Ginsenoside Rg3, a Gating Modifier of EAG Family K⁺ Channels

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ABSTRACT

Ginsenoside 20(S)-Rg3 (Rg3) is a steroid glycoside that induces human ether-à-go-go-related gene type 1 (hERG1, Kv11.1) channels to activate at more negative potentials and to deactivate more slowly than normal. However, it is unknown whether this action is unique to hERG1 channels. Here we compare and contrast the mechanisms of actions of Rg3 on hERG1 with three other members of the ether-à-go-go (EAG) K⁺ channel gene family, including EAG1 (Kv10.1), EAG3 (Kv11.3), and ELK1 (Kv12.1). All four channel types were heterologously expressed in Xenopus laevis oocytes, and K⁺ currents were measured using the two-microelectrode voltage-clamp technique. At a maximally effective concentration, Rg3 shifted the half-point of voltage-dependent activation of currents by ~14 mV for ERG1 (EC₅₀ = 414 nM), ~20 mV for ERG3 (EC₅₀ = 374 nM), ~28 mV for EAG1 (EC₅₀ = 1.18 μM), and more than ~100 mV for ELK1 (EC₅₀ = 197 nM) channels. Rg3 also induced slowing of ERG1, ERG3, and ELK1 channel deactivation and accelerated the rate of EAG1 channel activation. A Markov model was developed to simulate gating and the effects of Rg3 on the voltage dependence of activation of hELK1 channels. Understanding the mechanism underlying the action of Rg3 may facilitate the development of more potent and selective EAG family channel activators as therapies for cardiovascular and neural disorders.

Introduction

Ether-à-go-go (EAG) K⁺ channel family members are classified into three different subfamilies on the basis of their amino acid sequence and biophysical properties. The EAG subfamily includes two channels (Kv10.1, Kv10.2, or EAG1, EAG2) (Warmke et al., 1991; Saganich et al., 1999), the ether-à-go-go–related (ERG) subfamily includes three channels (Kv11.1–11.3, or ERG1–3) (Warmke and Ganetzky, 1994; Shi et al., 1997), and the ether-à-go-go–like (ELK) subfamily includes three channels (Kv12.1–12.3, or ELK1–3) (Warmke and Ganetzky, 1994; Engeland et al., 1998). As with other voltage-gated K⁺ (Kv) channels, EAG family channels are tetrameric complexes, formed by coassembly of four identical or highly similar subunits, each containing six transmembrane segments. The first four segments (S1–S4) of each subunit form a voltage sensor domain (VSD), and the S5 and S6 segments from all four subunits together form the pore domain. EAG family K⁺ channel subunits have unusually large N- and C-terminal cytoplasmic domains and a conserved K⁺ channel signature amino acid sequence (TSVGFG) that forms the selectivity filter.

The three EAG subfamilies have distinct patterns of tissue expression and diverse physiologic roles (Bauer and Schwarz, 2001). EAG channels are highly expressed in the central nervous system, where they contribute to threshold excitability in brainstem neurons (Hardman and Forsythe, 2009) and excitability of multiple sensory organs, such as the olfactory bulb (Hirdes et al., 2009). EAG channels are also expressed in a wide range of tumor cell lines, where they have a role in cell proliferation (Pardo et al., 1999; Agarwal et al., 2010). ELK channels modulate neuronal excitability, but little is known of their specific functions (Becchetti et al., 2002). EAG channels have diverse biologic and pathogenic roles in many tissues, including the heart, brain, smooth muscle, pancreas, and tumors (Babcock and Li, 2013). In the human heart, human ether-à-go-go–related gene type 1 (hERG1) channels contribute significantly to repolarization of cardiac action potentials. Reduced function of hERG1 channels, caused either by loss-of-function mutations in type 2 long QT syndrome (LQTS) (Curran et al., 1995) or pore block by several common medications, prolongs action potential duration in cardiomyocytes and increases the risk of lethal arrhythmia in susceptible individuals (Sanguinetti and Tristani-Firouzi, 2006).

