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Molecular Determinants of hERG Channel Block

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ABSTRACT

Drug-induced block of cardiac hERG K^+ channels causes acquired long QT syndrome. Here, we characterized the molecular mechanism of hERG block by two low-potency drugs (Nifekalant and bepridil) and two high-potency drugs 1-[2-(6-methyl-2pyridyl)ethyl]-4-(4-methylsulfonyl aminobenzoyl)piperidine (E-4031) and dofetilide). Channels were expressed in *Xenopus laevis* oocytes, and currents were measured using the two-microelectrode voltage-clamp technique. All four drugs progressively reduced hERG current during a 20-s depolarization to 0 mV after a 10-min pulse-free period, consistent with the preferential block of open channels. Recovery from block in response to pulses to −160 mV was observed for D540K hERG channels but not for wild-type hERG channels, suggesting that all four drugs are trapped in the central cavity by closure of the activation gate. The molecular determinants of hERG channel block were defined by using a site-directed mutagenesis approach. Mutation to alanine of three residues near the pore helix (Thr623, Ser624, and Val625) and four residues in Ser6 (Gly648, Tyr652, Phe656, and Val659) reduced channel sensitivity to block by dofetilide and E-4031, effects identical with those reported previously for two other methanesulfonanilides, (+)-*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 (MK-499) and ibutilide. The effect of nifekalant on mutant channels was similar, except that V659A retained normal sensitivity and I655A channels were less sensitive. Finally, mutation of the three residues near the pore helix and Phe656 in the Ser6 domain reduced channel block by bepridil. We conclude that the binding site is not identical for all drugs that preferentially block hERG in the open state.

Class III antiarrhythmic drugs are defined by their ability to block potassium channels and prolong the action potential duration of cardiomyocytes. Some of the most potent class III drugs such as dofetilide, E-4031, and MK-499 are structural analogs of the methanesulfonanilide sotalol, a compound with low potency. All of these drugs prolong action potentials by a relatively specific block of the rapid delayed rectifier K^+ current, I_{Kr} (Sanguinetti and Jurkiewicz, 1990). Sotalol is the only class III methanesulfonanilide that has been shown in clinical trials to improve prognosis; however, the potency of I_{Kr} block by sotalol is several hundred-fold weaker than dofetilide, E-4031, or MK-499. The discrepancy between the beneficial clinical profile and a low potency for I_{Kr} block has long been reported for sotalol and may indicate the greater importance of the β-adrenergic receptor blocking activity compared with I_{Kr} block.

The human I_{Kr} channel is encoded by *HERG* and mutations in this gene cause long QT syndrome (Curran et al., 1995), a disorder of cardiomyocyte repolarization that predisposes affected individuals to an increased risk of torsades de pointes and lethal ventricular fibrillation. The most common cause of prolonged QT interval is treatment with class III antiarrhythmic agents and side effects associated with treatment with certain noncardiac medications. For this reason, there is intense interest in determining the structural basis of hERG channel block to avoid this side effect during the discovery phase of new drug entities.

Pharmacophore models have defined some of the chemical features of drugs that determine hERG channel block (Cavalli et al., 2002; Ekins et al., 2002; Pearlstein et al., 2003). In an attempt to define the features of the receptor site for drugs on hERG, we alanine-scanned the inner pore region of the channel subunit and determined the sensitivity of individual

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ABBREVIATIONS: E-4031, 1-[2-(6-methyl-2pyridyl)ethyl]-4-(4-methylsulfonyl aminobenzoyl)piperidine; WT, wild type; 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; MES, 2-(*N*-morpholino)ethanesulfonic acid; hERG, human *ether-a-go-go*-related gene.

mutant channels to block by MK-499 (Mitcheson et al., 2000a). Several key residues were identified by this approach, including two aromatic residues located on the Ser6 domain (Tyr652 and Phe656) and several residues located at the base of the pore helix (Thr623, Ser624, and Val625). Homology modeling predicted that all of these residues faced the central cavity of the channel, consistent with earlier findings that hERG channels were preferentially blocked in the open state (Mitcheson et al., 2000b). A critical role for Phe656 was also proposed for binding of the antiarrhythmic agent dofetilide and quinidine (Lees-Miller et al., 2000), and the importance of both Tyr652 and Phe656 has been confirmed for several other drugs, including chloroquine (Sanchez-Chapula et al., 2002), quinidine (Sanchez-Chapula et al., 2003), halofantrine (Sanchez-Chapula et al., 2004), terfenadine and cisapride (Fernandez et al., 2004), lidoflazine (Ridley et al., 2004a), clofilium, and ibutilide (Perry et al., 2004). Some drugs, such as fluvoxamine (Milnes et al., 2003), propafenone (Witchel et al., 2004) and dronedarone (Ridley et al., 2004b), seem not to interact strongly with Tyr652 and/or Phe656; however, it is also not known whether these drugs interact with the other residues identified previously as part of the drug binding site of hERG. In this study, we extend our alanine-scan analysis of the Ser6 domain and pore helix region of hERG channels expressed in oocytes to four additional drugs (Fig. 1). First, we examined whether the pattern of residue interactions for dofetilide and E-4031 was similar to that for MK-499 as expected based on their similar chemical structures. Second, we examined the pattern of residue interactions for two low-potency and nonspecific hERG channel blockers, bepridil and nifekalant.

Materials and Methods

Mutagenesis and Channel Expression in Oocytes. Wild-type (WT) *HERG* subcloned into the pSP64 vector was prepared as described previously (Sanguinetti et al., 1995). Site-directed mutagenesis was performed using the megaprimer method (Sarkar and Sommer, 1990). Mutation constructs were confirmed by restriction enzyme and DNA sequence analyses. cRNAs for injection into oocytes were prepared with SP6 Cap-Scribe (Roche Applied Science, Indianapolis, IN) after linearization of the expression construct with EcoRI. Alanine-scanning mutagenesis of hERG was used to identify residues that interact with a specific drug (Mitcheson et al., 2000a).

