Full Paper

Molecular Determinants of hERG Channel Block by Terfenadine and Cisapride

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Abstract. Block of cardiac hERG K⁺ channels by the antihistamine terfenadine and the prokinetic agent cisapride is associated with prolonged ventricular repolarization and an increased risk of ventricular arrhythmia. Here, we used a site-directed mutagenesis approach to determine the molecular determinants of hERG block by terfenadine and cisapride. Wild-type and mutant hERG channels were heterologously expressed in *Xenopus laevis* oocytes and characterized by measuring whole cell currents with two-microelectrode voltage clamp techniques. Mutation of T623, S624, Y652, or F656 to Ala reduced channel sensitivity to block by terfenadine. The same mutations reduced sensitivity to cisapride. These data confirm our previous findings that polar residues (T623, S624) located near the base of the pore helix and aromatic residues (Y652, F656) located in the S6 domain are key molecular determinants of the hERG drug binding site. Unlike methanesulfonanilides (dofetilide, MK-499, E-4031, ibutilide) or clofilium, mutation of V625, G648, or V659 did not alter the sensitivity of hERG channels to terfenadine or cisapride. As previously proposed by molecular modeling studies (Farid R, et al. Bioorg Med Chem. 2006;14:3160–3173), our findings suggest that different drugs can adopt distinct modes of binding to the central cavity of hERG.

Keywords: hERG, long QT syndrome, oocyte, voltage clamp

Introduction

Methanesulfonanilide drugs such as dofetilide, E-4031, and MK-499 were developed as class III antiarrhythmic drugs based on their ability to prolong the duration of cardiac action potentials. These drugs are relatively specific and potent blockers of the rapid delayed rectifier K⁺ current, I_{Kr} (1), conducted by hERG channels. Inherited mutations in *HERG* cause long QT syndrome (2), a disorder of ventricular repolarization that increases the risk of lethal ventricular fibrillation. A more common cause of prolonged QT interval and ventricular arrhythmia is treatment with class III “antiarrhythmic” drugs and other noncardiac medications that block hERG channels as an unintended side effect. For example,

terfenadine is a histamine H₁-receptor blocker that was approved for clinical use in 1985 and soon became a widely used, over-the-counter drug to treat allergies. Terfenadine normally undergoes extensive first-pass metabolism in the liver to produce an active acidic metabolite. However, overdose or impaired metabolism resulted in rare cases of QT-interval prolongation, torsades de pointes ventricular arrhythmias, and sudden death (3). Only later was it discovered that terfenadine is a potent hERG blocker (IC₅₀ = 56–350 nM) (4, 5) and that its major metabolite (fexofenadine or Allegra[®]) is almost devoid of this activity (IC₅₀ = 65 μM) (6) and does not cause QT prolongation in extensive clinical trials (7). Proarrhythmic activity of terfenadine, plus the availability of alternative and safe antihistamine drugs, led to the removal from distribution and marketing of the drug by the manufacturers in 1998.

Ala-scanning mutagenesis studies have identified the molecular determinants of the binding site for several

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drugs that block hERG channels (8). Mutation of four residues located on the S6 domain (G648, Y652, F656, and V659) and three of the “K⁺-channel signature sequence” (9) residues (TSVGFG) located near the base of the pore helix (T623, S624, and V625) reduced block of hERG channels by the methanesulfonanilide class III drugs (MK-499, dofetilide, and E-4031). Homology modeling predicted that all these residues faced the central cavity of the channel, consistent with earlier findings that hERG channels were preferentially blocked in the open state (10). More limited mutagenesis studies have shown that F656 is a critical residue in the binding of dofetilide and quinidine (11) and confirmed the importance of Y652 and F656 for chloroquine (12), quinidine (13), halofantrine (14), terfenadine and cisapride (15), lidoflazine (16), clofilium and ibutilide (17), and cocaine (18).

