C-Linker Accounts for Differential Sensitivity of ERG1 and ERG2 K⁺ Channels to RPR260243-Induced Slow Deactivation

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Received February 12, 2015; accepted April 17, 2015

ABSTRACT

Compounds can activate human ether-à-go-go–related gene 1 (hERG1) channels by several different mechanisms, including a slowing of deactivation, an increase in single channel open probability, or a reduction in C-type inactivation. The first hERG1 activator to be discovered, RPR260243 ((3R,4R)-4-[3-(6-methoxyquinolin-4-yl)-3-oxo-propyl]-1-[3-(2,3,5-trifluorophenyl)-prop-2-ynyl]-piperidine-3-carboxylic acid) (RPR) induces a pronounced, voltage-dependent slowing of hERG1 deactivation. The putative binding site for RPR, previously mapped to a hydrophobic pocket located between two adjacent subunits, is fully conserved in the closely related rat ether-à-go-go–related gene 2 (rERG2), yet these channels are relatively insensitive to RPR. Here, we use site-directed mutagenesis and heterologous expression of channels in Xenopus oocytes to characterize the structural basis for the differential sensitivity of hERG1 and rERG2 channels to RPR. Analysis of hERG1–rERG2 chimeric channels indicated that the structural determinant of channel sensitivity to RPR was located within the cytoplasmic C-terminus. Analysis of a panel of mutant hERG1 and rERG2 channels further revealed that seven residues, five in the C-linker and two in the adjacent region of the cyclic nucleotide-binding homology domain, can fully account for the differential sensitivity of hERG1 and rERG2 channels to RPR. These findings provide further evidence that the C-linker is a key structural component of slow deactivation in ether-à-go-go–related gene channels.

Introduction

The rapid delayed rectifier K⁺ current (Iₖ,ᵣ) contributes to the repolarization phase of cardiomyocyte action potentials (Sanguinetti and Jurkiewicz, 1990, 1991). In the human heart, human ether-à-go-go–related gene 1 (hERG1) (Kv11.1) channels conduct Iₖ,ᵣ (Sanguinetti et al., 1995; Trudeau et al., 1995) and loss of function mutations in the hERG1 gene KCNH2 are a common cause of long QT syndrome (Curran et al., 1995), a disorder of ventricular repolarization that is associated with life-threatening arrhythmia (Jervell and Lange-Nielsen, 1957; Schwartz et al., 1991).

In response to repolarization of the cell membrane, ether-à-go-go–related gene (ERG) channels close (deactivate) slowly compared with the closely related ether-à-go-go (eag) channels and many other voltage-gated K⁺ channels. The structural basis of slow deactivation has been intensely investigated and together many studies indicate that it involves a cytoplasmic interaction between the amino- and carboxyl-termini of adjacent subunits within the tetrameric channel (Gustina and Trudeau, 2011; Gianulis et al., 2013; Li et al., 2014; Ng et al., 2014). The structures of amino- and carboxyl-termini of the ERG channel subunit have been solved (Morais Cabral et al., 1998; Adaixo et al., 2013; Brelidze et al., 2013). The N-terminus includes a Per-Arnt-Sim (PAS) domain that together are called the eag domain. The carboxyl-terminus contains a cyclic nucleotide-binding homology domain (CNBHD) that is coupled to the S6 α-helical transmembrane segment by the C-linker, a 77 amino acid structure containing four α-helices. Truncation of all (Schonherr and Heinemann, 1996) or a portion (Morais Cabral et al., 1998) of the eag domain accelerates hERG1 channel deactivation, as do specific point mutations in the PAS domain (Chen et al., 1999), PAS cap (Muskett et al., 2011), or CNBHD (Al-Owais et al., 2009).

Early in the drug discovery process, compounds are routinely screened for their propensity to block hERG1 channels, an undesirable side effect that can induce cardiac arrhythmia in susceptible individuals (Fenichel et al., 2004). Extensive screening fortuitously led to the discovery of several structurally diverse compounds that activate rather than block hERG1 channels. The first hERG1 channel activator, RPR260243 ((3R,4R)-4-[3-(6-methoxyquinolin-4-yl)-3-oxo-propyl]-1-[3-(2,3,5-trifluorophenyl)-prop-2-ynyl]-piperidine-3-carboxylic acid) (RPR) was discovered 10 years ago (Kang et al., 2005). The primary effect of RPR is a pronounced, voltage-dependent slowing of deactivation (Sanguinetti and Jurkiewicz, 1990, 1991). The putative binding site for RPR, previously mapped to a hydrophobic pocket located between two adjacent subunits, is fully conserved in the closely related rat ether-à-go-go–related gene 2 (rERG2), yet these channels are relatively insensitive to RPR. Here, we use site-directed mutagenesis and heterologous expression of channels in Xenopus oocytes to characterize the structural basis for the differential sensitivity of hERG1 and rERG2 channels to RPR. Analysis of hERG1–rERG2 chimeric channels indicated that the structural determinant of channel sensitivity to RPR was located within the cytoplasmic C-terminus. Analysis of a panel of mutant hERG1 and rERG2 channels further revealed that seven residues, five in the C-linker and two in the adjacent region of the cyclic nucleotide-binding homology domain, can fully account for the differential sensitivity of hERG1 and rERG2 channels to RPR. These findings provide further evidence that the C-linker is a key structural component of slow deactivation in ether-à-go-go–related gene channels.
voltage-dependent slowing of hERG1 deactivation (Kang et al., 2005; Perry et al., 2007), leading to a persistent outward current during cardiac repolarization that shortens the action potential duration. RPR has little or no effect on the voltage dependence of channel activation (Kang et al., 2005), and the modest increase in the amplitude of outward currents induced by RPR results from a small positive shift in the voltage dependence of channel inactivation (Perry et al., 2007). The effects of RPR on hERG1 channel gating have physiologic consequences in the heart. In isolated guinea pig ventricular myocytes, 10 μM RPR alone had little effect on the action potential shape, but reversed action potential prolongation caused by prior treatment of myocytes with dofetilide, a potent hERG1 channel blocker (Kang et al., 2005). In Langendorff-perfused guinea pig hearts, 5 μM RPR increased T-wave amplitude and shortened the QT interval (Kang et al., 2005). Thus, the slow deactivation of hERG1 channels induced by RPR accelerates the rate of cardiac repolarization.