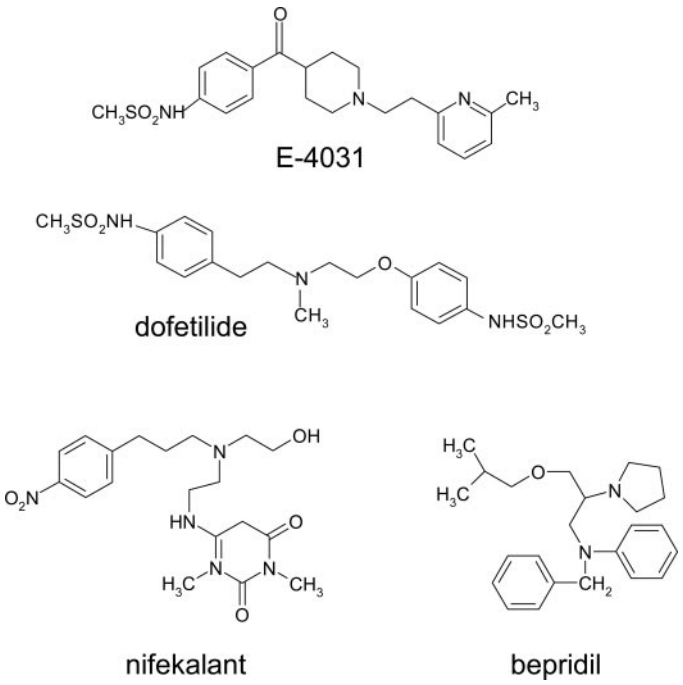


Fig. 1. Chemical structures of the four compounds investigated in this study.

Isolation and Injection of *Xenopus laevis* Oocytes. Oocytes were isolated by dissection from anesthetized adult *X. laevis*. Frogs were anesthetized by immersion in 0.2% tricaine (Sigma Chemical Co., St. Louis, MO) for 10 to 15 min. A small abdominal incision was made, and ovarian lobes containing oocytes were removed. The incision was sutured closed, and the frog was returned to its aquarium for a recovery period of at least 1 month before the procedure was repeated. After a maximum of three surgeries, tricaine-anesthetized frogs were killed by pithing. Clusters of oocytes were treated with 2 mg/ml type 2 collagenase (Worthington Biochemicals, Freehold, NJ) to remove follicle cells. Maintenance and cRNA injections into oocytes were performed as described previously (Stuehmer, 1992; Sanguinetti and Xu, 1999).

Voltage Clamp. Ionic currents were recorded at room temperature (22–24°C) using a GeneClamp 500 amplifier (Molecular Devices, Sunnyvale, CA) and standard two-microelectrode voltage-clamp techniques (Stuehmer, 1992). Digitized data were analyzed offline by using pCLAMP (Molecular Devices, Sunnyvale, CA) and ORIGIN (OriginLab Corp, Northampton, MA) software.

To attenuate endogenous outward chloride currents, Cl^- was replaced with MES in the external solution that contained 96 mM sodium-MES, 2 mM KMES, 2 mM $CaMES_2$, 5 mM HEPES, and 1 mM $MgCl_2$ adjusted to pH 7.6 with methane sulfonic acid. For T623A and G648A hERG channels that expressed poorly or were highly inactivated, KMES was increased to 96 mM with a similar reduction in NaMES. A small chamber containing a single oocyte was continuously superfused with solutions. Flow was maintained by gravity, and solutions were switched between separate lines using a solenoid-controlled switching device.

To assess the effects of drugs on WT and mutant hERG channels, currents were measured during repetitive 5-s depolarizing steps to 0 mV from a holding potential of −90 mV applied at a frequency of 0.166 Hz. Block was defined as the decrease in current measured at 0 mV and at the end of the 5-s pulses. This same protocol was used to define the IC_{50} value (the concentration of drug required to decrease WT hERG current by 50%). Concentration-effect data for currents normalized to the peak value (I_{norm}) were fitted to the Hill equation to determine the IC_{50} value and the Hill coefficient (h): $I_{norm} = ([drug])^h/\{(IC_{50})^h + ([drug])^h\}$.

The onset of channel block was assessed using concentrations of drug equivalent to 10 times their IC_{50} values. In the absence of drug, outward current was recorded during a single 20-s pulse to 0 mV. The oocyte was then held at a potential of −90 mV without pulsing while equilibrated with drug for 10 min. After the equilibration period, another test pulse to 0 mV was applied to the oocyte. The current recorded before drug was divided by the current recorded after drug to obtain the relative current versus time. This trace was fit to a single exponential using the Levenberg-Marquardt method in pCLAMP to obtain the time constant for the onset of drug block for each drug.

Data are presented as mean ± S.E.M. (n = number of cells), and statistical comparisons between experimental groups were performed using the Student's t test. Differences were considered significant at $P < 0.05$.

Drugs. Dofetilide was donated by Pfizer Pharmaceuticals Inc. (New York, NY) and prepared as a 10 mM solution in water. E-4031 was donated by Eisai-Pharmaceutical Inc. (Tokyo, Japan) and prepared as 30 mM stock solution in water. Bepridil was donated by Sankyo Co., Ltd. (Tokyo, Japan) and prepared as a 100 mM stock solution in methanol. Nifekalant was donated by Nihon Shering K.K.

(Osaka, Japan) and prepared as a 100 mM stock solution in water. Final drug concentrations were prepared daily by dilution of stock solutions kept at 20°C.