ABBREVIATIONS: [Ipeak], integral of leak-subtracted tail current; ANOVA, analysis of variance; APD, action potential duration; C-del, C-terminal-deleted; EAG, ether-à-go-go; G-V, conductance-voltage; g(mm), relative conductance; hEAG1, human ether-à-go-go gene type 1; hELK1, human ether-à-go-go–like gene type 1; hERG1, human ether-à-go-go–related gene type 1; Ipeak, peak tail current; Itp, tail current; Ipeak/Imax, peak outward current; K, slope factor for Boltzmann function; Kv, voltage-gated K⁺; LQT, long QT syndrome; N/C-del, lacking both the N- and C-termini; N-del, N-terminal-deleted; Rg3, 20(S)-ginsenoside Rg3; Rg3, (2S,3R,4S,5S,6R)-2-[[(2R,3R,4S,5R,6R)-4,5-dihydroxy-2-(3S,5R,8R,9R,10R,12R,13R,14R,17S)-12-hydroxy-17-[(2S)-2-hydroxy-6-methylhept-5-en-2-yl]-4,4,8,10,14-pentamethyl-4,5-triol]-τ, fast time constant; τs, slow time constant; V(0.5), half-point (mV) of Boltzmann function; V(pree), prepulse potential; V(r), return potential; VSD, voltage sensor domain; Vt, test potential; WT, wild-type.

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The most common cause of LQTS is a loss-of-function mutation in hERG1 or KCNQ1 channel subunits. The resulting reduction of repolarizing currents prolongs action potential duration (APD) of the ventricles and increases the risk of potentially lethal arrhythmia. Small-molecule activators of hERG1 channels can shorten cardiac APD and have been proposed as a novel approach for the treatment of LQTS (Sanguinetti, 2014). However, excessive shortening of APD can also increase the risk of ventricular arrhythmia as exemplified by gain-of-function mutations in ion channels that cause short QT syndrome (Bellocq et al., 2004, Hong et al., 2005, Priori et al., 2005). In recent years, several small-molecule activators of hERG1 channels have been discovered and their mechanisms of action were elucidated. RPR-260243 greatly slows the rate of channel deactivation (Kang et al., 2005b, Perry et al., 2007), PD-118057 (Zhoud et al., 2005) attenuates inactivation and increases channel open probability (Perry et al., 2009), ICA-105574 nearly eliminates inactivation (Gerlach et al., 2010, Garg et al., 2011), and mallotoxin causes a negative shift in the voltage dependence of activation (Zeng et al., 2006).

20(S)-ginsenoside Rg3 (Rg3), a steroid glycoside, activates hERG1 channels by a combination of mechanisms, including slowed deactivation and shifting the voltage dependence of channel activation by a maximum of −15 mV (Choi et al., 2011a). The relatively modest effects of Rg3 on hERG1 gating are a desirable feature of an activator to be used for treatment of LQTS, because unlike compounds that greatly attenuate inactivation (e.g., ICA-105574) it would not cause excessive shortening of APD or QT interval. Rg3 has been extensively studied and found to block or alter the gating of several other ion channels, but only at much higher concentrations than required to activate hERG1 (Kang et al., 2005a; Lee et al., 2006, 2007, 2008a,b, 2009). It is unknown if Rg3 affects gating of EAG K⁺ channel family members other than hERG1. It is important to further characterize channel specificity of Rg3 to fully evaluate its potential as a pharmacophore for further development of more potent and specific analogs. Thus, in the present study we have investigated the effects of Rg3 on the gating of representative EAG K⁺ channel family members, including ERG1, ERG3, EAG1, and ELK1 channels.

**Materials and Methods**

**Molecular Biology.** cDNAs for wild-type (WT) human ERG1 isoform 1a (NCBI reference sequence: NM_000238), rat ERG3 (GenBank accession number AF_016191), human EAG1 (NCBI reference sequence: NM_004633), human ELK1 (NCBI reference sequence: NM_002238), and human Kv1.5 (NCBI reference sequence: NM_000238), human EAG1 (NCBI reference sequence: NM_000238), rat ERG3 (GenBank accession number AF_016191), cRNAs were transcribed in vitro using mMgES-470 (Waltham, MA).