Site-directed mutagenesis and electrophysiological analysis of mutant channels heterologously expressed in Xenopus oocytes were used to identify the amino acid residues in hERG1 that form the putative binding site for RPR. This approach identified several residues in the S5 segment (Val549, Leu550, Leu553, or Phe557) and S6 segment (Asn658, Val659, Ile662, Leu666, or Tyr667) of the hERG1 subunit as key determinants of RPR activity. Mutation of any one of these residues attenuated or eliminated the effects of RPR on channel deactivation. Together, the side chains of these nine residues form a binding pocket for RPR that is located between two adjacent hERG1 subunits. Due to the 4-fold symmetry of voltage-gated K⁺ channels, a homotetrameric hERG1 channel should contain four identical RPR binding sites. Characterization of concatenated hERG1 tetramers containing a defined number and positioning of L533A mutant subunits that prevent RPR activity (Perry et al., 2007) revealed that occupancy of all four binding sites was required to achieve the maximal slowing of deactivation by RPR (Wu et al., 2015).

The key residues of the putative RPR binding site defined for hERG1 are conserved in human and rat ether-à-go-go–related gene type 3 (rERG3) channels. However, whereas RPR reduces the current magnitude at high concentrations, it has no effect on deactivation of human (Kang et al., 2005) or rat (Perry and Sanguinetti, 2008) ERG3 channels. Two residues in the S5 segment of hERG1 (Phe551 and Thr556) that are located within the putative RPR binding pocket, although not identified as interacting with RPR based on mutational analysis, are not conserved in rERG3. Phe551 and Thr556 in hERG1 are replaced by Met553 and Ile558 in rERG3. We previously reported that a Thr in the second position (as in hERG1) was required for RPR to activate channels, whereas an Ile in this position (as in rERG3) eliminated agonist activity in either channel type and revealed a secondary inhibitory effect of RPR (Perry and Sanguinetti, 2008).

In the present study, we further investigate the mechanistic basis of channel-selective pharmacology of RPR by comparing its effects on hERG1 and rat ether-à-go-go–related gene type 2 (rERG2). Similar to rERG3, rERG2 channels are nearly insensitive to high concentrations of RPR; however, unlike rERG3, the amino acid sequence of the putative RPR binding site of rERG2 is identical to hERG1. Thus, the structural basis for rERG2 insensitivity to RPR must be accounted for by a region of the channel outside of the putative ligand-binding pocket. In this study, we determined the effects of RPR on deactivation of hERG1-rERG2 chimeric channels and the cumulative effect of multiple point mutations on both hERG1 and rERG2 homotetramers to identify the specific residues that account for the differential RPR sensitivity of hERG1 and rERG2 channels.

Materials and Methods

Site-Directed Mutagenesis. cDNAs for rat kcnh6 (rERG2, NCBI Reference Sequence NP_446389), kindly provided by David McKinnon, and human KCNH2 (hERG1, isoform 1a, NCBI Reference Sequence NM_000238) were cloned into the pSP64 oocyte expression vector. Single or multiple point mutations in KCNH2 or kcnh6 were introduced into wild-type (WT) channels using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA).

Construction of hERG1/rERG2 Chimeras. Four hERG1/rERG2 chimeras channels were constructed by swapping between homologous regions of KCNH2 and kcnh6 cDNAs. The rERG2/N-S4/hERG1 chimera was composed of the N-terminus to the end of the S4 segment of rERG2 linked to the S4/S5 linker to the C-terminus of hERG1. A HindIII site was inserted into the multiple cloning site of both hERG1 and rERG2, and an AscI restriction site was inserted into hERG1 at Thr526-Leu529 (bp 1578–1585). rERG2 had an existing AscI site at Thr577-Leu580 (bp 1131–1138). The N-terminus to the S4 segment of hERG1 was excised and replaced by the homologous region of rERG2 using Ascl and BglII enzymatic cuts. The Ascl sites are described above, located on the 5’ end of the chimera swap. A pre-existing BglII site in hERG1 at Lys638-Phe640 (bp 1913–1918) was located on the 3’ end of the swap. A BglII site was introduced into rERG2 at Lys490-Phe492 (bp 1469–1474) and resulted in a V491I mutation in rERG2 (matching the residue in hERG1). The resulting chimera had Met1-Thr526 from rERG2 and Ala527-Lys639 from hERG1, and Ile640-Ser1160 from hERG1.

The hERG1/rERG2/S6-Cterm chimera was constructed by swapping the S6 segment to the C-terminus of hERG1 with the homologous region of rERG2. A BglII site was introduced into rERG2 as described above. An existing BglII site in hERG1 (bp 1913–1918) was located at the beginning of the S6. The S6 to C-terminus of rERG2 was excised from the pSP64 vector using BglII and EcoRI (located in the vector region, bp 2905–2910; rERG2 stop codon at bp 2851–2853), and inserted into hERG1 using BglII and EcoRI (also located in the pSP64 vector region, bp 3532–3537; hERG1 stop codon at 3478–3480) to remove the hERG1 S6 to the C-terminus. The resulting chimera had Met1-Lys639 from hERG1 and Ile639-Ser1160 from rERG2.

