depolarization and channel opening to block, but rapidly unblock upon membrane repolarization. Unblock is characterized by a slowing in the rate of current deactivation because channels are unable to close when occupied by drug, presumably because of steric interference with the activation gate. In contrast, channel block caused by trapping is not associated with an altered rate of current deactivation because only drug-free channels are capable of conducting current. MK-499 has no affect on the rate of channel deactivation (Spector et al., 1996a), consistent with our conclusion that when MK-499 is bound to its receptor it does not interfere with normal channel deactivation. If MK-499 binds to a site behind the activation gate, then the inner vestibule of a HERG channel must be $>20 \times 7$ Å, the size of a single MK-499 molecule. Our study provides no direct evidence that the site of MK-499 block of HERG channels is the vestibule. This would require knowing the crystal structure of HERG and mapping of residues important for high affinity drug binding. It is conceivable that the binding site is located outside the vestibule in a position that is allosterically affected by the D540K mutation in such a way that unbinding can occur in response to hyperpolarization in D540K, but not D540A or wild type HERG channels.

Other methanesulfonanilide compounds are also likely to block HERG channels by a trapping mechanism. Carmeliet (1992) studied the kinetics of I_{Kr} block by dofetilide in cardiac myocytes. Dofetilide is a methanesulfonanilide with similar potency and kinetics of HERG block as MK-499. Unblock of I_{Kr} by dofetilide was very slow at -50 mV, but was virtually irreversible at -75 mV. This pattern of unblock is consistent with drug trapping because some channels are open at -50 mV (but not at -75 mV) and would therefore allow drug to escape.

Channel State-dependent Binding of Methanesulfonanilides

It is clear that HERG channels must open before being blocked by MK-499 or other methanesulfonanilide compounds. However, it is not clear if drug then binds preferentially to the open or inactivated state of the channel. Some studies have suggested that inactivated HERG channels have a lower affinity for dofetilide than the open state (Kiehn et al., 1996; Snyders and Chaudhary, 1996). More recent studies using HERG mutants in which inactivation has been modified have reached the opposite conclusion. Wang et al. (1997) reported that block of wild-type HERG by E-4031 required 10-fold less compound than block of a mutant HERG (G628C:S631C) that removes inactivation. HERG/eag chimera channels that did not inactivate were also less sensitive to block by dofetilide (Ficker et al., 1998). eag channels do not inactivate and are relatively insensitive to methanesulfonanilides. These studies suggest that methanesulfonanilides preferentially bind to the inactivated state but do not rule out the possibility that reduced binding affinity to inactivation-deficient mutant channels results from an allosteric effect unrelated to channel state. Thus, it remains unclear whether channel inactivation alters the affinity of the methanesulfonanilide binding site.

Heterologous expression of HERG in *Xenopus* oocytes induces a K^+ current with properties very similar to the rapidly activating delayed rectifier K^+ current (I_{Kr}) that mediates repolarization of cardiac myocytes (Sanguinetti et al., 1995; Trudeau et al., 1995). However, it was recently reported that coassembly of HERG and MiRP1 (minK-related peptide) subunits induces a current with properties almost exactly like I_{Kr} recorded in cardiac myocytes (Abbott et al., 1999). Association of MiRP1 with HERG also alters the potency and kinetics of block by the methanesulfonanilide E-4031. Thus, association of MiRP1 with HERG may alter the state dependency or accessibility of drug to its receptor site.

In summary, our findings provide the most direct evidence to date that slow recovery from channel block by a charged drug is due to trapping of the compound in the inner vestibule by deactivation. The ability of HERG channels to trap MK-499, despite its large size, indicates that the vestibule of this channel is larger than the more well studied *Shaker* K^+ channel.

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