Terfenadine is a potent open-channel blocker of hERG. Block of current develops slowly in a pulse-dependent, but frequency-independent manner owing to ultra-slow recovery from block in the closed state (19). Slow recovery from block appears to be caused by a trapping of drug inside the central cavity behind the activation gate that closes when the channel deactivates (10, 20). Recovery from block by terfenadine can be strikingly accelerated when hERG channels contain a specific mutation (D540K) that allows the activation gate to reopen in response to membrane hyperpolarization (19). These characteristics of block are very similar to those of several other well studied and potent blockers of hERG, including MK-499, dofetilide, E-4031, and bepridil (19, 21). In contrast to these drugs, frequency-dependent block of hERG channels by cisapride develops rapidly and recovery from block is relatively fast (19). Regardless of the kinetics for onset and recovery from block, all the drugs discussed above have been shown to interact with residues that line the central cavity of hERG that are only accessible after the channel has opened (8, 10, 19, 21).

In this study, we extended our previous Ala-scanning mutagenesis approach to compare the pore residues critical for interaction with hERG by terfenadine and cisapride, drugs that differ dramatically in their rates of onset and recovery from channel block.

Materials and Methods

Molecular biology

Wild-type (WT) *HERG* subcloned into the pSP64 vector was prepared as described (14). Site-directed mutagenesis was performed using the megaprimer method (22) and mutation constructs were confirmed by restriction enzyme and DNA sequence analyses. As

described previously (8), we mutated multiple residues in the S6 domain (L646 – Y667) and the few residues near the C-terminal end of the pore helix (L622 – V625) that are predicted to face the central cavity. Complementary RNAs (cRNAs) for injection into oocytes were prepared with SP6 Cap-Scribe (Roche Applied Science, Indianapolis, IN, USA) following linearization of the expression construct with *EcoRI*.

Xenopus oocytes

Oocytes were isolated by dissection from anesthetized adult *Xenopus laevis*. Frogs were anesthetized by immersion in 0.2% tricaine (Sigma Chemical Co., St. Louis, MO, USA) for 10–15 min. A small abdominal incision was made and ovarian lobes containing oocytes were removed. The incision was sutured closed and the frog returned to its aquarium for a recovery period of at least one 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, USA) to remove follicle cells. Maintenance and cRNA injections into oocytes were performed as previously described (23).

Two-microelectrode voltage clamp

Ionic currents in oocytes were recorded by using standard two microelectrode voltage-clamp techniques (23) and a GeneClamp 500 amplifier (Axon Instruments, Burlingame, CA, USA). Oocytes were placed in a small (10 mL) Plexiglas chamber that was continuously superfused at a rate of 2 ml/min with external bath solution. Recording electrodes were fabricated from 1.0-mm o.d. borosilicate glass tubing. When filled with 3 M KCl, recording electrodes had a resistance of 0.8–1.0 Mohms. Experiments were performed at room temperature (22°C–24°C).

hERG channel currents were recorded during repetitive 5-s depolarizing steps to 0 mV, applied at a frequency of 0.166 Hz. The holding potential was –90 mV and tail currents were measured at –80 mV. After an initial period of “run-up”, currents reached a stable level and the bathing solution was switched from control to one containing a concentration of drug equal to 10× the IC₅₀ value determined for WT channels. Block of hERG currents at the end of the 5-s pulse is expressed as a fractional value ($I_{\text{drug}}/I_{\text{control}}$).

Data analysis

Digitized data were analyzed off-line by using pCLAMP (Molecular Devices, Sunnyvale, CA, USA) and ORIGIN (OriginLab Corp. Northampton, MA,

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

Drugs and solutions

The normal external bath solution ("2 K") contained 96 mM NaMes (2-[*N*-morpholino]ethanesulfonic acid), 2 mM KMes, 2 mM CaMes₂, 5 mM Hepes, and 1 mM MgCl₂, adjusted to pH 7.6 with methane sulfonic acid. For G648A hERG channels that expressed poorly and were highly inactivated, KMes was increased to 96 mM with a similar reduction in NaMes ("96 K"). Terfenadine was purchased from Sigma. Cisapride was purchased from Research Diagnostics, Inc. (Flanders, NJ, USA). Both drugs were prepared in 10 mM methanol stock solution. Final drug concentrations were prepared daily by dilution of stock solutions kept at -20°C .

