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# Structural Determinants of HERG Channel Block by Clofilium and Ibutilide

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## ABSTRACT

Block of human ether-a-go-go related gene (HERG) $K^+$ channels by a variety of medications has been linked to acquired long QT syndrome, a disorder of cardiac repolarization that predisposes to lethal arrhythmias. The drug-binding site is composed of residues that face into the central cavity of the channel. Two aromatic residues located on the S6 domain (Tyr652 and Phe656) are particularly important structural determinants of drug block. The role of pore helix residues (Thr623, Ser624, Val625) is less clear. In this study, we compared the pharmacological properties of two structurally related compounds, ibutilide and clofilium. Both compounds are charged amines with a single phenyl ring. Clofilium, a chlorobenzene derivative, is a potent blocker of HERG channels, but has a remarkably slower time course for recovery from block than ibutilide, a methanesulfonanilide. The difference in the rate of recovery from block can be explained simply by variation in drug trapping. There is little recovery from clofilium block with D540K HERG channels that permit untrapping at hyperpolarized potentials. Alanine-scanning mutagenesis of the S6 domain and a portion of the pore helix revealed that the binding site residues were the same for both compounds. However, S624A, located at the base of the pore helix, was the only HERG mutation that enabled rapid recovery from clofilium block. In summary, the pore helix residues are important components of the HERG drug binding site, and may be particularly important for drugs with polar substituents, such as a halogen (e.g., clofilium) or a methanesulfonamide (e.g., ibutilide).

HERG channels mediate the rapidly activating delayed rectifier K current ($I_{Kr}$) in the heart (Sanguinetti et al., 1995). $I_{Kr}$ has a key role in repolarization of the cardiac action potential and in controlling action potential duration (for review, see Keating and Sanguinetti, 2001; Tseng, 2001; Vandenberg et al., 2001). Inherited mutations of HERG are a major cause of congenital long QT syndrome (LQTS), a disorder characterized by lengthening of the cardiac action potential and increased incidence of ventricular arrhythmias and sudden death (Keating and Sanguinetti, 2001). However, LQTS is more commonly an acquired disorder caused by drug-induced block of $I_{Kr}$ channels (Redfern et al., 2003; Roden et al., 1996; Roy et al., 1996). A number of medications have been removed from the market because of drug-induced ventricular arrhythmia (Fermini and Fossa, 2003). These drugs are structurally diverse and include a variety of different therapeutic agents, but they all preferentially block $I_{Kr}$. Understanding the mechanisms of block of HERG by drugs may facilitate the rational design of safer drugs without the LQTS side effect (Mitcheson et al., 2000a).

Significant progress has been made in identifying the structural basis for the unusual sensitivity of HERG channels to drugs (Mitcheson and Perry, 2003). The S6 domains of most voltage-gated $K^+$ channels contain a Pro-*X*-Pro motif that may cause a kink in the inner helices resulting in a smaller central cavity compared with KcsA (del Camino et al., 2000). In contrast, the S6 domains of HERG subunits lack this motif and therefore may have a larger central cavity.

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**ABBREVIATIONS:** HERG, human *ether a go-go* related gene; LQTS, long QT syndrome; MK-499, (+)-*N*-[1′-(6-cyano-1,2,3,4-tetrahydro-2(*R*)-naphthalenyl)-3,4-dihydro-4(*R*)-hydroxyspiro(2*H*-1-benzopyran-2,4′-piperidin)-6-yl]methanesulfonamide] monohydrochloride; E-4031, 1-[2-(6-methyl-2-pyridyl)ethyl]-4-(methylsulfonyl-aminobenzoyl)piperidine; S6, sixth transmembrane segment; WT, wild-type; MES 2-[*N*-morpholino]ethanesulfonic acid; clofilium, 4-chloro-*N*,*N*-diethyl-*N*-heptylbenzenebutanaminium.

This is supported by drug trapping experiments indicating that HERG is able to accommodate much larger molecules than Shaker channels, which have the Pro-*X*-Pro motif (Mitcheson et al., 2000b). The amino acid residues important for high-affinity block of HERG by many compounds have been described previously (Lees-Miller et al., 2000; Mitcheson et al., 2000a; Kamiya et al., 2001; Sanchez-Chapula et al., 2002, 2003). These residues are located on S6 and at the C-terminal end of the pore helices at positions predicted to project into the central cavity (Lees-Miller et al., 2000; Mitcheson et al., 2000a).

Two S6 residues, Tyr652 and Phe656, are critical for channel block. These aromatic residues are unique to the eag channel family; most other $K_v$ channels have Val or Ile residues in homologous positions. Aromatic residues can bind drug molecules by $\pi$-stacking interactions with phenyl groups and cation-$\pi$ interactions with protonated nitrogens (Dougherty, 1996). So far, the HERG channel binding site for high-affinity compounds has only been investigated for relatively complex molecules, such as MK-499 and dofetilide, that have multiple phenyl rings and more than one charged amine group (Lees-Miller et al., 2000; Mitcheson et al., 2000a). To determine which parts of the drug molecules are interacting with Tyr652 and Phe656 and whether these aromatic residues function together or independently within the inner cavity will require the study of drugs with relatively simple, asymmetric structures.

In addition to the S6 aromatic residues, three residues (Thr623, Ser624, and Val625) located at the C-terminal end of the pore helices just before the GFG selectivity filter were found to be involved in drug block (Mitcheson et al., 2000a). Residues in these positions are highly conserved in $K^+$ channels; the first two positions are polar (either Ser or Thr) and the next position is either Val or Ile. Unlike Phe656 and Tyr652, which are important components of the binding site for nearly all compounds, the effect of mutation of the pore helix residues differs considerably among compounds (Mitcheson et al., 2000a).

In this study, we investigated the molecular determinants of HERG channel block by clofilium and ibutilide, two structurally related compounds (Fig. 1) with quite different time dependent kinetics of block. Ibutilide has a methanesulfonanilide group found in many other potent HERG channel blockers such as MK-499, E-4031, and dofetilide (Yang et al., 1995). Clofilium, a chlorobenzene derivative, is also a potent blocker of HERG channels but has remarkably slow time constants for onset and recovery from block (Gessner and Heinemann, 2003). The aims of this study were to compare the high-affinity binding sites of clofilium and ibutilide using Ala-scanning mutagenesis and to explore the structural basis for the difference in recovery from block by ibutilide and clofilium. Our results indicate an important role for pore helix residues in clofilium and ibutilide binding.

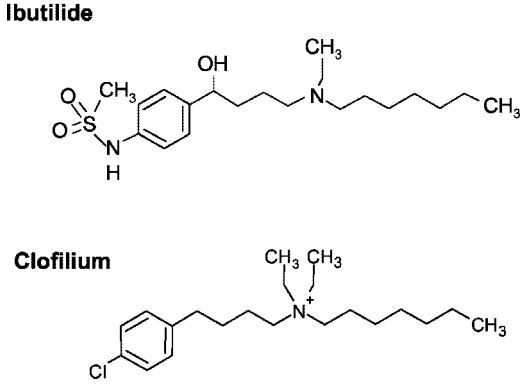


**Fig. 1.** Comparison of chemical structures of ibutilide and clofilium. Ibutilide is a tertiary amine with a methanesulfonamide group in the *para* position on the phenyl ring. Clofilium is a quarternary amine with an electronegative chlorine in the *para* position of the phenyl ring.