Voltage clamp of oocytes. The procedure used to harvest oocytes from Xenopus laevis was as described (Garg et al., 2012) and was approved by the University of Utah Institutional Care and Use Committee. Oocytes were cultured at 17°C in Barth’s solution. Individual oocytes were injected with 1–20 ng cRNA encoding WT and mutant hERG1, rat ether-a-go-go-related gene type 3 (rERG3), human EAG1 (hEAG1), human ELK1 (hELK1), and human Kv1.5 K⁺ channels. Ionic currents were measured from oocytes 1–4 days after cRNA injection using the two-electrode voltage-clamp technique (Schreibmayer et al., 1994). Current and voltage signals were acquired using a GeneClamp 500 amplifier, Digidata 1322A data acquisition system, and pCLAMP 8.2 software (Molecular Devices, Inc., Sunnyvale, CA).

Data analysis. pCLAMP 8.2 software (Molecular Devices) was used for data acquisition and analysis. Origin (version 8.6) software (OriginLab Corp., Northampton, MA) was used for further data analysis and to prepare graphs and figures. Data are expressed as mean ± S.E.M. (n = number of oocytes) and, where appropriate, analyzed by two-way analysis of variance (ANOVA) or paired t test. A p value < 0.05 was considered significant.

Concentration-response relationships for Rg3 effects on several measured parameters of channel current were fitted with a logistic equation:

\[
y = \frac{A_{\text{max}}}{1 + \frac{(Rg3)/EC_{50}}{A_{\text{max}}}}
\]

where \(A_{\text{max}}\) and \(A_{\text{min}}\) are the maximum and minimum values of the measured parameter, EC_{50} is the concentration required for half maximal effect, and \(n_H\) is the Hill coefficient.

**Voltage Dependence of Channel Activation.** For WT hERG1 and rERG3 channels, the voltage dependence of activation was determined by measuring the peak tail currents (I_{tail-peak}) elicited at a return potential (V_{0.5}) of −70 mV. The relative conductance–voltage (g/g_{max} vs. V) relationship for hERG1 and rERG3 channel activation was determined by plotting the normalized value of I_{tail-peak} versus the preceding test voltage (V_{0.5}). The data were fitted with a Boltzmann function to obtain the half-point (V_{0.5}) and slope factor (k) for channel activation:

\[
g_{\text{max}} = \frac{1}{1 + e^{(V_{0.5} - V)/k}}
\]

For hEAG1 or Kv1.5 channels, the rate of deactivation was too fast to accurately quantify I_{tail-peak}. Therefore, to determine the voltage dependence of activation for these channels, the peak outward current (I_{out-max}) at each potential divided by the electrical driving force for K⁺ (I_{out-max} / (V_{0.5} − E_{K})) was normalized to its maximum value and plotted as a function of V_{0.5}. The resulting conductance-voltage (G-V)
relationship was fitted with a Boltzmann function to obtain $V_{0.5}$ and $k$ for channel activation.

The $G$-$V$ relationship for split hERG1 channels was poorly fit with a standard Boltzmann function but well described by a model of channel gating that assumes four independent and identical voltage-dependent closed state transitions followed by a final concerted transition to the open state (Koren et al., 1990, Zagotta and Aldrich, 1990). The state diagram and equilibrium constants for this gating model were as previously described (Gonzalez et al., 2000):

$$
K_{0} \leftrightarrow C_{1} \leftrightarrow C_{2} \leftrightarrow C_{3} \leftrightarrow C_{4} \leftrightarrow O
$$

with the equilibrium constant for closed stated transitions, $K = K(0)$ exp($-z_{F}FV/RT$), and the equilibrium constant for the final concerted transition between the fourth closed state ($C_{4}$) and the open ($O$) state, $K_{0} = K_{0}(0)$ exp($-z_{F}FV/RT$). The normalized $G$-$V$ relationship is described by:

$$
\frac{g}{g_{\text{max}}} = \left( 1 + K_{0} + 4K_{1}K + 6K_{1}K^{2} + 4K_{1}K^{3} + K_{1}K^{4} \right)^{-1}
$$