Results

Terfenadine

The IC₅₀ for block of WT hERG channel current by terfenadine was 350 nM in oocytes bathed in a solution containing an external K⁺ concentration ([K⁺]_e) of 2 mM. Elevated [K⁺]_e is known to reduce the sensitivity of WT hERG to drug block (8); and accordingly, we found that the IC₅₀ for terfenadine was increased to 2.8 μM when using a solution containing an [K⁺]_e of 96 mM (Fig. 1). WT and most mutant channels were screened with 3.5 μM terfenadine, a concentration equal to 10 times the IC₅₀ value measured in 2 K solution. Examples of WT and three mutant hERG channel currents recorded before and after block by 3.5 μM terfenadine are shown in Fig. 2. At this concentration, terfenadine reduced WT hERG current measured at the end of the 5-s pulse by approximately 80%. Tail currents were reduced by a similar amount. The sensitivity of S660A hERG channels was similar to WT, whereas S624A and Y652A channels were only reduced by ~50% and 35%, respectively (Fig. 2). These data suggest that S624 and Y652 interact with terfenadine, whereas S660 does not.

F656A, V625A, and T623A hERG channels express poorly in oocytes. Therefore, to enhance current magnitude, we measured tail currents of these mutant channels at -140 mV. F656A and T623A, but not V625A, greatly reduces the sensitivity of hERG to terfenadine (Fig. 3). G648A hERG channels express poorly in oocytes and exhibit enhanced C-type inactivation. To diminish inactivation and enhance current magnitude of G648A hERG channels, oocytes were bathed in a solution containing an [K⁺]_e of 96 mM.

Fig. 1. Concentration-response relationships for terfenadine block of hERG current in oocytes bathed in 2 K or 96 K solution. For 96 K, the IC₅₀ was $2.8 \pm 0.3 \mu\text{M}$ and the Hill coefficient was 0.94 ($n = 4$). For 2 K, the IC₅₀ was $0.35 \pm 0.10 \mu\text{M}$ and the Hill coefficient was 0.89 ($n = 4$).

Fig. 2. Effects of terfenadine on ionic currents measured in *Xenopus* oocytes expressing WT or mutant hERG channels. Superimposed current traces were recorded during a single pulse to 0 mV under control conditions and again after block by 3.5 μM terfenadine reached a steady-state level. Sensitivity of S660A hERG channel current to terfenadine was similar to WT hERG, whereas S624A and Y652A hERG channels were less sensitive to the drug.

Under these conditions, 28 μM terfenadine ($10 \times \text{IC}_{50}$ concentration) reduced tail currents by approximately 80% (Fig. 3).

To fully characterize the putative binding site for terfenadine, we performed an Ala-scan of the rest of the pore helix base and the portion of the S6 domain that forms the central cavity of the hERG channel. The results of the Ala-scanning mutagenesis are summarized in Fig. 4. In confirmation of our previous, but limited study of mutant channels (8), Y652 and F656 are important determinants of hERG-channel block by

Fig. 3. Effect of terfenadine on V625A, T623A, F656A, and G648A hERG channels. Each panel shows pulse protocol and associated currents recorded before and after exposure to the indicated drug concentration. An expanded view of the tail current traces (outlined by the boxed region) is shown. The $[K^+]_o$ (in mM) of the bathing solution (2 K or 96 K) is also indicated.