## Materials and Methods

**Molecular Biology and Oocyte Injection.** Complementary RNA for wild-type (WT) and mutant HERG channel expression in *Xenopus laevis* oocytes was prepared by in vitro transcription using SP6 RNA polymerase (mMessage mMachine kit, Ambion) after linearization with EcoRI of the HERG expression construct subcloned into pSP64. *X. laevis* oocytes were isolated, maintained in culture, and injected with cRNA as described previously (Sanguinetti and Xu, 1999).

**Voltage Clamp Recordings.** The two microelectrode voltage clamp technique was used to record membrane currents 1 to 7 days after cRNA injection as described previously (Mitcheson et al., 2000b). A low-chloride, 2 mM potassium extracellular recording solution, in which chloride was replaced with MES, was used to attenuate endogenous chloride currents. The extracellular solution contained (in mM): 96 mM NaMES, 2 mM KMES, 2 mM $CaMES_2$, 5 mM HEPES, and 1 mM $MgCl_2$, pH adjusted to 7.6 with NaOH. In some experiments, a high-$K^+$ solution was used that contained 96 mM KMES and 2 mM NaMES, with all other constituents remaining the same. Oocytes were impaled with microelectrodes filled with 3M KCl and resistances of 1 to 2 MΩ. Extracellular solutions were applied from a solution-switching device described previously (Mitcheson et al., 2000b), the barrel of which was placed close to the oocyte so that solution flow was directed around the oocyte to minimize extracellular $K^+$ accumulation and allow bulk solution changes in less than 10 s. Currents were recorded with an Axoclamp 500B, digitized with a Digidata 1320A (Axon Instruments, Inc., Union City, CA) and saved to computer for off-line analysis. pClamp 8.1 software (Axon Instruments, Inc.) was used for voltage clamp data acquisition and analysis.

Clofilium (4-chloro-*N*,*N*-diethyl-*N*-heptylbenzenebutanaminium) was purchased from Sigma (St. Louis, MO), dissolved in dimethyl sulfoxide to make a 5 mM stock and stored at −20°C. Ibutilide (methanesulfonamide; *N*-{4-{4-(ethylheptylamino)-1-hydroxybutyl} phenyl}) was purchased as Corvert (Pfizer, New York, NY) an intravenous preparation containing (per milliliter) 0.1 mg of ibutilide fumarate, 8.9 mg of NaCl, and 0.189 mg of sodium acetate trihydrate, and stored at 4°C. Both drug stocks were diluted to the required concentration in extracellular solution on each experimental day. Before drug application, cells were repetitively depolarized and the currents carefully monitored until the amplitudes had fully stabilized. Leak subtraction was performed by stepping briefly to −70 mV from the holding potential and subtracting the resulting current from peak tail currents measured at the same potential. The normalized current after steady-state block by drug ($I_{drug}/I_{control}$) was plotted as a function of drug concentration. The concentration of drug that achieved half-maximal inhibition ($IC_{50}$) was obtained by fitting this relationship with a Hill equation. Time constants for the time course of recovery from block were obtained by fitting exponential functions to $I_{drug}/I_{control}$ data plotted as a function of time after the commencement of blocker wash off. Curve fitting and statistical analyses were performed with Prism 3.01 (GraphPad Software Inc., San Diego, CA). Data are presented as mean ± S.E.M. ($n$ = number of cells) unless otherwise stated. Statistical analysis was performed with a Student's $t$ test. Differences were considered significant for $P < 0.05$.

**Molecular Modeling.** Clofilium was docked into the vestibule of a HERG homology model based on the crystal structure of KcsA [Protein Data Bank entry 1bl8 (Doyle et al., 1998)]. The docking program GOLD (version 1.2; CCDC Software, Cambridge, UK) was used to obtain 20 different binding modes within the vestibule. The modeling package SYBYL (version 6.9; Tripos Inc., St. Louis, MO) was used to visualize and examine the interactions with various parts of the vestibule. One binding mode is shown in Fig. 9.

## Results

To assess the effects of ibutilide and clofilium on HERG channels, outward currents were elicited by 5-s depolarizations to 0 mV from a holding potential of −90 mV and tail currents were recorded during a 400-ms repolarizing pulse to −70 mV (Fig. 2A). This pulse protocol was repeated at 6-s intervals and ensured that channels were open most of the time. Ibutilide inhibited HERG currents in a concentration-dependent manner (Fig. 2B) with no significant effects on the time-dependent kinetics of activation or deactivation. On average, 300 nM clofilium reduced HERG currents by 93 ± 1.8% ($n$ = 6), which is similar to the amount of block induced by the same concentration of ibutilide (87 ± 1.5%, $n$ = 8; Fig. 2C). The $IC_{50}$ for block of HERG currents by ibutilide was 28 ± 2.5 nM (Fig. 2D). We were not confident that we could obtain a precise measure of $IC_{50}$ for clofilium for WT HERG, because at low concentrations (10 to 30 nM), the onset of block was extraordinarily slow and steady-state block was not reached after extended periods of time (>1 h). Gessner and Heinemann (2003) also reported low accessibility of clofilium to its binding site on human *ether a-go-go* 1 channels expressed in mammalian cell lines, but block was more rapid when clofilium was applied to the intracellular rather than extracellular side of excised membrane patches. In our hands, even preincubation for 1 h with 30 nM clofilium did not enable inhibition to reach steady-state in whole-cell oocyte recordings after 30 min of continuous pulsing. Ultraslow onset of $K^+$ channel block by clofilium has also been reported in mammalian cell lines and rat ventricular myocytes (Castle, 1991; Gessner and Heinemann, 2003) and is therefore not a problem that is unique to oocytes. These findings are consistent with clofilium blocking from the intracellular side, with the permanent charge of the quaternary amine being responsible for slow equilibration of clofilium across the membrane.

Recovery from block after washing off ibutilide was almost complete after 10 mins (Fig. 2A). However, there was very little recovery from block by clofilium (Fig. 2C). Figure 3 shows representative time courses for the onset and recovery from block by 300 nM of each drug. Peak tail current amplitudes in the presence of drug were normalized to the amplitude of steady-state currents in control solution ($I_{drug}$ /$I_{control}$). The time course for onset of block by clofilium and ibutilide were well fitted with single exponential functions. Mean time constants were 45 ± 2.4 s for clofilium ($n$ = 5) and 39 ± 3.3 s for ibutilide ($n$ = 8). To assess the time course of recovery from block, the drugs were washed out of the bath for 1 to 2 min, and repetitive pulsing resumed for >20 min. Recovery from block by clofilium was very slow with a quasi-linear time course, whereas recovery from block by ibutilide was virtually complete and had an exponential time course with a mean time constant of 461 ± 29 s. After 10 min, currents recovered to 72 ± 1.5% of the control value for ibutilide and 19 ± 5.1% for clofilium. Thus, despite the similarities in drug potencies and rates of onset, the time courses for recovery from block by ibutilide and clofilium were very different.