Results

Onset of and Recovery from Drug Block. The IC_{50} values for block of WT hERG channels, determined by repetitive pulsing to 0 mV for 20 min were 0.42 ± 0.11 μM ($n = 5$) for dofetilide, 0.57 ± 0.13 μM ($n = 5$) for E-4031, 3.0 ± 0.4 μM ($n = 5$) for bepridil, and 5.8 ± 0.32 μM ($n = 5$) for nifekalant. The onset of channel block was assessed using concentrations of drug equivalent to 10 times these IC_{50} values. In the absence of drug, outward current was recorded during a single depolarizing pulse to 0 mV (20 s for dofetilide and E-4031 and 10 s for bepridil and nifekalant). After a 10-min drug equilibration period during which the transmembrane potential of the oocyte was held at −90 mV without pulsing, another 20-s test pulse to 0 mV was applied. An example of the currents recorded before and after application of 4.2 μM dofetilide is illustrated in Fig. 2A. In the absence of drug, current was fully activated within a few seconds and remained constant thereafter. The tail current measured upon repolarization to −80 mV was larger than the current during the pulse as channels first recovered from inactivation before deactivating. In the presence of dofetilide, the current amplitude was unchanged during the initial ~100 ms of the depolarizing pulse but decreased slowly throughout the 20-s pulse as channel block developed. The rate of block onset for dofetilide was well described by a single exponential function (Fig. 2B) with a time constant of 4.56 ± 0.33 s ($n = 3$). These data suggest that dofetilide can only block hERG channels after they have opened, similar to our previous finding with a chemically related methanesulfonanilide, MK-499 (Spector et al., 1996).

To investigate the recovery of WT hERG channels from block by dofetilide, we used the voltage protocol illustrated at the top of Fig. 2C. Voltage pulses (5-s duration) were repetitively applied to 0 mV at 6-s intervals. Once current amplitudes had stabilized under control conditions, 4.2 μM dofetilide was applied to the cell chamber. When block reached a near steady-state value, hyperpolarizing pulses to −160 mV were applied (also 5-s duration) in the continued presence of dofetilide. These repetitive hyperpolarizing pulses were applied at 6-s intervals for 4 min followed by a depolarizing pulse to 0 mV to assess potential recovery from block (Fig. 2C, middle). The percentage of recovery from block was calculated from the difference in peak current amplitude between current traces labeled as "e" and "b" divided by peak current labeled as "a". A trace diary for one oocyte is plotted at the bottom of Fig. 2C. Using this protocol, there was no recovery from block of WT hERG channels (mean change = −0.1 ± 0.1%, $n = 3$). The same protocol was used to examine the kinetics of recovery from block of D540K hERG channels (Fig. 2D). As described previously, D540K channels are unusual because they reopen in response to hyperpolarization of the membrane (Mitcheson et al., 2000b). The unique gating behavior of D540K hERG allows drugs that are otherwise trapped inside the central cavity (by closure of the activation gate) to be released when the channels open at negative potentials. Thus, a comparison between drug block/unblock

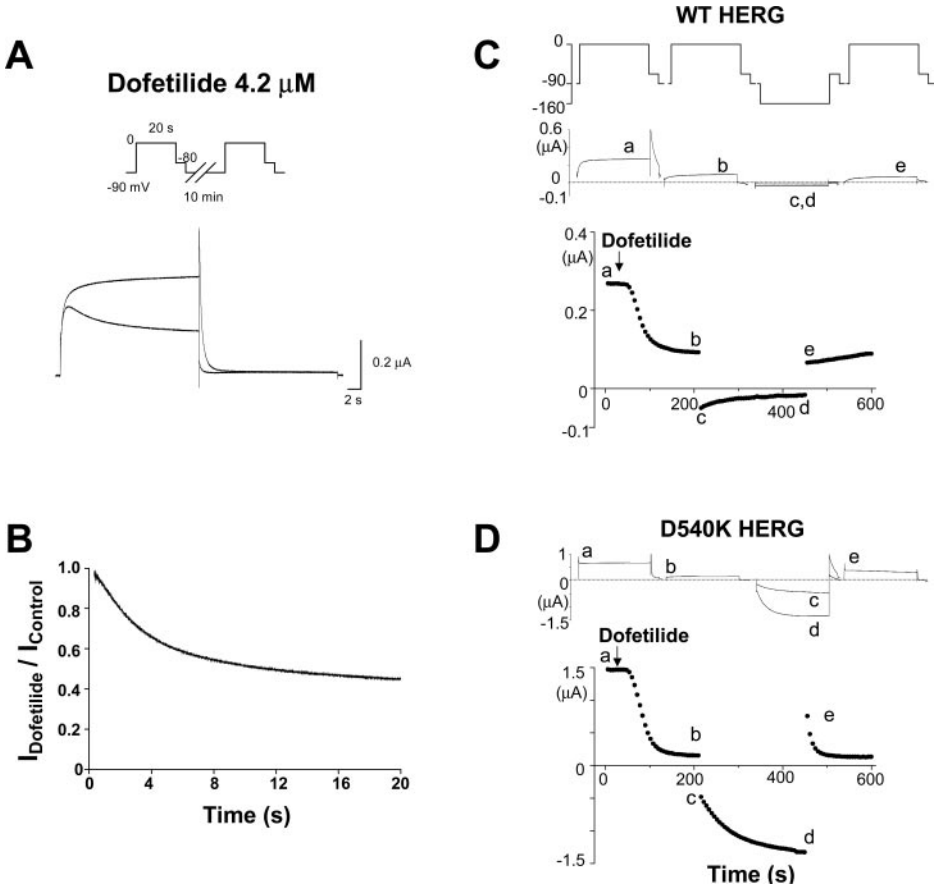


Fig. 2. Onset and recovery from block of hERG channels by dofetilide. A, current traces showing onset of open channel block by dofetilide during a 20-s pulse to 0 mV after a 10-min pulse-free period of equilibration with drug. B, the onset of hERG channel block by dofetilide expressed as relative current. Block of current developed exponentially with a time constant of 4.08 s. C, block of WT hERG by dofetilide (4.2 μM) induced by repetitive pulsing to 0 mV is not recovered by repetitive pulsing to −160 mV. Top, voltage pulse protocol (5-s pulses to 0 or −160 mV, applied every 6 s; tail currents were induced by steps to −70 mV). Middle, representative current traces. Bottom, plot of peak outward hERG current as a function of time (s). D, block of D540K hERG by dofetilide (4.2 μM) induced by repetitive pulsing to 0 mV is partially recovered by repetitive pulsing to −160 mV. The same pulse protocol described in C was used here.