Fig. 4. Ala-scanning mutagenesis of hERG identifies amino acid residues that modulate channel block by terfenadine. Normalized current ($I_{\text{terfenadine}}/I_{\text{control}}$) measured after steady-state block by terfenadine at a concentration of $3.5 \mu\text{M}$ for all mutant channels except for G648A (where oocytes were bathed in 96 K solution and terfenadine was increased to $28 \mu\text{M}$). The number of oocytes tested for each mutant channel is indicated in parentheses. Error bars are $\pm$ S.E.M.; N.T., residues that were not tested; N.E., mutant channels that lacked functional expression. * $P < 0.05$.

terfenadine, whereas G648 and V625 are not important. The new findings are that mutation of T623 and S624 located at the base of the pore helix attenuated block by terfenadine and that of the 18 S6 mutant channels examined, only Y652A and F656A significantly attenuated block. Thus, unlike some of the other potent hERG channel blockers (8, 17, 21), terfenadine block is not affected by mutation of V625, G648, or V659.

Cisapride

The IC_{50} for block of WT hERG channel tail current by cisapride was 630 nM in oocytes bathed in 2 K

solution and $1.1 \mu\text{M}$ when bathed in 96 K solution (Fig. 5). We examined the response to cisapride for a limited set of mutant hERG channels, specifically single Ala substitutions of the 7 residues proposed to be essential for interaction with MK-499 (8), E-4031, and dofetilide (21). WT and mutant channels were screened with a cisapride equal to 10 times its IC_{50} value. Cisapride at $6.3 \mu\text{M}$ reduced WT hERG channel currents by 80%, but only reduced S624A current by approximately 50% and Y652A current by approximately 15% (Fig. 6). V625A and G648A hERG channels retained normal sensitivity to cisapride, whereas T623A and

Fig. 5. Concentration–response relationships for cisapride block of hERG current in oocytes bathed in 2 K or 96 K solution. For 2 K, the IC_{50} was $0.63 \pm 0.1 \mu M$ and the Hill coefficient was 1.13 ($n = 3 - 5$). For 96 K, the IC_{50} was $1.1 \pm 0.2 \mu M$ and the Hill coefficient was 0.61 ($n = 4$).

Fig. 6. Effects of cisapride on ionic currents measured in *Xenopus* oocytes expressing wild type or mutant hERG channels. Superimposed current traces were recorded during a single pulse to 0 mV under control conditions and again after block by $3.5 \mu M$ cisapride had reached a steady-state level. Sensitivity of V659A hERG channel current to cisapride was similar to WT hERG, whereas S624A and Y652A hERG channels were less sensitive to the drug.

F656A hERG channels exhibited reduced block compared to WT hERG (Fig. 7). A summary of the limited Ala-scan is presented in Fig. 8. Similar to the results with terfenadine, T623, S624, Y652, and F656 are important determinants for block, whereas mutation of V625, G648, or V659 did not alter channel sensitivity to cisapride.

Discussion

Binding studies of radiolabeled dofetilide or astemizole binding studies (24–26) suggest that many structurally diverse drugs compete for a common binding site on the hERG channel. An alanine scanning mutagenesis approach has been used to map the putative binding site of these drugs to specific residues that form the central cavity (inner pore) of the channel. Specifically, site-directed mutagenesis was used to identify residues in the S6 domain and base of the pore helix of the hERG subunit that were important for channel block by the methanesulfonanilide class III antiarrhythmic drugs MK-499 (8), E4031 and dofetilide (21). For these structurally related drugs, mutation of three residues near the pore helix (T623, S624, V625) and four residues in S6 (G648, Y652, F656, and V659) reduced channel sensitivity to block. Not all hERG blocking drugs interact with these residues. For example, bepridil appears not to interact with G648, Y652, or V659 (21) and mutation of F656 and/or Y652 only slightly reduced hERG block by fluvoxamine and dronedarone (27, 28). Thus, although a wide variety of drugs bind to a site located within the central cavity of the channel, the interacting residues differ between drugs.