Slow recovery from block is typical of potent HERG channel blockers such as MK-499 and is caused by slow unbinding and drug trapping within the central cavity of the channel by closure of the activation gate upon membrane repolarization (Carmeliet, 1992; Carmeliet, 1993; Mitcheson et al., 2000b). We previously studied the phenomenon of drug trapping

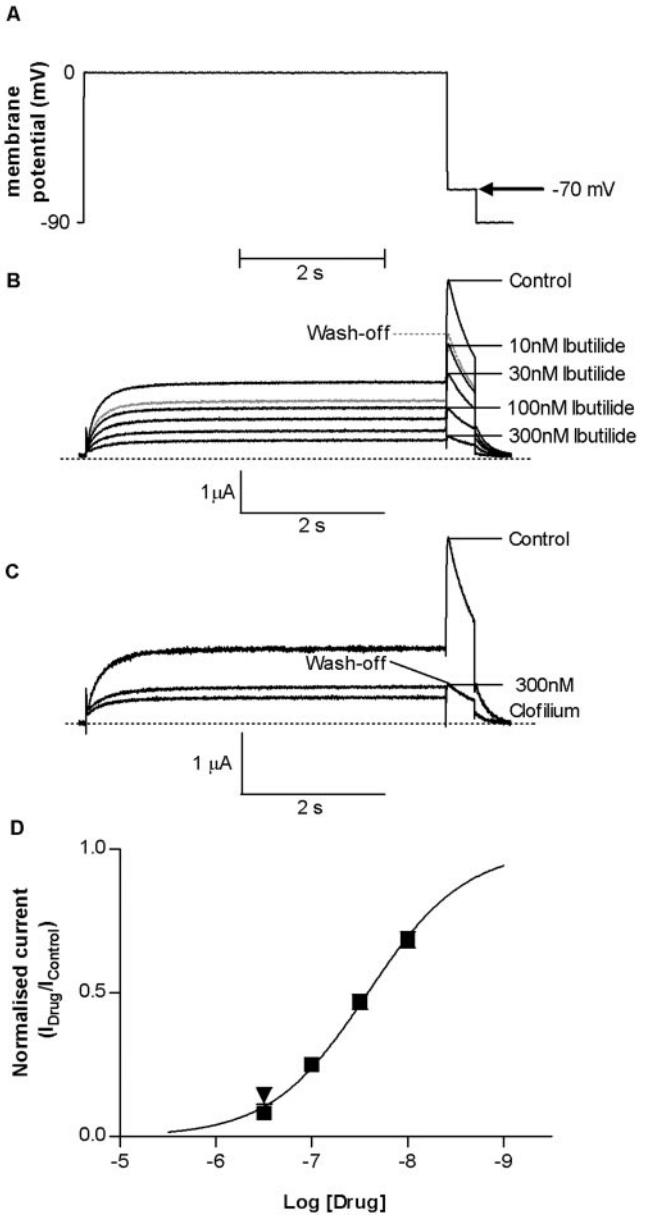


**Fig. 2.** Inhibition by clofilium and ibutilide of WT HERG currents expressed in *X. laevis* oocytes. A, currents were recorded with 5-s test depolarizations from the holding potential of −90 to 0 mV, with tail currents recorded upon repolarization to −70 mV for 400 ms. Start-to-start interval was 6 s. B and C, currents before (control) and after attaining steady-state block with 10, 30, 100, and 300 nM ibutilide (B) and 300 nM clofilium (C). Note the difference in recovery from ibutilide and clofilium block after 10 min of drug wash-off. D, concentration-response relationship for the block of HERG tail current by ibutilide (■) and clofilium (▼). The mean $IC_{50}$ value for block by ibutilide was 28 ± 1.1 nM ($n$ = 8).

using MK-499 and a mutant HERG channel that exhibits the unusual property of opening in response to either depolarization or hyperpolarization (Mitcheson et al., 2000b). D540K HERG channel currents recorded at hyperpolarized potentials exhibit rapid recovery from block by MK-499 (un-trapping of drug), which is not observed with WT HERG currents. The differences in rates of recovery are a result of hyperpolarization-dependent opening of D540K (but not WT) channels, which enables MK-499 to exit the central cavity (Mitcheson et al., 2000b). To determine whether the differences in rates of recovery from block of WT HERG by ibutilide and clofilium were caused by differences in drug trapping, we investigated block of WT and D540K HERG currents by these compounds using the protocols illustrated in Fig. 4A. WT and D540K HERG currents were elicited with repetitive 5-s depolarizations to 0 mV, applied at 6-s intervals. When currents had stabilized in control solution, 300 nM concentrations of drug were applied, and pulsing to 0 mV continued until steady-state inhibition was achieved (Fig. 4B, left). Recovery from block at hyperpolarized potentials was determined by applying 40 to 50 5-s pulses to −160 mV. The first and last currents elicited by the hyperpolarizing pulses are shown (Fig. 4B, middle). No inward WT HERG currents were seen at −160 mV, because these channels are closed at hyperpolarized potentials. In contrast, small D540K HERG currents were seen with the first hyperpolarizing pulse and these currents were larger with the last hyperpolarization. In particular, D540K HERG currents in the presence of ibutilide were much larger during the last hyperpolarization as recovery from block occurred. An instantaneous component of current through channels that hadn't fully deactivated during the interval between hyperpolarizations was seen at the start of the pulse. The extent of recovery from block was determined with a depolarizing pulse to 0 mV in the continued presence of drug (Fig. 4B, right). The peak current amplitudes normalized to control current amplitude ($I_{control,0mV}$) and plotted as a function of time for WT (i) or D540K (ii and iii) HERG channels are illustrated in Fig. 5A. Recovery from block in response to membrane hyperpolarization was calculated by dividing the difference between peak current amplitudes just before (△) and after (□) the hyperpolarizing pulses, by the amount of current inhibited during repetitive depolarizations (difference between currents at △ and ○). WT HERG currents showed almost no recovery from ibutilide block (Fig. 5Ai). However, there was a dramatic difference in the amount of recovery from block of D540K HERG channels. In the presence of ibutilide, inward D540K currents induced by hyperpolarizing pulses increased in amplitude with successive voltage pulses, and a comparison of current amplitudes immediately before and after the hyperpolarizations reveals that there was almost complete recovery from block (Fig. 5Aii), similar to the recovery previously reported for MK-499 (Mitcheson et al., 2000b). Surprisingly however, D540K HERG currents inhibited by clofilium showed very little change in amplitude when activated by repetitive hyperpolarizations (Fig. 5Aiii) and mean recovery from block was only 25.8 ± 6%. Thus, compared with ibutilide, there was little recovery from clofilium block with drug wash-off or in D540K HERG channels reopened with hyperpolarizations. These results, summarized in Fig. 5B, suggest that the slow recovery from block of HERG by clofilium is not caused by drug trapping.