The voltage dependence of hELK1 channel currents was analyzed by yet another method. From a holding potential of $-110$ mV, currents were elicited by pulsing to a prepulse potential ($V_{\text{prep}}$) that was varied in 10-mV increments from $-150$ to $-10$ mV. Each prepulse was followed by a test pulse to $+20$ mV. The onset of currents activated by the pulse to $+20$ mV were fitted with a single exponential function and extrapolated back to the initial time of the step in voltage to obtain $I_{\text{init}}$, a measure of the extent of channel activation induced at each $V_{\text{prep}}$. $I_{\text{init}}/I_{\text{test-max}}$ was plotted as a function of $V_{\text{prep}}$ and the resulting relationship fitted with a Boltzmann function to obtain the $V_{0.5}$ and $k$ for hELK1 channel activation. In the presence of Rg3, the normalized $G$-$V$ relationships were fit by the sum of two Boltzmann functions:

$$
\frac{g}{g_{\text{max}}^1} = \frac{A_{\text{min1}} - A_{\text{max1}}}{1 + \exp \left[ \frac{V - V_{0.51}}{k_{1}} \right]} + \frac{A_{\text{max2}} - A_{\text{max2}}}{1 + \exp \left[ \frac{V - V_{0.52}}{k_{2}} \right]}
$$

$A_{\text{min1}}$ and $A_{\text{min2}}$ were fixed to zero, $A_{\text{max1}} + A_{\text{max2}} = 1$, and $V_{0.51}$ and $k_{1}$ were fixed to the values determined under control conditions. The $G$-$V$ relationships for C-del and N/C-del hERG1 channel currents were also fitted with a double Boltzmann function.

Voltage Dependence of Channel Inactivation. The fully activated current-voltage relationship for hERG1 and rERG3 channel currents was determined by applying a 2-second prepulse to $+40$ mV, followed by repolarization to a $V_{\text{prep}}$ that varied in 10-mV increments from $-140$ to $+30$ mV. As described previously (Sanguinetti et al., 1995), the voltage dependence of hERG1 inactivation was determined by fitting the normalized values of $I_{\text{tail-peak}}/(V_{\text{ref}} - E_{K})$ as a function of $V_{\text{ref}}$ with a Boltzmann function:

$$
\frac{g}{g_{\text{max}}} = \frac{1}{1 + \exp \left[ \frac{V_{\text{ref}} - V_{0.5}}{k} \right]}
$$

For hELK1 channel currents, $I_{\text{test-max}}$ at $+20$ mV was normalized to its peak value, plotted as a function of $V_{\text{prep}}$, and fitted with the Boltzmann function to obtain the $V_{0.5}$ and $k$ for channel inactivation.

Deactivation Kinetics. For the kinetic analysis of hERG1, rERG3, and hELK1 deactivation (or hEAG1 activation in presence of Rg3), the rate of tail currents was quantified by fitting current decay (or increase) to a biexponential function:

$$
I_{\text{tail}}(t) = A_{f}e^{-t/\tau_{f}} + A_{s}e^{-t/\tau_{s}} + C
$$

to obtain the fast and slow time constants ($\tau_{f}, \tau_{s}$) and relative amplitude of the slow component of deactivation, $A_{s}/(A_{f} + A_{s})$. hERG1 deactivation was also quantified by measuring the integral of leak-subtracted 3.5-second and 40-second tail currents ($\int I_{\text{tail}}$) at $-70$ mV.