of WT and D540K hERG channels can be used to test whether channel closure can "trap" drugs inside the central cavity, as first described for quaternary ammonium compounds and squid K channels (Armstrong, 1971). D540K hERG channel current was blocked >85% by repetitive pulsing to 0 mV in the presence of 4.2 μM dofetilide (compare traces a and b, Fig. 2D). Unlike WT hERG, the mutant channel conducts when the membrane was hyperpolarized and current was gradually increased during the initial 5 s pulse to −160 mV (trace c, Fig. 2D). After 40 pulses to −160 mV, currents at the end of the pulse and peak tail currents were substantially larger (trace d, Fig. 2D). Recovery from block that developed during the repetitive pulses to −160 mV (in this example, 48%) was assessed with a single depolarizing pulse to 0 mV (trace e, Fig. 2D). A trace diary for this representative experiment is plotted at the bottom of Fig. 2D. The mean recovery with 40 hyperpolarizing pulses was 37.3 ± 5.8% (n = 3). These findings suggest that drug molecules trapped within the central cavity by deactivation can escape D540K (but not WT) channels that are reopened at hyperpolarized potentials.

The same pulse protocols used to estimate the rate of block onset for WT channels and recovery from block of D540K hERG channels by dofetilide was repeated using E-4031. E-4031 at 5.7 μM blocked open channels with a time constant of 3.79 ± 0.17 s (n = 3) during a 20-s depolarization to 0 mV (Fig. 3, A and B). Repetitive hyperpolarization to −160 mV did not enable recovery from block of WT hERG by E-4031 (Fig. 3C). After 30 hyperpolarizing pulses (3 min), the percentage of recovery from block of WT hERG was −0.1 ± 0.1%. In contrast, recovery from block of D540K hERG channels (Fig. 3D) was 85.3 ± 10.8% (n = 3).

The onset of block and recovery from block of hERG were also evaluated for bepridil (Fig. 4) and nifekalant (Fig. 5). These drugs were less potent than the methanesulfonanilides dofetilide or E-4031, and the rates for block onset of WT hERG and recovery from block of D540K hERG were much faster. The onset of block of WT hERG current was 3.79 ± 0.17 s (n = 3) for bepridil (Fig. 4, A and B) and 0.73 ± 0.19 s (n = 3) for nifekalant (Fig. 5, A and B). Repetitive hyperpolarizations to −160 mV did not facilitate recovery from block of WT channels by bepridil (Fig. 4C) or nifekalant (Fig. 5C), whereas recovery from block of D540K hERG at −160 mV was rapid and nearly complete at the end of a single pulse (trace c in Figs. 4D and 5D). The first pulse to 0 mV that was applied after the hyperpolarizations (trace e in Figs. 4D and 5D) elicited a current that was initially similar to the predrug value (trace b), but block redeveloped quickly. The peak current during trace e indicated a recovery from block of 104 ± 1.5% for bepridil and 88 ± 5.9% for nifekalant.

In summary, all four drugs examined in this study share common features of blocking activity. First, hERG channels must open before the drugs can block. Second, the drugs are trapped inside the central cavity when the activation gate closes. Third, escape of the trapped drug can be observed for D540K but not WT hERG channels.

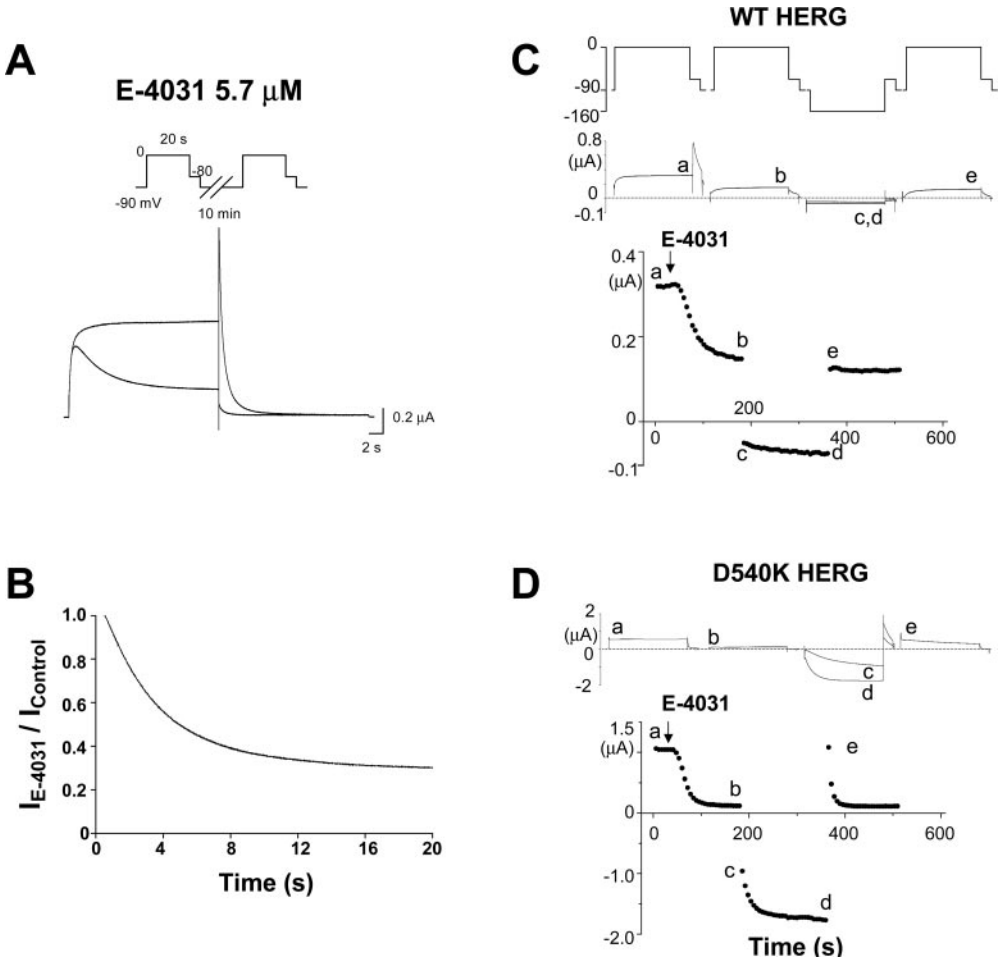
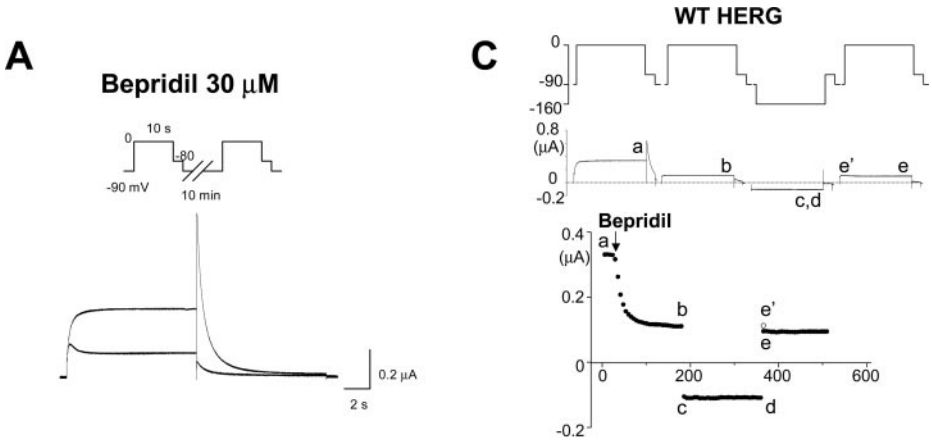