To determine whether ibutilide and clofilium bind to the same residues as identified for MK-499, we used an Ala-scanning mutagenesis approach described previously (Mitcheson et al., 2000a). Based on homology between HERG and the solved crystal structure of the KcsA channel, Ala

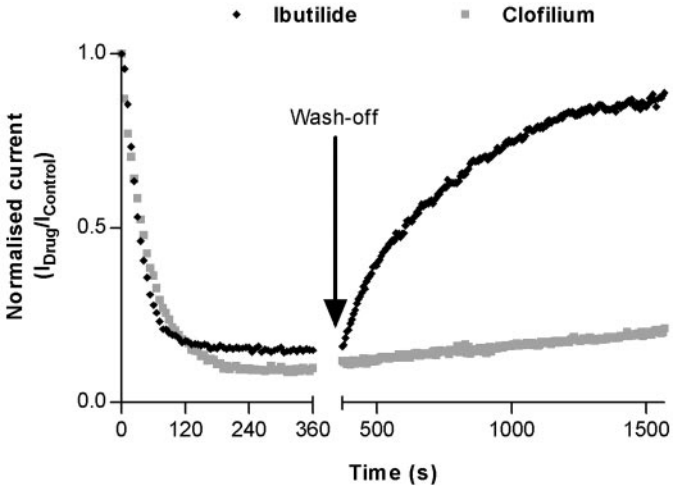


**Fig. 3.** Time dependence of WT HERG block and recovery from block in response to ibutilide and clofilium. Oocytes were repetitively depolarized using protocol described in Fig. 2. Peak tail currents during drug application (300 nM) and after wash-off were normalized to control currents and plotted against time. The onset of block was slow for both ibutilide and clofilium. However, whereas complete recovery from block was observed with ibutilide washout, there was almost no recovery from clofilium block.

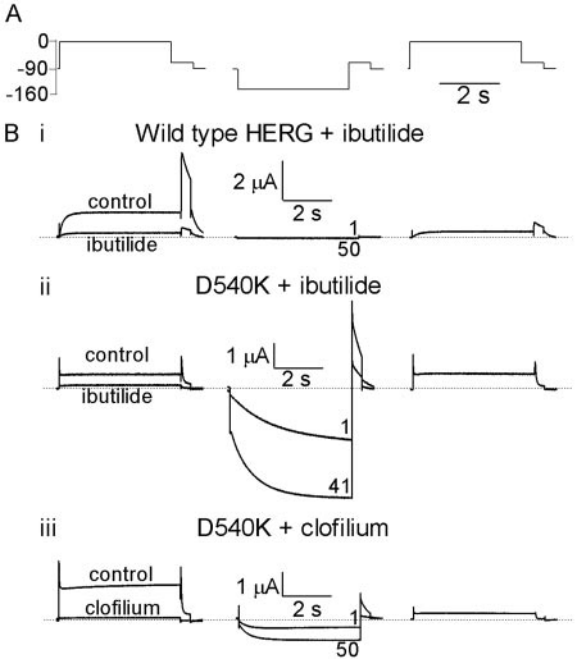


**Fig. 4.** D540K HERG current block and recovery from block in response to ibutilide and clofilium. A, voltage pulse protocol. B, WT (i) and D540K (ii, iii) HERG currents. *X. laevis* oocytes were repetitively depolarized at 0.166 Hz with 5-s voltage steps to 0 mV until currents reached steady state (control) before switching to a solution containing either ibutilide (i,ii) or clofilium (iii). Recovery from block at hyperpolarized potentials was investigated by applying 5-s pulses to −160 mV. Currents elicited by the first and last hyperpolarizations are shown (middle). Recovery from block was assessed with a depolarizing pulse to 0 mV (right). Tail currents were recorded with 400-ms steps to −70 mV.

mutations were introduced into residues of S6 (from Leu646 to Tyr667) and the pore helix (Leu622 to Val625) predicted to line the central cavity of HERG. The mutant channels were individually tested for their sensitivity to block by 300 nM of each drug (Fig. 6). Currents were measured during repetitive pulsing to 0 mV using the protocol described for determination of the $IC_{50}$ for ibutilide (Fig. 2). Clofilium and ibutilide inhibited WT HERG currents by 93% and 86%, respectively, at a concentration of 300 nM. Both drugs blocked most S6 mutant channels to an extent similar to that of the WT channels. However, G648A, Y652A, F656A, and V659A HERG currents were relatively insensitive to inhibition by 300 nM of either drug. These findings were very similar to the results observed previously with MK-499 (Mitcheson et al., 2000a). Three pore helix mutant channels (T623A, S624A, V625A) also exhibited reduced sensitivity to block by these compounds. In contrast, S624A was only slightly less sensitive than WT HERG to MK-499 (Mitcheson et al., 2000a).

To further explore features of the drug binding site, we characterized the biophysical properties and determined the concentration-response relationships for ibutilide and clofi-

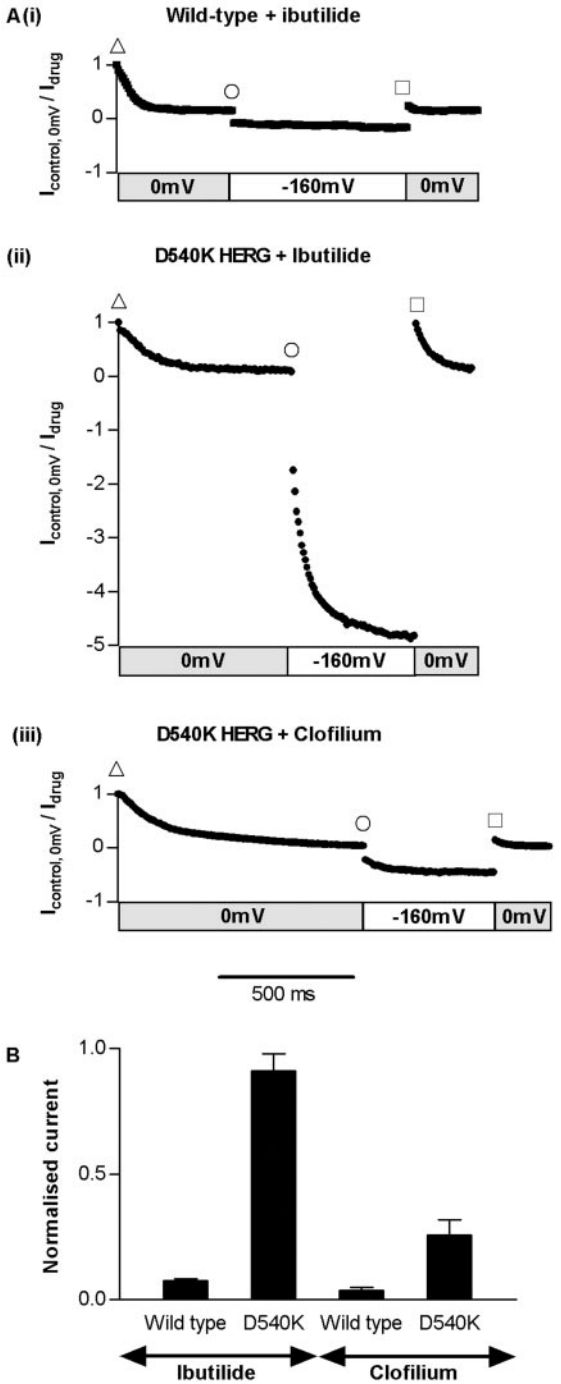


**Fig. 5.** D540K HERG channels recover from ibutilide but not clofilium block. A, normalized currents from experiments in Fig. 4. Peak current magnitudes in the presence of drug ($I_{drug}$) normalized to peak current magnitude with a depolarization to 0 mV in the control solution ($I_{control,0mV}$). A, i, WT HERG currents before and after applying ibutilide. A, ii and iii, D540K HERG currents before and after applying ibutilide (ii) or clofilium (iii). Each data point represents current elicited by a single voltage pulse. △, control current; ○, current after steady-state block was achieved; □, current with first depolarization to 0 mV after the train of hyperpolarizations. B, mean recovery from ibutilide or clofilium block for WT and D540K HERG after 40 to 50 repetitive hyperpolarizations to −160 mV.