Solutions and Drugs. Barth’s solution contained (in mM): 88 NaCl, 1 KCl, 0.41 CaCl$_{2}$, 0.33 Ca(NO$_{3}$)$_{2}$, 1 MgSO$_{4}$, 2.4 NaHCO$_{3}$, 10 HEPES, 1 pyruvate, plus gentamicin (50 mg/liter), amikacin (100 mg/liter), and ciprofloxacin (25 mg/liter); pH adjusted to 7.4 with NaOH. The extracellular salt solution used for voltage-clamp experiments contained in (mM): 98 NaCl, 2 KCl, 1 CaCl$_{2}$, 1 MgCl$_{2}$, 5 HEPES; pH adjusted to 7.6 with NaOH. Split hERG1 channel currents were recorded using a high extracellular K$^{+}$ solution that contained (in mM): 104 KC$_{1}$, 1 CaCl$_{2}$, 1 MgCl$_{2}$, 1 HEPES; pH adjusted to 7.6 with NaOH. 20(S)-ginsenoside Rg3 (Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide to make a 1 or 10 mM stock solution that was stored at $-20^\circ$C. Before each experiment, a stock solution was diluted with extracellular solution to produce a final [Rg3] of 0.1–10 $\mu$M.

Modeling of hELK1 Currents. Computational models were developed to reconstruct hELK1 channel currents in the absence and presence of Rg3. The models include a Markovian description of channel gating and a description of $K^{+}$ current through channels on the basis of the Nernst equation. The Markovian model of hELK1 currents in the absence of Rg3 comprises four closed states, one open and one inactivated state (Fig. 11A). Transition between these states was described by membrane voltage ($V_{m}$)-dependent forward rate $\alpha$ and backward rate $\beta$:

$$
\alpha = \alpha_{0}e^{-\gamma V_{m}/F/RT}
$$

$$
\beta = \beta_{0}e^{-\gamma V_{m}/F/RT}
$$

with the conductance $G_{\text{ELK}}$, the Nernst voltage $E_{K}$, and the probability that channels are in the open state $O$. $T$ was set at 293$^\circ$K. $E_{K}$ was calculated for $[K^{+}]_{o} = 2$ mM and $[K^{+}]_{i} = 114$ mM. Parameters of the Markov models were determined using a previously developed stochastic multiscale fitting approach (Abbruzzese et al., 2010, Wu et al., 2014). In short, we defined a fit error $E$ on the basis of feature vectors extracted from experimental data $f$ and simulation data $f_{\text{sim}}$:

$$
E = \sqrt{\sum_{i} \left( f_{\text{sim}, i} - f_{, i} \right)^{2}} + (1 - \text{Max} (C_{0} + C_{2} + C_{4}) + \text{area} (1 - \text{Max} (O + 1)))
$$

with the number of features $n$, the Euclidean norm $\mathbf{\ldots}$, the maximal probability of closed states at the end of a $-100$ mV pulse, the maximal probability of open and inactivated state during a 60-mV pulse, and weighting factors $w_{\text{area}}$ and $w_{\text{Max}}$. Measured features $f_{\text{m}}$ included maxima and time constants from exponential fits (Supplemental Table 1).

A multiscale procedure was applied to estimate initial values of the fitting parameters. A first estimate was determined by fitting to reduced data, i.e., features from traces for every third voltage step. The estimates were used for a second fitting using features from traces for every second voltage step. The resulting estimates were applied for a final fitting using all voltage steps. Perturbations were initially in the range of 50–150% of the original parameter value. After each iteration, the perturbation range was decreased to a final range of 98–102%. We used 32 iterations per scale. In each iteration, 960 perturbed parameter sets were evaluated. Subsequently, the 96 parameter sets with the smallest fit error were optimized using the steepest descent method (Press et al., 1992) and used for parameter set perturbation in the next iteration.