Fig. 3. Onset and recovery from block of hERG channels by E-4031. A, current traces showing the onset of open channel block by 5.7 μM E-4031 during a 20-s pulse to 0 mV after a 10-min pulse-free period of equilibration with drug. B, the onset of hERG channel bock by E-4031 expressed as relative current. Block of current developed exponentially with a time constant of 3.5 s. C, block of WT hERG by E-4031 induced by repetitive pulsing to 0 mV is not recovered by repetitive pulsing to −160 mV. Top, voltage pulse protocol (pulses applied every 6 s for 5 s to 0 or −160 mV; tail currents were induced by steps to −70 mV). Middle, representative current traces. Bottom, plot of peak outward hERG current as a function of time (s). D, block of D540K hERG by E-4031 induced by repetitive pulsing to 0 mV is recovered by repetitive pulsing to −160 mV. The same pulse protocol described in C was used here.



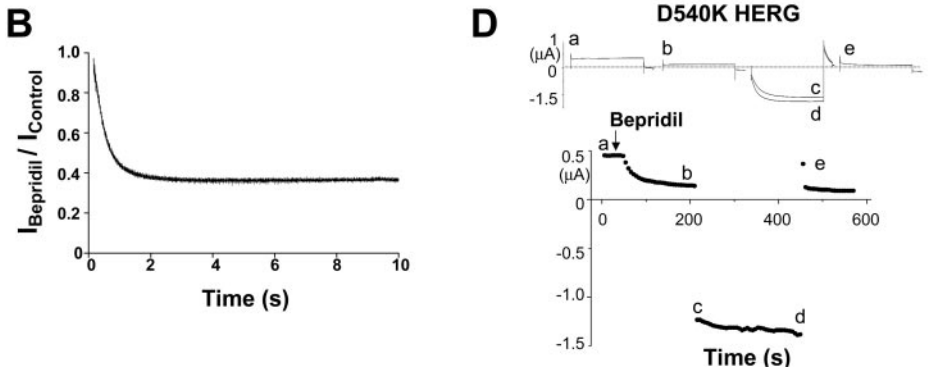
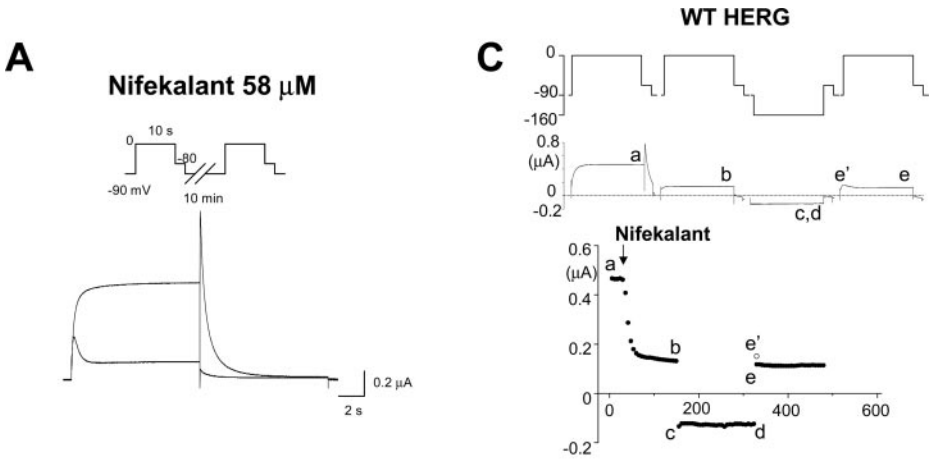


Fig. 4. Onset and recovery from block of hERG channels by 30 μM bepridil. A to D, same procedure as described for Figs. 2 and 3, except 10-s instead of 20-s depolarization to 0 mV was used in A.