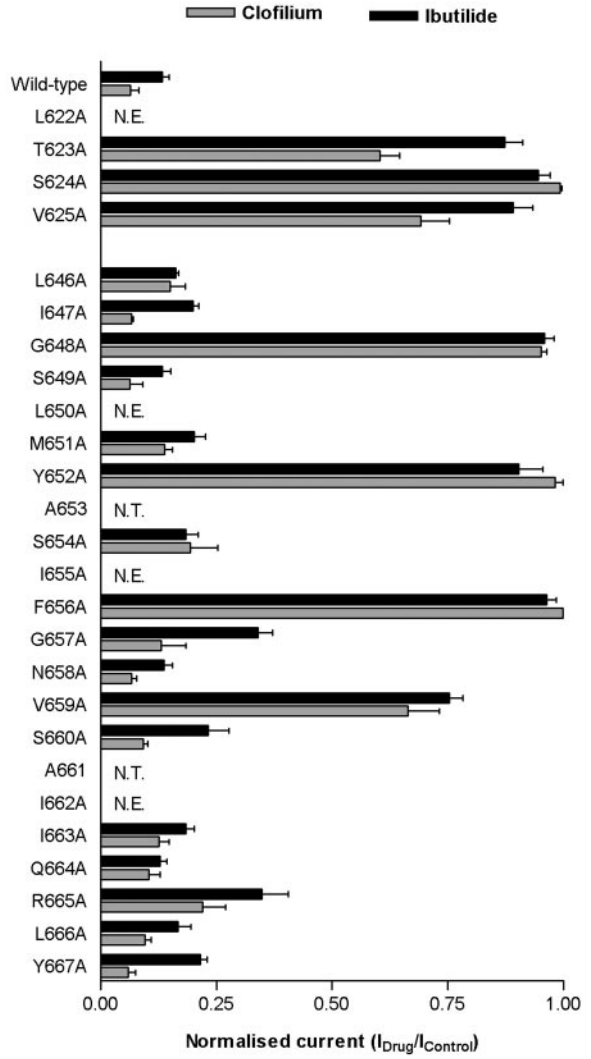


**Fig. 6.** Alanine scanning mutagenesis to identify binding sites for clofilium and ibutilide. HERG mutants were individually expressed in *X. laevis* oocytes and currents recorded with repetitive depolarizations to 0 mV before (control) and during clofilium or ibutilide (drug) application. The bars represent mean normalized current ($I_{drug}/I_{control}$) once steady-state block with 300 nM clofilium (gray) or ibutilide (black) has been achieved. A value near 1 indicates the mutation confers low sensitivity to drug block. N.T., residues were not tested; N.E., no functional expression.

lium of each of the pore helix mutant channels. HERG currents were elicited by membrane depolarization to test potentials ranging from −60 to +50 mV. T623A HERG currents inactivated more than WT and outward currents were extremely small when measured using the standard extracellular solution containing 2 mM $K^+$. To reduce inactivation and increase the amplitude of inward tail currents, T623A HERG currents were measured using a high (96 mM) $K^+$ solution (Fig. 7A). The slope and potential for half-maximal activation ($V_{0.5}$) of T623A HERG using this solution was 5.5 ± 0.23 mV and −24.2 ± 1.16 mV, respectively ($n$ = 6). V625A and S624A HERG currents were measured using the standard (2 mM) extracellular $K^+$ solution. S624A HERG currents (Fig. 7B) were similar to WT currents; depolarization-activated currents peaked at −10 mV and the slope and $V_{0.5}$ for activation was 8.8 ± 0.16 mV and −23.5 ± 0.48 mV, respectively (Fig. 7B, $n$ = 4). Val625 is located next to the GFG motif that forms the $K^+$ selectivity filter (Mitcheson et al., 2000a). It is not surprising that mutation of Val625 caused a reduced $K^+$ selectivity, resulting in a positive shift in the current reversal potential such that currents were inward at potentials negative to −10 mV (Fig. 7C). The slope and $V_{0.5}$ for V625A HERG channel activation was 5.72 ± 0.12 mV and −38.5 ± 5.5 mV, respectively ($n$ = 4). The current-voltage relationship of V625A HERG current at positive potentials was nearly linear, and tail currents elicited by repolarization did not have the characteristic hooks (Fig. 7C), indicating that these channels did not appreciably inactivate at potentials up to +50 mV. In summary, the pore helix mutant channels all activated at similar potentials and at slightly more negative potentials than WT HERG. However, whereas S624A inactivation properties were similar to those of WT, V625A HERG channels did not inactivate, and the voltage dependence of T623A HERG channel inactivation was shifted in the negative direction.

The concentration-response relationships for T623A, S624A, and V625A HERG channels to block by ibutilide and clofilium were determined (Fig. 7D). All three mutations greatly reduced channel sensitivity to block by both drugs (Table 1). The rank order of importance of residues for ibutilide binding was Val625 > Ser624 > Thr623. V625A was very insensitive to ibutilide (>300 times WT HERG $IC_{50}$), and mutation of the polar residues (Thr623 and Ser624) reduced block by 55- to 90-fold relative to WT HERG. For clofilium, the rank order of importance was Ser624 > Val625 > Thr623. The $IC_{50}$ values for T623A, S624A, and V625A HERG were 0.64 ± 0.25, 11.6 ± 0.87, and 7.2 ± 0.5 μM, respectively. Thus, despite the relative spatial proximity of Thr623 and Ser624 and the similarity in side chain physicochemical properties, there was a substantial difference in the relative importance of Ser624 over Thr623 for clofilium binding. Moreover, mutation of Val625 reduced sensitivity to block by ibutilide far more than clofilium. These findings indicate a highly specific and distinct interaction of the two drugs with residues located at the base of the pore helix.

Further support for the importance of residue Ser624 for clofilium block of HERG came from comparing the rates of recovery from block of the pore helix mutant channels (Fig. 8). Steady-state block was attained by repetitively pulsing to 0 mV in the presence of 10 μM clofilium (>300 times WT HERG $IC_{50}$). Clofilium was then washed off and repetitive pulsing to 0 mV resumed after 1 to 2 min. Representative normalized current amplitudes plotted against time are shown in Fig. 8A. WT and T623A HERG channels were profoundly blocked by 10 μM clofilium, and there was only a small recovery from block during the washout period. Despite the decreased inhibition of V625A currents relative to WT HERG, the response to drug wash-off was qualitatively similar. In contrast, S624A channels recovered from block relatively rapidly. The percentage recovery from block after 10 min of drug washout for WT and the three pore helix mutant channels are summarized in Fig. 8B. S624A was the only pore helix mutant studied that demonstrated rapid and complete recovery from block with clofilium wash-off. The decreased drug sensitivity of S624A channels and the rapid recovery from block suggest that clofilium interactions with Ser624 are important for drug binding at depolarized potentials and for the exceptionally slow rates of recovery from block in WT HERG channels.