In this study we mapped the residues important for block of hERG channels by terfenadine and cisapride. For both drugs, only 4 residues were identified as critical for block: T623 and S624 located at the base of the pore helix and Y652 and F656 located in the S6 domain. Unlike methanesulfonanilides, block of hERG by terfenadine or cisapride was not affected by mutation of the K^+ signature sequence residue V625 or the S6 residues G648 and V659. These findings are consistent with the predicted docking of terfenadine to a homology model of hERG based on the bacterial channel KvAP (29). Based on an induced fit model, Farid and colleagues proposed that the piperidine N and the two hydroxyl groups of terfenadine are located nearest the propeller-shaped hydrophilic space formed by the intracellular base of the pore helix and selectivity filter. Hydrogen bonding to the side chains of S649 and S624 is predicted at this interface. A similar docking was favored by cisapride. Our findings are consistent with the importance of S624, but not interaction with S649. The model and our data also agree on the lack of importance of V625 for both drugs. The docking model (29) also predicts two T-shaped π - π interactions with two Y652 and two F656 residues and two additional hydrophobic interactions with Y652 in the homotetrameric hERG channel. These predictions are consistent with our finding that mutation of these two aromatic residues caused the greatest decrease in

Fig. 7. Effects of cisapride on V625A, T623A, F656A, and G648A hERG channels. Each panel shows pulse protocol and associated currents recorded before and after exposure to the indicated drug concentration. An expanded view of the tail current traces (outlined by the boxed region) is shown. The $[K^+]_e$ (in mM) of the bathing solution (2 K or 96 K) is also indicated.

Fig. 8. Summary of effects of cisapride on mutant hERG channels. Normalized current ($I_{cisapride}/I_{control}$) measured after steady-state block by cisapride at a concentration of $6.3 \mu M$ in 2 K solution (WT, S624A, V625A, Y652A, F656A, V659A) or $11 \mu M$ and 96 K solution (G648A). The number of oocytes tested is indicated in parentheses. Error bars are $\pm$ S.E.M., * $P < 0.05$.

channel sensitivity to terfenadine and cisapride and with our previous finding that an aromatic residue is essential at position 652 in the S6 domain (15).

Elevated $[K^+]_e$ shifts the voltage dependence of fast inactivation to more positive potentials, essentially reducing the extent of channel inactivation (30). Most hERG-channel blockers preferentially block the inactivated state of the channel (30, 31). Thus, reduced inactivation results in decreased drug sensitivity. We found that the $[K^+]_e$ -dependent shift in the concentration response curve was greater for terfenadine (8-fold) compared to cisapride (1.7-fold). This suggests that the preference for binding to the inactivation state is greater for terfenadine than cisapride. A disconnect between hERG inactivation and drug sensitivity has also been reported for other drugs, including disopyramide (32), propafenone (33), and halofantrine (34).

A recent study by Stork et al. (19) compared the rates of onset and recovery from hERG block by several drugs. Of particular interest was the finding that cisapride ($3 \mu M$) exhibited a fast, frequency-dependent onset of block. Recovery from block by cisapride was complete in approximately 2 min. In contrast, the onset of block by $1 \mu M$ terfenadine was very slow and frequency-independent, and recovery from block was extremely slow (>30 min without pulsing and approximately 15 min when cells were pulsed at 1 Hz). The kinetics of block and unblock by terfenadine were nearly the same as that observed for the methanesulfonanilide E-4031. As noted by Stork et al. (19), the differences in blocking kinetics cannot be predicted based on simple measures of physicochemical properties of the drugs. Our results also indicate that the variable kinetics cannot be explained by differences in the putative binding site because we found that the same residues were crucial for sensitivity to block by both drugs. Thus, the mechanism for slow recovery from channel block by terfenadine relative to the rapid recovery for cisapride remains to be determined.

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