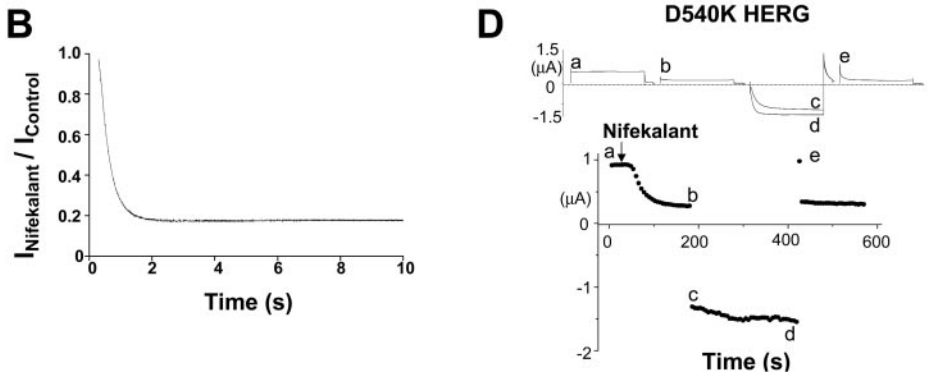


Fig. 5. Onset and recovery from block of hERG channels by 58 μM nifekalant. A to D, same procedure as described for Fig. 4.

Alanine-Scanning Mutagenesis to Define Binding Site. An alanine-scanning mutagenesis approach was used to determine residues important for block of hERG by drugs. As described previously (Mitcheson et al., 2000a), we mutated multiple residues in the Ser6 domain (Leu646-Tyr667) and the few residues near the C-terminal end of the pore helix (Leu622-Val625) that are predicted to face the central cavity based on homology with the solved crystal structure of the KcsA channel (Doyle et al., 1998). The sensitivity to block by a single concentration of each drug (10 times its IC_{50} value) was determined for each mutant channel.

Dofetilide (4.2 μM) and E-4031 (5.7 μM) caused approximately 85% inhibition of WT hERG current when measured during repetitive 5-s depolarizing steps to 0 mV and applied at a frequency of 0.166 Hz. The pattern of block of mutant channels by these two methanesulfonanilides was strikingly similar to the pattern we reported previously for MK-499 (Mitcheson et al., 2000a). Mutation (to alanine) of Thr623, Ser624, and Val625 located at the base of the pore helix or Gly648, Tyr652, Phe656, and Val659 located in the Ser6 inner helix attenuated block by dofetilide (Fig. 6A). Examples of WT and three mutant hERG channel currents recorded before and after block by dofetilide are shown in Fig. 7. S649A hERG was as sensitive to block by dofetilide as WT hERG channels, whereas S624A was less sensitive and Y652A hERG was very insensitive to dofetilide. The effect of E-4031 on mutant hERG channels (Fig. 6B) was nearly identical with the results obtained with dofetilide (Fig. 6A). Thus, the same residues of hERG were identified as important for interaction with four different methanesulfonanilides, MK-499, ibutilide, dofetilide, and E-4031.

Nifekalant exhibited a pattern of block of mutant hERG channels that was comparable with the methanesulfonanilides. However, unlike the methanesulfonanilides, nifekalant sensitivity was retained by V659A channels, whereas I655A channels were less sensitive to block (Fig. 8A). Sensitivity to block by bepridil was also altered by mutation of the three residues near the pore helix, but the only mutation in the S6 helix that altered block by bepridil was F656A (Fig. 8B).

In summary, the blocking activity of all four hERG blockers was attenuated to varying extents by mutation of any one of three residues located at the base of the pore helix (Thr623, Ser624, and Val625), but larger differences were noted for mutation of residues located in the S6 domain.

Discussion

In this study, we compared the kinetics of block and mapped the residues important for block of hERG channels by four different drugs. The goal was to determine whether structurally diverse hERG blockers interact with the same binding site for MK-499 that was proposed to be located within the central cavity of the channel (Spector et al., 1996; Mitcheson et al., 2000a). Competition among a wide spectrum of drugs for a common site on hERG has been implied by radiolabeled ligand (e.g., [^{3}H]dofetilide or [^{3}H]astemizole) binding studies (Finlayson et al., 2001; Chiu et al., 2004; Diaz et al., 2004). However, although the binding studies indicate