## Discussion

**Features of the HERG Drug Binding Site Revealed by Site-Directed Mutagenesis.** The principle underlying mechanism for drug-induced LQTS is inhibition of cardiac $I_{Kr}$ (Roden, 1998; Crumb and Cavero, 1999). Therefore, understanding the molecular determinants of drug binding to HERG could have a major impact in the identification of new compounds without this undesirable side effect. Previous studies have shown that the central cavity is the location of the binding site of HERG. Several structural features of the cavity make it susceptible to block. The lack of a Pro-*X*-Pro motif suggests the central cavity of HERG may be larger than most other voltage-gated $K^+$ channels. The S6 domain of HERG subunits also contain aromatic residues (Tyr652 and Phe656) that face into the central cavity and form a crucial component of the binding site for diverse chemical structures (Mitcheson et al., 2000a,b; Kamiya et al., 2001; Sanchez-Chapula et al., 2002, 2003). The present study provides further evidence that residues at the base of the pore helices, which also face into the central cavity, can form interactions with drugs that may facilitate high-affinity binding and, in the case of clofilium, can prevent unbinding from open D540K HERG channels at negative potentials. For this reason, the pore helix residues must also be considered an important site of drug interaction.

Mutation of T623A and S624A resulted in ~90- and ~60-fold increases of ibutilide $IC_{50}$ values. Ibutilide at 10 μM had little effect on V625A HERG currents, indicating that mutation of this residue results in a >300-fold increase of $IC_{50}$. We were unable to attain steady-state block of WT HERG at low concentrations of clofilium and therefore could not obtain a precise measure of $IC_{50}$ for clofilium. Nonetheless, it was clear that mutation of Thr623, Ser624, and Val625 HERG substantially increased the amount of clofilium required to inhibit HERG currents.

**Hyperpolarization-Dependent Recovery from Block of D540K HERG Occurs with Ibutilide but Not Clofilium.** Slow recovery from block could be caused by trapping of unbound drugs within the inner cavity by closure of the activation gate. Carmeliet showed that recovery from block of $I_{Kr}$ by almokalant could be facilitated by using a more positive holding potential, presumably because open probability was higher and drug was able to exit more rapidly than at

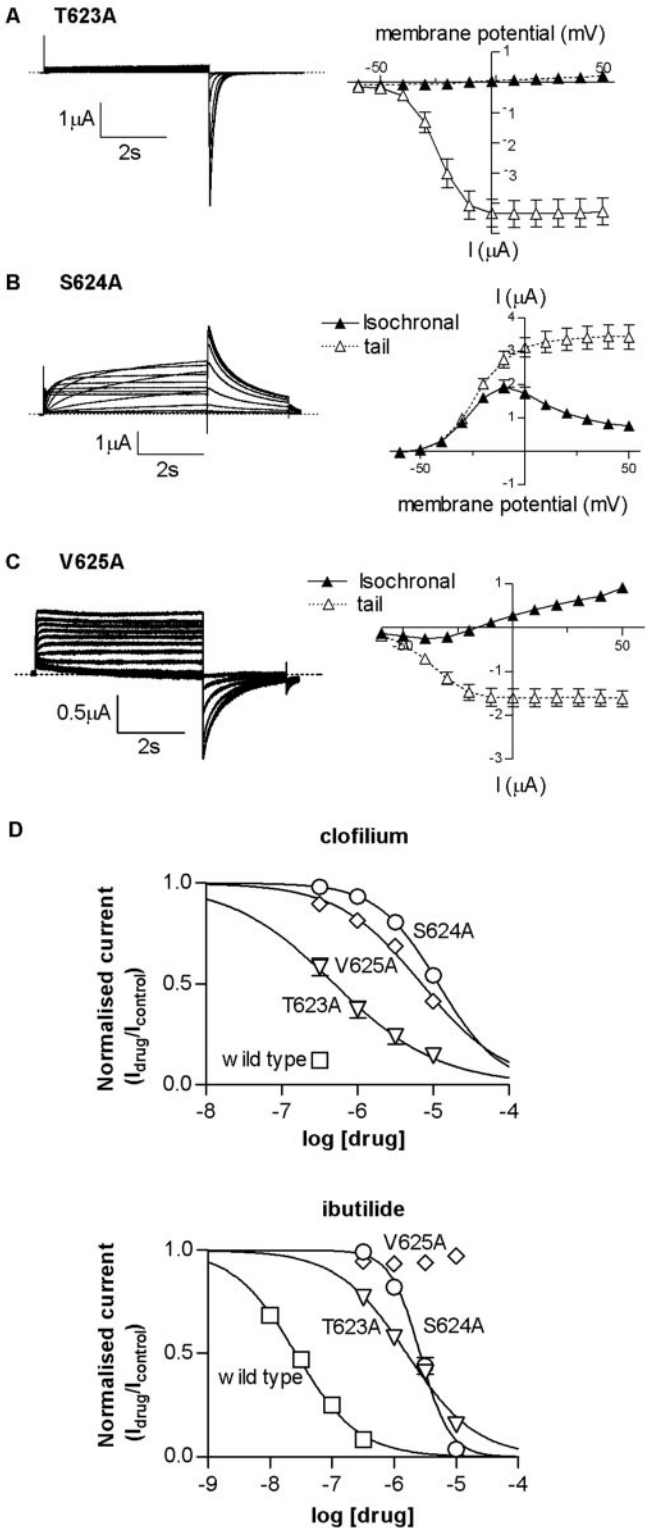


**Fig. 7.** Electrophysiological and pharmacological properties of pore helix mutants. A-C, representative currents (left) and mean steady-state (▲) and tail (△) current-voltage relationships (right) for cells expressing T623A (A), S624A (B), and V625A (C) HERG channels. Currents were elicited by 5-s depolarizations from −60 to +50 mV from a holding potential of −90 mV, with tail currents measured upon repolarization back to −70 mV. T623A HERG currents were recorded in high extracellular $K^+$ solution, with a tail current potential of −90 mV. D and E, concentration-response relationships for block of T623A, S624A, and V625A HERG by clofilium (D) and ibutilide (E). The $IC_{50}$ values for block by clofilium and ibutilide are given in Table 1. $n$ = 4 to 6 cell recordings for all channels.

negative potentials (Carmeliet, 1992, 1993). We demonstrated rapid recovery of D540K HERG channels (which open with hyperpolarization) from MK-499 block. Recovery from block was facilitated by the high open probability and negative transmembrane charge field that helps drive positively charged drugs out of the channel at hyperpolarized potentials (Mitcheson et al., 2000b). In the present study, hyperpolarization-dependent opening of D540K also facilitated untrapping of ibutilide. However, channels blocked with clofilium did not rapidly recover from block, suggesting that

TABLE 1
Comparison of $IC_{50}$ values for block of WT HERG and pore helix mutants by clofilium and ibutilide

| Channel | Clofilium | | Ibutilide | |
|---|---|---|---|---|
| | $IC_{50}$ ± S.E.M. | $n$ | $IC_{50}$ ± S.E.M. | $n$ |
| | $\mu M$ | | $\mu M$ | |
| Wild-type | N.D. | | 0.028 ± 0.0025 | 6 |
| T623A | 0.64 ± 0.25 | 6 | 1.50 ± 0.74 | 6 |
| S624A | 11.57 ± 0.87 | 6 | 2.61 ± 0.23 | 5 |
| V625A | 7.24 ± 0.49 | 4 | >10 | 4 |

N.D., not determined (see *Results* for explanation).