The hERG1/rERG2/S45L-PL chimera was constructed by swapping the S4/S5 linker (S45L) to the pore linker (PL) of hERG1 with the homologous region of rERG2 using Ascl and BglII enzymatic cuts. The Ascl sites are described above, located on the 5’ end of the chimera swap. A pre-existing BglII site in hERG1 at Lys638-Phe640 (bp 1913–1918) was located on the 3’ end of the swap. A BglII site was introduced into rERG2 at Lys490-Phe492 (bp 1469–1474) and resulted in a V491I mutation in rERG2 (matching the residue in hERG1). The resulting chimera had Met1-Thr526 of hERG1, Ala527-Lys639 from rERG2, and Ile640-Ser1160 from hERG1.

The hERG1/rERG2/S6-Cterm chimera was constructed by swapping the S6 segment to the C-terminus of hERG1 with the homologous region of rERG2. A BglII site was introduced into rERG2 as described above. An existing BglII site in hERG1 (bp 1913–1918) was located at the beginning of the S6. The S6 to C-terminus of rERG2 was excised from the pSP64 vector using BglII and EcoRI (located in the vector region, bp 2905–2910; rERG2 stop codon at bp 2851–2853), and inserted into hERG1 using BglII and EcoRI (also located in the pSP64 vector region, bp 3532–3537; hERG1 stop codon at 3478–3480) to remove the hERG1 S6 to the C-terminus. The resulting chimera had Met1-Lys639 from hERG1 and Ile639-Ser1160 from rERG2.

The hERG1/rERG2/CNBHD(pseu-Cterm) chimera was constructed by swapping a portion of CNBHBD to the C-terminus of hERG1 with the homologous region of rERG2. A BglII site was introduced into rERG2 as described above. An existing BglII site in hERG1 (bp 1913–1918) was located at the beginning of the S6. The S6 to C-terminus of rERG2 was excised from the pSP64 vector using BglII and EcoRI (located in the vector region, bp 2905–2910; rERG2 stop codon at bp 2851–2853), and inserted into hERG1 using BglII and EcoRI (also located in the pSP64 vector region, bp 3532–3537; hERG1 stop codon at 3478–3480) to remove the hERG1 S6 to the C-terminus. The resulting chimera had Met1-Lys639 from hERG1 and Ile639-Ser1160 from rERG2.

All mutant constructs were verified by DNA sequence analysis. To prepare complementary RNA (cRNA) for use in oocyte expression studies, pSP64 plasmids were linearized with EcoRI prior to in vitro transcription using the mMessage mMachine SP6 kit (Ambion, Austin, TX).

Isolation, cRNA Injection, and Voltage Clamp of Xenopus Oocytes. Procedures used for the surgical removal of ovarian lobes from Xenopus laevis and isolation of oocytes was approved by the
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University of Utah Institutional Animal Care and Use Committee and performed as described previously (Abbruzzese et al., 2010). Single oocytes were injected with 5–40 ng cRNA encoding WT or mutant ERG subunits and studied 2–7 days later. Ionic currents in oocytes were recorded using standard two-electrode voltage-clamp techniques (Goldin, 1991; Stühmer, 1992) and agarose-cushion microelectrodes (Schreibmayer et al., 1994), with tip resistances that ranged from 0.6 to 1.9 MΩ when back-filled with a 3 M KCl solution. A GeneClamp 500 amplifier, Digitida 1322A data acquisition system, and pCLAMP 8.2 software (Molecular Devices, Inc., Sunnyvale, CA) were used to produce command voltages and record current and voltage signals.

Voltage Pulse Protocols and Data Analysis. ERG channel deactivation was quantified by comparing the integral of leak-subtracted tail currents (\(I_{\text{tail}}\)) before and after treatment of an oocyte with a variable concentration of RPR. Channel currents were initially activated with a 1-second pulse to +40 mV, and tail currents were elicited by 9-second pulses to a return potential (\(V_{\text{ret}}\)) that was applied in 10-mV increments from -60 to -110 mV. The holding potential was either -80 or -90 mV. A 9-second pulse was long enough for channels to completely deactivate for each \(V_{\text{ret}}\) under control conditions. However, deactivation was often incomplete after 9 seconds in the presence of high concentrations of RPR, especially when \(V_{\text{ret}}\) was more positive than -90 mV. An analysis of digitized data, including fitting of \(I_{\text{tail}}\) kinetics and determination of \(I_{\text{tail}}\), were performed with pCLAMP 8.2 software. For some experiments, \(I_{\text{tail}}\) was fitted with a two-exponential function

\[
I_{\text{tail}}(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} + C
\]

where \(A_1\) and \(\tau_1\) are the amplitude and time constant for the fast component, \(A_2\) and \(\tau_2\) are the amplitude and time constant for the slow time component of \(I_{\text{tail}}\) deactivation, and \(C\) is the maintained current component. The relative amplitude of the fast component of \(I_{\text{tail}}\) was defined as \(A_1/(A_1+A_2)\).

ORIGIN 8.5 software (OriginLab, Northhampton, MA) was used to construct graphs and analyze concentration-effect relationships for RPR. The fold change in \(I_{\text{tail}}\) was plotted as a function of [RPR], and the relationship was fitted with the logistic equation

\[
f_c = f_{\text{max}} + (f_{\text{min}} - f_{\text{max}})/(1 + [\text{RPR}]/EC_{50})^H
\]

where \(f_c\) is the fold change in \(I_{\text{tail}}\), \(EC_{50}\) is the concentration of RPR that produced a half-maximal effect, and \(H\) is the Hill coefficient. Plots of fold change in \(I_{\text{tail}}\) versus time constants of deactivation at -70 mV were analyzed by linear regression, and the adjusted \(R^2\) was used to estimate the degree of relationship between the two variables. All data in the text and figures are presented as mean ± S.E.M. (\(n = \)

Solutions and Drugs. The extracellular solution used for voltage-clamp experiments contained (in millimolar): 96 NaCl, 2 KCl, 1 CaCl₂, 1 MgCl₂, and 5 HEPES, and the pH was adjusted to 7.6 with NaOH. RPR 269243 (ChemShuttle, Wuxi, China) was dissolved in dimethylsulfoxide to make a 10 mM stock solution, and final drug concentrations (3–50 \(\mu\)M) were obtained by dilution of the stock solution with the extracellular solution.