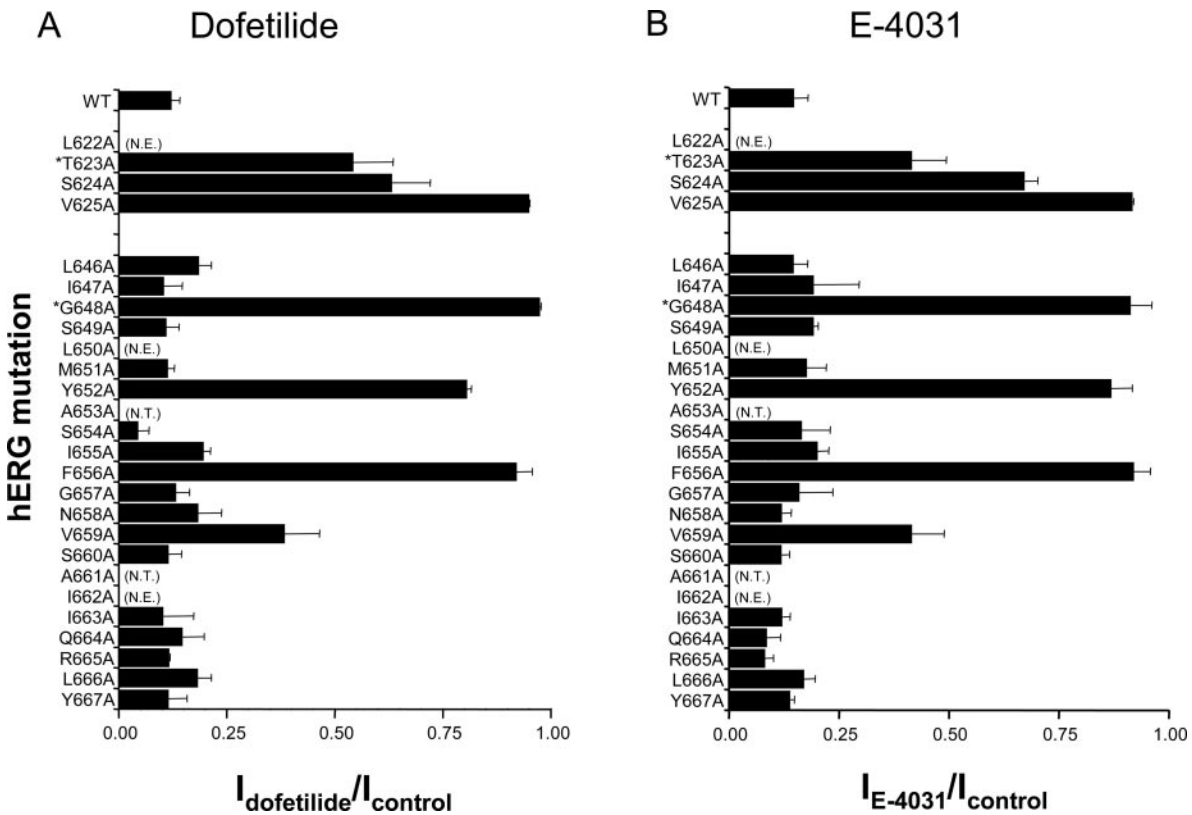


Fig. 6. Alanine-scanning mutagenesis of hERG to identify amino acid residues that modulate channel block by dofetilide and E-4031. A, normalized current ($I_{dofetilide}/I_{control}$) measured after steady-state block by 4.2 μM dofetilide (n = 4–5; error bars, ± S.E.M.). B, alanine-scanning mutagenesis of hERG to define binding sites for E-4031. Normalized current ($I_{E\text{-}4031}/I_{control}$) measured after steady-state block by 5.7 μM E-4031 (n = 4–6; error bars, ± S.E.M.). N.T., residues that were not tested; N.E., mutant channels that lacked functional expression. ∗, mutants in which recordings were made in 96 mM KMES external solutions.

that many drugs compete with dofetilide or astemizole for binding to the channel, these studies cannot determine whether these structurally diverse compounds bind to the same residues on the hERG channel.

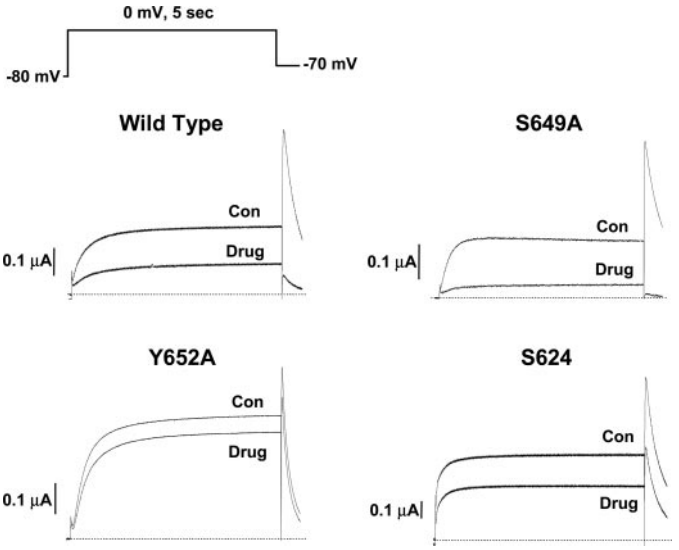


Fig. 7. Effects of dofetilide at 4.2 μM on WT and three mutant (Y649A, Y652A, and S624A) hERG channel currents.

The compounds examined in this study were found to require channel opening before block of hERG was apparent. The initial outward current recorded in response to a depolarizing pulse to 0 mV was the same before and after a 10-min incubation of oocytes with a high concentration of any of the four drugs. A progressive block developed during a 20-s depolarization to 0 mV. This finding is consistent with a preferential block of hERG channels in the open state and further suggests that these drugs do not block closed channels.

The onset of WT hERG channel block by the methanesulfonanilides (dofetilide and E-4031) was slower than the rate observed for nifekalant and bepridil at concentrations that result in nearly equivalent steady-state levels of block. This difference could be caused by a faster on-rate of binding for nifekalant and bepridil or a more limited access of the methanesulfonanilides to the binding site located in the central cavity. The rate of unblock of D540K hERG channels induced by channel openings at −160 mV was also faster for nifekalant and bepridil. It would seem that this difference is related to a faster off-rate for these compounds compared with the methanesulfonanilides.

We previously used an alanine-scanning mutagenesis approach to identify residues in the Ser6 domain and base of the pore helix of the hERG subunit that were important for