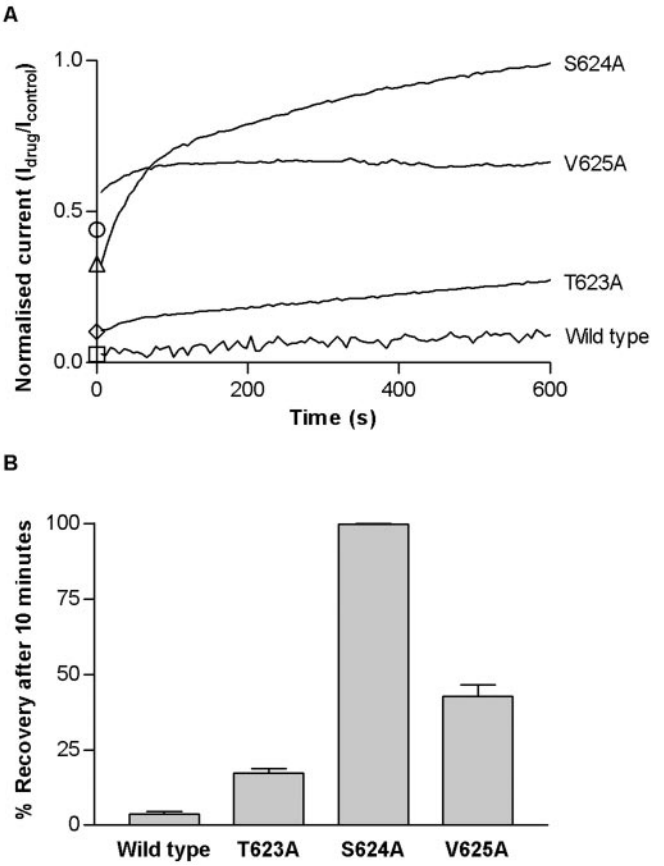


**Fig. 8.** S624A HERG channels demonstrate relatively rapid recovery from clofilium block. A, typical time courses of recovery from block by 10 $\mu$M clofilium of WT HERG and pore helix mutants. Cells were repetitively depolarized to 0 mV, and 10 $\mu$M clofilium was applied until steady-state block was achieved. The extent of steady-state block for each mutant is indicated by the open symbols (□, WT HERG; ◇, T623A; △, S624A; ○, V625A). The clofilium was washed off for 1 to 2 min and pulsing resumed for 10 min. B, mean percentage recovery from clofilium block, calculated by measuring the difference in current amplitude after steady-state block and 10 min wash-off and expressing as a percentage of current blocked by 10 $\mu$M clofilium.

channel repolarization does not result in clofilium unbinding and that significant differences exist in the mechanism of clofilium block relative to other drugs investigated to date.

**Ser624 Is a Critical Residue for Slow Recovery from Block by Clofilium.** S624A HERG was the only pore helix mutant to show rapid recovery from clofilium block with drug wash off. Recovery did not proceed during a wash-out period when the channels were closed and only occurred once repetitive depolarizations were applied to open the channels, indicating that although S624A allows unbinding upon repolarization, the drug remains trapped until channels are opened. Mutation of neighboring residues (Thr623 and Val625) decreased drug sensitivity without a change in recovery rates, suggesting that a specific interaction with Ser624 is responsible for the slow off-rates that characterize clofilium block of WT and D540K HERG channels. S624A currents were similar to WT HERG, suggesting that the pharmacological properties were not altered by modified gating properties or allosteric effects on the drug binding site. Polar interactions between the −OH of Ser and the Cl of clofilium may help stabilize clofilium binding. A homology model of HERG, based on the KcsA crystal structure provides support for this hypothesis (Fig. 9). In several of the most energetically favorable docking conformations, there are close interactions between the chlorine atom and the −OH group of Ser624 and $\pi$-stacking interactions between the phenyl ring of clofilium and Tyr652. An allosteric effect on the binding site would be expected to reduce block by most compounds, but this is not the case. There are only small effects of S624A on block by MK-499 (Mitcheson et al., 2000a). In contrast, much larger responses to this mutation are observed for ibutilide, clofilium, and vesnarinone (Kamiya et al., 2001). In contrast to Ser624, several lines of evidence suggest Val625 is not part of the drug-binding site. $K^+$ channel crystal structures show the side chain of the Val analogous to position 625 of HERG buried within the hydrophobic core surrounding the selectivity filter and not facing into the inner cavity (Doyle et al., 1998; Jiang et al., 2002). Val625 is part of the $K^+$ channel signature sequence (**V**GFG), and mutation of this residue to Ala alters channel selectivity and inactivation properties. Therefore, an allosteric effect of this mutation, resulting from repositioning of residues near Val625 (i.e., Thr623 and Ser624), cannot be discounted. Consistent with this idea, compounds that are sensitive to mutation of Ser624 and Thr623 are also sensitive to V625A and vice versa.