Results

RPR Has Differential Effects on hERG1 and rERG2 Channels. The effects of RPR on hERG1 channel currents have been characterized in detail (Kang et al., 2005; Perry et al., 2007; Wu et al., 2015). For this study, we did not analyze the effects of RPR on the kinetics of activation or the voltage dependence of activation or inactivation. Instead, we focused on how RPR dramatically slows the rate of deactivation of hERG1 channels but has very little effect on the deactivation of closely related rERG2 channels. Deactivation of ERG channel currents in the absence of RPR is best described as a two exponential process; however, slow deactivation induced by RPR is characterized by complex kinetics that are not amenable to analysis by exponential fitting. Therefore, to simplify our analysis of the effects of RPR on deactivation, we measured the integral of tail currents (\(I_{\text{tail}}\)) over a limited voltage range (-60 to -110 mV) and duration (9 seconds) before and after treatment of an oocyte with a solution containing RPR at a concentration ranging from 3 to 50 \(\mu\)M. At 10 \(\mu\)M, RPR slowed the rate of deactivation of hERG1 channels measured as outward \(I_{\text{tail}}\) in response to repolarization of the membrane potential to -60, -70, -80, and -90 mV or inward \(I_{\text{tail}}\) in response to a \(V_{\text{ret}}\) of -100 or -110 mV (Fig. 1, A and B). \(I_{\text{tail}}\) was integrated (Fig. 1, C and D), and the value of \(I_{\text{tail}}\) at 9 seconds was normalized to that measured at -60 mV under control conditions and plotted as a function of \(V_{\text{ret}}\) (Fig. 1E). At -60 mV, 10 \(\mu\)M RPR increased \(I_{\text{tail}}\) of hERG1 channels by a factor of 3.2 ± 0.2 (\(n = 7\)). Using the same experimental protocol and method of analysis indicated that deactivation of rERG2 channel currents are barely affected by 10 \(\mu\)M RPR (Fig. 1F–I). At -60 mV, \(I_{\text{tail}}\) of rERG2 channels was only increased by 16 ± 6\% (\(n = 5\); Fig. 1J). At the highest soluble concentration of 50 \(\mu\)M, RPR dramatically slowed the deactivation of hERG1 and reduced peak \(I_{\text{tail}}\), measured a few milliseconds after repolarization to a \(V_{\text{ret}}\) that is more negative than -60 mV (Fig. 2A). A plot of the average normalized \(I_{\text{tail}}\) of hERG1 for several oocytes as a function of \(V_{\text{ret}}\) is presented in Fig. 2B. At -60 mV, \(I_{\text{tail}}\) for hERG1 was increased by a factor of 4.1 ± 0.5 (\(n = 5\)). For rERG2 channels, peak \(I_{\text{tail}}\) was also reduced, but deactivation was only modestly slowed by 50 \(\mu\)M RPR (Fig. 2C). At -60 mV, \(I_{\text{tail}}\) for rERG2 was increased by only 27 ± 9\% (\(n = 5\); Fig. 2D). Thus, even a high concentration of RPR has only a small effect on deactivation of rERG2 compared with hERG1 channels. Reduced RPR sensitivity was also evident for \(I_{\text{tail}}\) measured at more negative potentials, where the rate of rERG2 channel deactivation is much faster (Fig. 2, C and D).

The effect of cumulative concentrations (3–50 \(\mu\)M) of RPR on the fold change in \(I_{\text{tail}}\) of hERG1 and rERG2 channels as a function of \(V_{\text{ret}}\) is summarized in Fig. 3. For hERG1, \(I_{\text{tail}}\) was increased as a function of \(V_{\text{ret}}\) from -60 to -90 mV (Fig. 3A). The apparent reduced effect of RPR on \(I_{\text{tail}}\) at the less negative potentials is because \(I_{\text{tail}}\) was measured for only 9 seconds and not at steady-state levels, which can take >30 seconds (Wu et al., 2015). The fold change in \(I_{\text{tail}}\) for hERG1 plotted as a function of [RPR] at five different voltages is presented in Fig. 3B. Although the efficacy of RPR (peak response) was greater when determined at the more negative voltages, where deactivation was faster and neared completion even in the presence of RPR, the potency (\(EC_{50}\), summarized in Table 1) did not vary much over the voltage range of -60 to -90 mV. To determine if the potency or efficacy of RPR was voltage dependent when measured under near steady-state conditions, \(I_{\text{tail}}\) was measured using 60-second pulses at a \(V_{\text{ret}}\) of -70 and -110 mV (Fig. 3C). The fold change in \(I_{\text{tail}}\) for these long pulses was larger when measured at -70 mV compared with -110 mV (Fig. 3D), but there was no voltage-dependent difference between the values when normalized to the fold change in \(I_{\text{tail}}\) at 50 \(\mu\)M RPR (Fig. 3E).