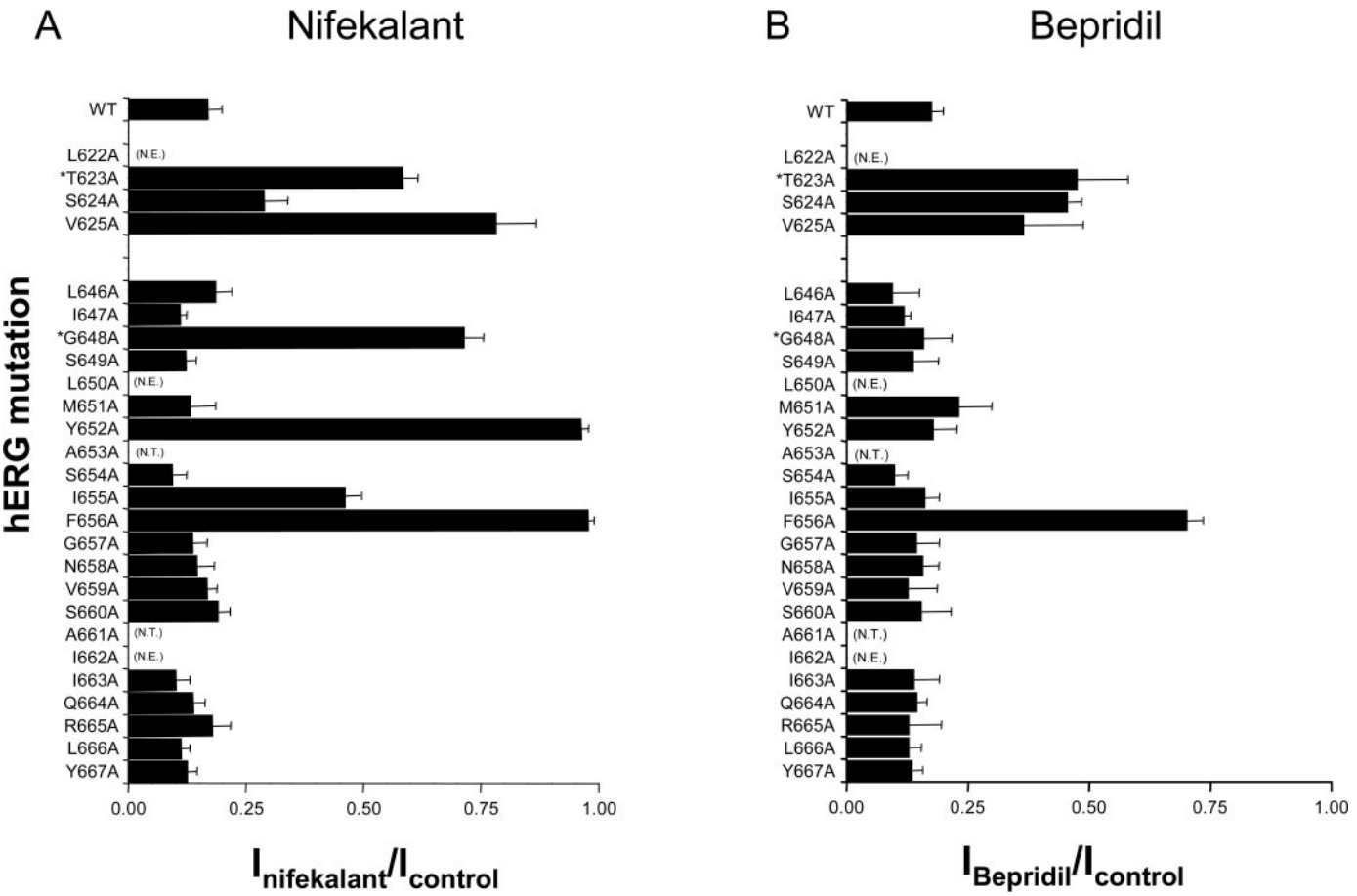


Fig. 8. Alanine-scanning mutagenesis of hERG to identify amino acid residues that modulate channel block by bepridil and nifekalant. A, normalized current ($I_{nifekalant}/I_{control}$) measured after steady-state block by 58 μM nifekalant (n = 4–6; error bars, ± S.E.M.). B, alanine-scanning mutagenesis of hERG to define binding sites for bepridil. Normalized current ($I_{bepridil}/I_{control}$) measured after steady-state block by 30 μM bepridil (n = 4–6; error bars, ± S.E.M.). N.T., residues that were not tested; N.E., mutant channels that lacked functional expression. ∗, mutants in which recordings were made in 96 mM KMES external solutions.

channel block by the class III antiarrhythmic drug MK-499 (Mitcheson et al., 2000a). In the present study, we used the same alanine mutations to determine the molecular determinants of hERG channel block by an additional four drugs. E-4031 and dofetilide are close structural analogs of MK-499. Thus, it was not surprising that, similar to previous findings with MK-499, mutation to alanine of three residues near the pore helix (Thr623, Ser624, and Val625) and four residues in Ser6 (Gly648, Tyr652, Phe656, and Val659) reduced channel sensitivity to block by dofetilide and E-4031. Nifekalant was also similar to the methanesulonanilides except that in addition, I655A channels were less sensitive, and V659A retained normal sensitivity. Finally, only a few mutations affected bepridil block of hERG. Mutation of the three residues near the pore helix (Thr623, Ser624, and Val625) and only one residue in the Ser6 domain (Phe656) reduced channel block by bepridil. All four drugs were sensitive to the pore helix residues, although the pattern of block varied. Recent studies suggest that Thr623 and Ser624 form electrostatic interactions with drugs. These are most favorable for drugs with a phenyl ring with polar or electronegative *para*- substituents (Perry et al., 2004, 2005). It is unlikely that Gly648 is a binding site for any of these drugs; however, mutation of this residue to alanine may prevent normal binding of the methanesulfonanilides and nifekalant, but not bepridil, by an allosteric effect (Mitcheson et al., 2000a). The mutation Y652A also had no discernible effect on hERG block by bepridil. We reported previously that the effect of Tyr652 mutations on hERG block is variable even within the same chemical class. For example, the IC_{50} value for block of Y652A hERG was shifted by 500-fold for chloroquine but only by 3-fold for another quinoline, quinidine (Sanchez-Chapula et al., 2002, 2003). Moreover, block of F656A and/or Y652A hERG by other drugs such as fluvoxamine and dronedarone is only slightly reduced (Milnes et al., 2003; Ridley et al., 2004b). Together, these findings indicate that only a few specific residues form the drug binding site located within the central cavity of the hERG channel and that the combination of residues that define binding are not identical for all drugs.

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