**Tyr652 and Phe656 Are Both Required for Block of HERG by Ibutilide and Clofilium.** Despite differences in

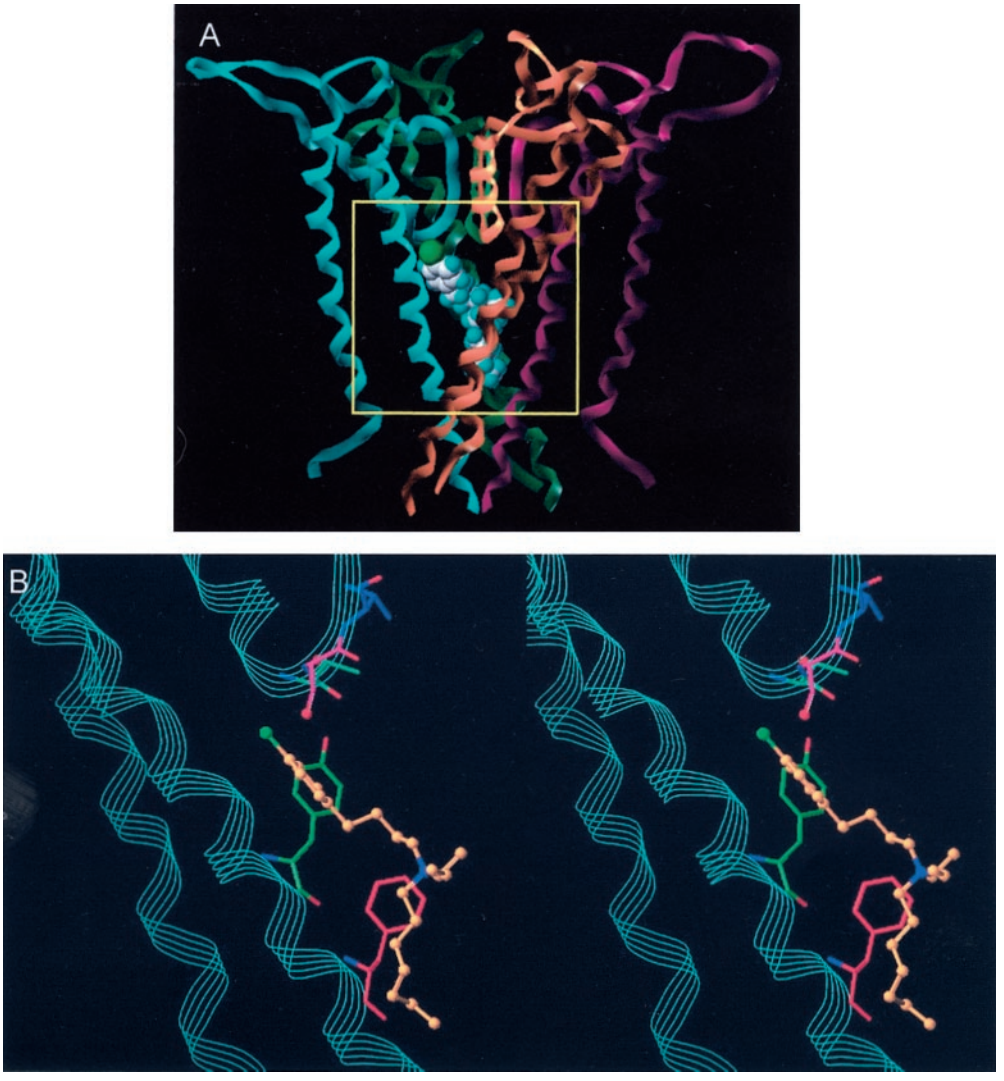


**Fig. 9.** Docking of clofilium within the inner cavity of a homology model of the HERG channel. Twenty different binding modes were generated with either the chlorophenyl group or aliphatic tail of clofilium oriented toward the selectivity filter end of the cavity. A, side view of one possible binding mode that is highly consistent with the experimental results. The S5–S6 domains of the channel are represented by shaded ribbons, with the four subunits distinguished by different colors. Clofilium is shown with the chlorine (green) oriented toward the selectivity filter. Clofilium carbon atoms are shown in gray and the hydrogen atoms in cyan. B, stereoview of the region outlined by the yellow box in A. The model in A was rotated about the vertical axis to get the best view of the perpendicular $\pi$-stacking interactions of the phenyl group of clofilium with Tyr652 and hydrophobic interactions of the aliphatic tail of clofilium with Phe656. Clofilium is represented by ball and sticks, with the chlorine atom in green, nitrogen atom in blue, and carbon atoms in orange. In this binding mode, the clofilium is interacting with residues from the cyan subunit only, which is shown in line-ribbon mode with key residues highlighted with colored sticks: Tyr652 (green), Phe656 (red), Thr623 (blue-green), Ser624 (pink), and Val625 (blue). The OH groups of Ser624 and Thr623 are shown as capped sticks to distinguish them from carbonyl oxygens. The OH group of Ser624 is near the chlorine of clofilium, whereas the OH group of Thr623 is pointing away.

the relative importance of pore helix residues for drug interactions, the amino acids on S6 involved in drug binding seem to be similar for most drugs. Ibutilide or clofilium (300 nM) had almost no effect on G648A, Y652A, and F656A HERG currents (Fig. 5). Mutation of Tyr652 and Phe656 greatly reduced the $IC_{50}$ for block of HERG by nearly all other drugs investigated so far, including MK-499, cisapride, terfenadine, and chloroquine (Lees-Miller et al., 2000; Mitcheson et al., 2000a; Sanchez-Chapula et al., 2002, 2003), although exceptions have been noted. For example, vesnarinone block was sensitive to mutation of Phe656 but not Tyr652 (Kamiya et al., 2001), and fluvoxamine has been reported to be relatively insensitive to mutation of either aromatic residue (Milnes et al., 2003).

Clofilium and ibutilide block was disrupted by mutation of either Tyr652 or Phe656. This suggests either that these residues interact with different parts of the drug molecule (e.g., phenyl ring and charged amine) at the same time or that the final high-affinity binding conformation isn't reached without interactions involving both aromatic residues, perhaps at different stages during binding.

**Model of HERG Channel Block.** Our findings provide further support for the importance of interactions between aromatic groups of residue side-chains and drug molecules. An additional feature of many drugs that induce LQTS is a polar group (such as a halogen atom or methanesulfonamide) that is usually attached to the phenyl ring at one end of the drug molecule. These polar groups are highlighted in a three dimensional quantitative structure-activity relationship pharmacophore model that correlates the physicochemical characteristics of drugs with their HERG channel-blocking potencies (Cavalli et al., 2002). The results from the present study suggest that interactions between Thr623 and Ser624 with polar moieties on the drug molecules are an important component of the drug-channel interaction. Based on this data we propose that the aromatic and polar groups of clofilium and ibutilide are oriented toward the selectivity filter end of the central cavity, and the aliphatic tail of the drugs are pointed toward the intracellular opening of the inner helices. The evidence in support of this model is 4-fold. First, clofilium interacts with the pore helix residues (principally Ser624) of the inner cavity. This interaction is most likely to be a polar interaction with the chlorine atom of clofilium, rather than an interaction with the aliphatic, hydrophobic tail of the molecule. Second, there are significant differences in the relative importance of Thr623, Ser624, and Val625 for channel block by ibutilide and clofilium. These are more likely to reflect differences between interactions with the chlorine and methanesulfonamide groups than with the aliphatic tails of ibutilide and clofilium, which are the same in both molecules. Third, the suggested orientation allows $\pi$-stacking and cation-$\pi$ interactions between Tyr652 and Phe656 residues and the phenyl ring and charged amine groups on the drugs. If the drug molecules were oriented in the opposite direction, the interactions with Tyr652 in particular would be predicted to be less favorable. Fourth, the suggested orientation is most consistent with the three-dimensional quantitative structure-activity relationship model of Cavalli et al. (2002). This model is characterized by regions of the pharmacophore, where increasing positive and negative charge of groups attached to the primary aromatic ring increases HERG blocking activity. In our suggested orientation, this would be consistent with interaction of these regions with Ser624 and Thr623 at the selectivity filter end of the inner cavity. At the opposite end of the pharmacophore model, where the aliphatic tails of ibutilide and clofilium would align, is a region in which increasing volume increases blocking activity. This agrees well with crystal structures of $K^+$ channels in the open state, which show a large aperture at the cytosolic end of the cavity (Jiang et al., 2002, 2003) and the seminal studies of Armstrong (1968) using quarternary amine compounds to probe $K^+$ channel structure, which showed that increasing the size of the quarternary amine side chains increased compound potency.

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