In contrast to the maximum 30-fold increase in \(I_{\text{tail}}\) of hERG1 induced by 50 \(\mu\)M RPR at -110 mV, \(I_{\text{tail}}\) of rERG2...
was only enhanced by a maximum of 2.3-fold (Fig. 3F). The fold change in $I_{\text{tail}}$ for rERG2 plotted as a function of $[\text{RPR}]$ at the different voltages is presented in Fig. 3G, with EC50 and Hill coefficient values summarized in Table 1. The average EC50 for RPR across all voltages, when measured with 9-second pulses, was similar for rERG2 channels (13.5 \pm 1.9 \mu M) compared with hERG1 (9.3 \pm 1.2 \mu M; t test, $P > 0.05$).

We next examined hERG1-rERG2 chimeric channels to determine the structural domain(s) that might account for the differential sensitivity of hERG1 and rERG2 channel gating to RPR.

C-Linker Is a Critical Determinant of Differential ERG Channel Sensitivity to RPR. The protein sequences of hERG1 and rERG2 channel subunits are 55.2% identical. Four different hERG1/rERG2 chimeric channel subunits (Fig. 4) were constructed by substituting the coding sequence of a specific region of KCNH2 with the homologous coding sequence from kcnh6. The first chimera, rERG2(N-S4)/hERG1, contained rERG2 from the N-terminus to the end of the S4 segment. $I_{\text{tail}}$ was increased by 10 \mu M RPR at all potentials examined, and the increase was 1.6-fold at a $V_{\text{ret}}$ of 260 mV (Fig. 4A), which is about half of the effect observed for WT hERG1 channels (Fig. 1E). The second chimera, hERG1/rERG2(S45L-PL), contained the rERG2 sequence from the end of the S4 segment to the start of the S6 segment. Under control conditions, these chimeric channels deactivated about 10 times faster than WT hERG1 channels. The fast and slow time constants for deactivation at −60 mV were 38 ± 3 and 219 ± 24 milliseconds for the chimeric channel ($n = 5$), as compared with 471 ± 19 milliseconds and 2.92 ± 0.11 seconds for WT hERG1 ($n = 7$). The accelerated rate of deactivation resulted in a larger than normal (7-fold) RPR-induced increase in $I_{\text{tail}}$ at a $V_{\text{ret}}$ of −60 mV (Fig. 4B). Thus, the hERG1/rERG2(S45L-PL) chimera retained RPR sensitivity. The third chimera, hERG1/rERG2(S6-Cterm), contained the rERG2 sequence from the start of the S6 segment to the

![Fig. 1.](image1.png)

Effect of 10 \mu M RPR on deactivation of hERG1 and rERG2 channel currents. (A) Voltage pulse protocol and representative hERG1 current traces under control conditions. (B) Currents from the same cell after treatment with 10 \mu M RPR. (C and D) $I_{\text{tail}}$ determined from current traces shown in (A and B). In (A–D), colors correspond to the voltages shown in the inset of (A). Small horizontal arrow indicates 0 current level. (E) Effect of RPR on $I_{\text{tail}}$ determined over a 50-mV range of $V_{\text{ret}}$. $I_{\text{tail}}$ was normalized to the value determined at $V_{\text{ret}}$ of −60 mV under control conditions ($n = 7$). (F) Voltage pulse protocol and representative rERG2 current traces under control conditions. (G) Currents from same cell after treatment of oocyte with 10 \mu M RPR. (H and I) $I_{\text{tail}}$ determined from current traces shown in (F and G). In (F–I), colors correspond to the voltages shown in the inset of (F). Small horizontal arrow indicates 0 current level (F and G) or charge level (H and I). (J) RPR has only a minimal effect on $I_{\text{tail}}$ determined over a 50-mV range of $V_{\text{ret}}$. $I_{\text{tail}}$ was normalized to the value determined at $V_{\text{ret}}$ of −60 mV under control conditions ($n = 5$). In (E and J), the S.E. bars are smaller than the symbol size for some of the data points.

![Fig. 2.](image2.png)

Effect of 50 \mu M RPR on deactivation of hERG1 and rERG2 channel currents. (A) Superimposed hERG1 current traces recorded before and after treatment of an oocyte with 50 \mu M RPR. Small horizontal arrow indicates 0 current level. (B) Effect of RPR on $I_{\text{tail}}$ of hERG1 as a function of $V_{\text{ret}}$. $I_{\text{tail}}$ was normalized to the value determined at $V_{\text{ret}}$ of −60 mV under control conditions ($n = 7$). (C) Superimposed rERG2 current traces recorded before and after treatment of an oocyte with 50 \mu M RPR. (D) Effect of RPR on $I_{\text{tail}}$ of rERG2 as a function of $V_{\text{ret}}$. $I_{\text{tail}}$ was normalized to the value determined at $V_{\text{ret}}$ of −60 mV under control conditions ($n = 7$). In (B and D), the S.E. bars are smaller than the symbol size for some of the data points.
increase in (D) Effect of RPR on fold change in hERG1 pulses to pulse protocol. The lower panel shows outward tail currents in response to chimera was insensitive to 10 \(^a\) r es m a l l e r th a n t h e s y m b o l s i z ef o r s o m eo ft h ed a t ap o i n t s .

and Hill coefficient values are presented in Table 1. In all panels, the S.E. bars measured for 60 seconds at

Concentration and voltage-dependent effects of RPR on deactivation change in rERG2

therefore not included in these plots. (G) \([RPR]\)-response relationship for fold change in hERG1

of RPR (near the \(I_n\)–roll-Cterm) contained rERG2 from the start of the

Tail reversal potential of

\(70\) mV) was extremely variable and

I\(_{\text{tail}}\) at a

\(100\) mV. Upper panel depicts voltage

ret. Data were fitted with a logistic equation (smooth curves) to determine EC\(_{50}\) and the Hill coefficient at each

position in rERG2. We first examined the functional con-

between hERG1 and rERG2 within this structure are

highlighted in Fig. 5B, where the

sequences of substituting residues located in the C-linker.

Mutation of both

A helix of the CNBHD to the C-terminus. RPR at 10 \(\mu\)M increased \(I_{\text{tail}}\) of this chimera by 2.9-fold at a \(V_{\text{ret}}\) of –60 mV (Fig. 4D), similar to that observed for WT hERG1 channels (Fig. 1E). Together, these findings suggest that the structural component that accounts for the differential sensitivity of hERG1 and rERG2 channels to RPR is located between the S6 segment and the C-terminus.

The region of ERG subunits bounded by the start of the S6 segment and the C-terminus is long and significantly divergent between hERG1 and rERG2. The region contains 521 residues in hERG1 and 460 residues in rERG2, with only a 48.5% identity. However, the S6 segments for hERG1 and rERG2 differ by only a single residue (Ile639 in hERG1 versus Val491 in rERG2) located at the extracellular end, and the S6 to the end of the CNBH is 87% identical for the two subunits. Moreover, most of the sequence divergence in the S6-CNBH region occurs in the \(\beta\)-roll of the CNBHD (17 residues differ), whereas the C-linker and all regions of the CNBH other than the \(\beta\)-roll are more similar. Only 7 of the 77 residues are not identical. Therefore, we concentrated our efforts on examining the residues highlighted in Fig. 5A that differ between hERG1 and rERG2: five located in the C-linker and two in the \(\alpha\)A helix of the CNBH. The structure of the C-linker plus the CNBH of the Anopheles gambiae ERG channel subunit was recently solved by X-ray crystallography (Breidt et al., 2012). The initial seven residues that differ between hERG1 and rERG2 within this structure are highlighted in Fig. 5B, where the \(\alpha\)A gambiae residues were substituted to match the residues in hERG1.

hERG1 Sensitivity to RPR Can Be Incrementally Reduced by Cumulative Amino Acid Substitutions in the C-Linker to Match the Sequence of rERG2. The seven residues, located in the C-linker and the proximal region of the CNBH domain of hERG1, were mutated in pairs or singly to match the residues in the homologous positions in rERG2. We first examined the functional consequences of substituting residues located in the C-linker. Two adjacent residues in hERG1 (Lys741 and Pro742) are not identical. Therefore, we concentrated our efforts on examining the residues highlighted in Fig. 5A that differ between hERG1 and rERG2: five located in the C-linker and two in the \(\alpha\)A helix of the CNBH. The structure of the C-linker plus the CNBH of the Anopheles gambiae ERG channel subunit was recently solved by X-ray crystallography (Breidt et al., 2012). The initial seven residues that differ between hERG1 and rERG2 within this structure are highlighted in Fig. 5B, where the \(\alpha\)A gambiae residues were substituted to match the residues in hERG1.

<table>
<thead>
<tr>
<th>(V_{\text{ret}}) (mV)</th>
<th>hERG1</th>
<th>rERG2</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>6.8 ± 0.3</td>
<td>2.2 ± 1.1</td>
</tr>
<tr>
<td>70</td>
<td>8.2 ± 0.5</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>80</td>
<td>9.1 ± 0.2</td>
<td>1.7 ± 0.03</td>
</tr>
<tr>
<td>90</td>
<td>8.4 ± 1.2</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>110</td>
<td>13.9 ± 5.3</td>
<td>1.5 ± 0.3</td>
</tr>
</tbody>
</table>
The addition of a fifth mutation (R681K) produced a C-linker that fully matched the corresponding region in rERG2. This mutant channel (R681K/N733H/S735A/K741P/P742A hERG1) was even less sensitive to RPR. RI tail at a V_m of 260 mV was only increased by 1.2-fold (Fig. 6C). The change associated with the addition of the R681K mutation was unanticipated given the conserved nature of the substitution. Therefore, we examined the effect of this isolated mutation. The single mutation R681K was equally or more effective at reducing the RPR response as that obtained with the quadruple mutant, with a 1.7-fold increase in RI tail at 260 mV (Fig. 6D). We next substituted Met756, located in the α A helix of the CNBHD, with a Val, and added this mutation to the five-mutant subunit to obtain the six amino acid substitution subunit R681K/N733H/S735A/K741P/P742A/M756V. This mutant channel was as insensitive to RPR as the WT rERG2 channel, with only a 9 ± 5% (n = 5) increase in |Itail| at −60 mV (Fig. 6E). Finally, we mutated another residue in the αA helix of the CNBHD (T747S) to obtain a seven-mutant subunit: R681K/N733H/S735A/K741P/P742A/T747S/M756V. The seven substitutions rendered the hERG1 channel insensitive to 10 μM RPR. |I_tail| at −60 mV was increased by only 2 ± 2% (n = 5; Fig. 7A). At the higher concentrations of 30 and 50 μM RPR, caused both a reduction in current magnitude and a slight slowing of deactivation and together these effects resulted in only a minimal change in |I_tail| for hERG1 containing the seven mutations (Fig. 7, B and C), which is similar to the effects on rERG2 channels (Fig. 3C). Thus, the differences in the sequence of the C-linker and the adjoining initial α-helix of the CNBHD account for the differential sensitivities of hERG1 and rERG2 channel gating to modification by RPR.

rERG2 Sensitivity to RPR Can Be Incrementally Enhanced by Cumulative Amino Acid Substitutions in the C-Linker to Match the Sequence of hERG1. If the C-linker and adjoining initial α-helix of the CNBHD account for the relative insensitivity of rERG2 channels to RPR, then we should be able to make cumulative substitutions of these key residues in rERG2 to match hERG1 and enhance RPR sensitivity. To test this hypothesis, we used the same approach described above for hERG1 channels, but instead, we introduced paired or single mutations into the rERG2 subunit to match the corresponding residues in hERG1. The mutant rERG2 channels expressed poorly in oocytes and limited our ability to accurately record currents at negative potentials without interference from endogenous currents. Therefore, we only analyzed I_tail recorded at a V_rest of −60 and −70 mV. Representative current traces for each of the mutant rERG2 channels, containing two, four, five, six, or seven amino acid substitutions, before and after treatment of an oocyte with 10 μM RPR, are presented in Fig. 8.
appreciably alter the kinetics of channel deactivation, but the ability of RPR to slow deactivation was incrementally enhanced by cumulative addition of two to seven mutations. As illustrated in Fig. 9A, 50 μM RPR had dramatic effects on $I_{\text{tail}}$ of rERG2 channels containing the seven amino acid substitutions. In addition to slower deactivation, peak $I_{\text{tail}}$ at −60 mV was increased by 68 ± 2% ($n = 5$), which is twice as much as the 32% observed for WT hERG1 channel currents. The EC$_{50}$ for the RPR-induced increase in $I_{\text{tail}}$ at −60 mV for the rERG2 plus seven amino acid substitutions channel was 11.6 ± 0.7 μM (Fig. 9B), which is higher than the EC$_{50}$ for WT hERG1 (6.8 μM) (Table 1) determined at the same $V_{\text{ret}}$. To verify that this activity resulted from RPR binding to the homologous site previously defined for hERG1, we introduced an eighth mutation (L404A) into rERG2. This mutation is equivalent to the L553A substitution in hERG1, that abolishes the agonist effects of RPR, presumably because it disrupts the RPR binding site (Perry et al., 2007). As shown in Fig. 9C, deactivation of rERG2 containing all eight mutations was not slowed by 50 μM RPR. The only obvious effect was a decrease in peak $I_{\text{tail}}$ (e.g., −38 ± 5% at −60 mV; $n = 6$). Thus, analogous to our finding with hERG1 (Wu et al., 2015), mutation of a specific residue in the S5 segment (L404A in rERG2) eliminates the effects of RPR on deactivation and reveals an inhibitory effect of the compound.

The fold change in $I_{\text{tail}}$ induced by RPR at a $V_{\text{ret}}$ of −70 mV for WT channels and five mutant hERG1 and five mutant rERG2 channels is summarized in Fig. 10. This figure illustrates that substitution of five residues in the C-linker of hERG1 to match the corresponding residues in rERG2 is sufficient to account for almost all of the reduced sensitivity to RPR of WT rERG2 relative to WT hERG1 channels. Conversely, substitution of five residues in the C-linker of rERG2 to match the corresponding residues in hERG1 is sufficient to account for almost all of the enhanced sensitivity to RPR of WT rERG2 relative to WT hERG1 channels. Further substitution of a sixth and seventh residue in the αhelix of the CNBH domain of hERG1 channels completes the transformation of channel sensitivity to RPR in either channel. The altered sensitivity of mutant hERG1 and rERG2 channels was not correlated with the rate of current deactivation measured in the absence of the drug. This is illustrated in Fig. 11A, where the fast and slow time constants of deactivation (upper panel) and the relative amplitude of the fast component of deactivation (lower panel) at −70 mV for WT and mutant hERG1 channels are plotted as a function of the fold change in $I_{\text{tail}}$ induced by 10 μM RPR. A similar plot for WT and mutant rERG2 channels is presented in Fig. 11B. These data indicate that the reduced response of WT rERG2 and some of the mutant hERG1 and rERG2 channels cannot be simply attributed to a slower rate of deactivation under control conditions.

![Fig. 6](image1.png)

![Fig. 7](image2.png)
large effect given the conserved nature of this point mutation. RPR at 10 μM induced a 2.4-fold increase in $I_{\text{tail}}$ at $V_{\text{ret}}$ of −70 mV, which is a greater reduction in efficacy than the 2.9-fold change that was obtained by swapping all four of the remaining nonconserved residues (Asn733, Ser735, Lys741, and Pro742) located in the C-linker. Second, addition of a seventh amino acid swap (S599T) in rERG2 reduced the response to RPR relative to the six substitutions. The reason why such conserved mutations resulted in relatively large changes in the pharmacological response is unclear, but suggests that even subtle changes in the C-linker can have significant effects on the ability of RPR to slow ERG channel deactivation.

Several recent studies have provided strong evidence for physical association between the cytoplasmic N- and C-termini and the role such an interaction has in determining the kinetics of transitions between the open and closed state of ERG channels (Gustina and Trudeau, 2011; Gianulis et al., 2013; Li et al., 2014; Ng et al., 2014). The N-terminal eag domain has also been proposed to modulate the deactivation rate by a direct interaction with the S4-S5 linker (Li et al., 2010; de la Pena et al., 2011; Ng et al., 2011); however, another study concluded that the eag domain associates with the C-linker/CNBHD, but not the S4-S5 linker (Gianulis et al., 2013). Interactions between specific residues in the eag domain and the C-linker have recently been investigated. Based on homology modeling and site-directed mutagenesis results, Ng et al. (2014) proposed that Arg56 of the PAS domain and Asp803 in the CNBHHD form a stable interaction, whereas Arg4 and Arg5 of the PAS-cap domain transiently interact with Glu698 and Glu699 of the C-linker (yellow

Discussion

ERG channels differ in their response to the agonist effects of RPR. We previously found that the reduced sensitivity to RPR of rERG3 compared with hERG1 channels could be explained by a single residue in the S5 segment located within the putative RPR binding pocket. Substitution of Ile558 in rERG3 with a Thr, the homologous residue in hERG1, conferred hERG1-like sensitivity to RPR. The converse substitution had the opposite effect, making hERG1 insensitive to RPR (Ferry and Sanguinetti, 2008). However, because the residues that compose the binding pocket for RPR are fully conserved between rERG2 and hERG1, it was clear that the mechanism of reduced response of rERG2 to RPR differed from that previously described for rERG3. Analysis of hERG1-rERG2 chimeric channels, followed by multiple amino acid swaps in hERG1 and rERG2 to match the other channel sequence revealed that differential sensitivity to RPR could be fully accounted for by a few residues in the C-linker and the initial region of the adjoining CNBHHD. Based on the effects achieved by cumulative amino acid substitutions, the C-linker appears to be more important than the CNBHHD as a structural determinant of the efficacy of RPR. Swapping the five nonconserved residues in the C-linker reduced the sensitivity of hERG1 to that of rERG2, whereas the equivalent substitutions in rERG2 made this channel almost as sensitive to RPR as hERG1. There were two anomalies associated with these observations. First, substitution of Arg681 with Lys (R681K) in the αA’ helix of the C-linker had an unexpectedly
colored side chains in Fig. 5B) in the open state, and this association slows the rate of deactivation. CNBHD-eag domain interactions have also been investigated using purified proteins and NMR spectroscopy. Thr747 (the seventh hERG1 amino acid substituted in our study) was among the many residues in the CNBHD identified as interacting with the eag domain (Li et al., 2014). Finally, a disulfide bridge can be formed between a Cys introduced into the PAS cap and either Cys723 in the C-linker (de la Pena et al., 2013, 2014) or an introduced Cys (Y542C) in the S4/S5 linker (de la Pena et al., 2011). Together these studies suggest that the PAS cap can interact with both the S4/S5 linker and the C-linker regions of the hERG1 channel and that these interactions alter the kinetics of channel deactivation.

We recently studied concatenated hERG1 tetromers to investigate the stoichiometry of altered channel gating induced by RPR. Concatenmers were constructed to contain a variable number of WT subunits and subunits containing the L553A mutation that presumably diminishes RPR binding affinity (Wu et al., 2015). RPR-induced slowing of deactivation was directly proportional to the number of WT subunits (and by inference, the number of high affinity sites available to bind RPR) incorporated into a concatenated tetrameric channel. In contrast, normal slow deactivation is an all-or-none process with regard to the requirement of full-length N-terminal domains (Thomson et al., 2014). Although our current findings indicate that differential sensitivity to the allosteric effects of RPR on channel gating can be swapped between hERG1 and rERG2 by mutating the few key nonconserved residues in the C-linker, the structural basis of RPR-induced slowing of hERG1 deactivation remains elusive. We previously reported that RPR has no effect on the kinetics, magnitude, or voltage dependence of hERG1 gating currents (Abbruzzese et al., 2010), indicating that the compound dissociates movement of the voltage sensor domain from the opening and closing of the activation gate. RPR could directly or by an allosteric mechanism stabilize the association between the PAS cap and the C-linker (Ng et al., 2014) or interfere with the coupling between the S4/S5 linkers and the C-terminal region of the S6 segments (Ferrer et al., 2006). Further experimentation will be required to distinguish between these and other possible mechanisms of RPR action.

Our study has a few limitations. First, we did not examine the effects of RPR on inactivation of WT ERG2 or the many hERG1 and rERG2 mutant channels. However, the peak of \( I_{\text{tail}} \) of mutant rERG2 channels containing the seven amino acid substitutions in the C-terminus to match hERG1 was increased by high concentrations of RPR (Fig. 9), an effect previously shown to be associated with a positive shift in the voltage dependence of inactivation in WT hERG1 channels (Perry et al., 2007). Second, poor expression of mutant ERG2 channels coupled with interference from endogenous currents at negative potentials in oocytes prevented analysis of deactivation kinetics over a broad range of voltages. Third, a Hill coefficient >1 for the [RPR]-response relationships could indicate positive ligand-binding cooperativity or could result from cooperative subunit interactions, as we previously found for two other hERG1 activators, PD-118057 [2-[[4-[[2-[3,4-dichlorophenyl]ethyl]phenyl]amino]benzoic acid] and ICA-105574 [3-nitro-N-(4-phenoxyphe-nyl)-benzamide]. In our previous study of the effects of PD-118057 and ICA-105574 on concatenated hERG1 tetromers (Wu et al., 2014), we found that although subunit cooperativity contributes to the effects of these two hERG1 agonists, there was no change in EC\(_{50}\) or the Hill coefficient as the number of drug binding sites was increased from one to four. This finding indicates that occupancy of one binding site did not lead to an enhancement of PD-118057 and ICA-105574 affinity of other sites. In the [RPR]-response relationships presented in Fig. 3, the fold change in \( I_{\text{tail}} \) was determined using 9-second pulses, which is an insufficient duration to measure the full effect of the drug on deactivation. Thus, EC\(_{50}\) values are underestimated and Hill coefficients cannot be used as an
indicator of the extent of either subunit or drug binding cooperativity.

In summary, together with previous findings (Perry and Sanguinetti, 2008), our studies indicate that differential sensitivity of ERG channels to the slowing of deactivation induced by RPR is not explained by a common mechanism. Reduced sensitivity of ERG3 channels to RPR is explained by a single residue in the putative RPR binding pocket, whereas differences in the C-linker can account for the reduced effect of RPR on ERG2 compared with hERG1 channels. Our findings may also have clinical implications as it is clear that activators that display substantial selectivity among the different ERG channels can be developed. Activation of ERG1 channels can be achieved (e.g., for the treatment of congenital long QT syndrome) in the relative absence of effects on the related ERG2 and ERG3 channels that are prominently expressed in the central nervous system.

Authorship Contributions

Participated in research design: Gardner, Sanguinetti.
Conducted experiments: Gardner.
Performed data analysis: Gardner, Sanguinetti.
Wrote or contributed to the writing of the manuscript: Gardner, Sanguinetti.

References


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