To investigate effects of Rg3 on the G$-$V relationship, we implemented a two-compartment model (Supplemental Fig. 4A). One compartment was derived from the developed six-state model for current in the absence of Rg3. The other compartment lumped together behavior of channels affected by Rg3. For this compartment, we determined forward rates between C$_{0}$, C$_{1}$, C$_{2}$, C$_{3}$, and O to reproduce $V_{0.5}$ from fitting of Boltzmann functions to measured
Results

Rg3 Induces a Negative Shift in the Voltage Dependence of Activation and Slows the Rate of Deactivation of ERG Channels. As previously reported (Choi et al., 2011a), Rg3 enhances hERG1 current magnitude and induces a profound slowing of tail-current ($I_{tail}$) decay (Fig. 1A). $I_{test-max}$, the current measured at the end of a 4-second test pulse, was augmented by Rg3 in a voltage- and concentration-dependent fashion (Fig. 1B). To investigate whether the voltage dependence of channel activation was altered by Rg3, $I_{tail-peak}$ was plotted as a function of $V_m$ and the resulting relationship was fitted to the Boltzmann function (Fig. 1C). Rg3 shifted the voltage dependence of activation to a more negative voltage in a concentration-dependent manner (Table 1). The $EC_{50}$ for the Rg3-induced shift in the half-maximal voltage required for activation ($V_{0.5}$) was $414 \pm 49 \text{nM}$ ($n = 7$), and the maximum shift was $-13.7 \pm 0.7 \text{mV}$ in response to $3 \mu\text{M}$ Rg3 (Fig. 1D). Rg3 also increased the maximum value of $I_{tail-peak}$ in a concentration-dependent manner (Fig. 1D) with an $EC_{50}$ of $255 \pm 34 \text{nM}$ ($n = 7$).

To investigate the action of Rg3 on hERG1 deactivation gating, channels were fully activated by applying a 2-second prepulse to $+40 \text{mV}$, followed by a variable $V_m$ that ranged from $–60$ to $–140 \text{mV}$ to elicit tail currents (Fig. 2A). The time-dependent decay of $I_{tail}$ at each $V_m$ was fitted with a biexponential function to define the kinetics of channel deactivation. In the presence of $3 \mu\text{M}$ Rg3, both the slow and fast time constants ($\tau_s$, $\tau_f$) were increased significantly compared with control (Fig. 2B). The voltage-dependent variation of both $\tau_s$ and $\tau_f$ was shifted about $–50 \text{mV}$ by $3 \mu\text{M}$ Rg3, far more than the $–13.7 \text{mV}$ shift in the $V_{0.5}$ for activation observed for this concentration of Rg3. The relative amplitude of the slow component of current deactivation was also enhanced by $3 \mu\text{M}$ Rg3 (Fig. 2C). The concentration-dependent effects of Rg3 (0.1–$10 \mu\text{M}$) on hERG1 deactivation was investigated at a single $V_m$ of $–70 \text{mV}$ (Fig. 2D). Effects on $I_{tail}$ were quantified by calculating the tail current integral ($\langle I_{tail} \rangle$ at 3.5 and 40 seconds and by fitting the full 40-second traces to a biexponential function to estimate the slow and fast time constants for deactivation ($\tau_s$, $\tau_f$) before and after exposure of oocytes to each Rg3. The maximum fold increase in $\langle I_{tail} \rangle$ was $5.1 \pm 0.4$ ($EC_{50} = 250 \pm 22 \text{nM}$) at 3.5 seconds and $41 \pm 6$ ($EC_{50} = 556 \pm 47 \text{nM}$) at 40 seconds (Fig. 2E, $n = 4–5$), indicating the pronounced effect of Rg3 on deactivation. When the decay of $I_{tail}$ was fitted over the full 40 seconds of recording time, the fold increase in $\tau_f$ at $–70 \text{mV}$ was $5.2 \pm 0.3$ ($EC_{50} = 272 \pm 90 \text{nM}$), whereas the fold increase in $\tau_s$ was $11 \pm 1.7$ ($EC_{50} = 275 \pm 51 \text{nM}$). Rg3 increased the relative amplitude of the slow component of deactivation, $A_s/(A_s + A_f)$, in a concentration-dependent manner with an $EC_{50}$ of $196 \pm 24 \text{nM}$ (Fig. 2G). The similar $EC_{50}$ values for the multiple effects of Rg3 on the biophysical properties of hERG1 (Fig. 1D, Table 1).

Fig. 1. Concentration and voltage-dependent effects of Rg3 on hERG1 channel currents. (A) Representative hERG1 currents recorded from a single oocyte before (control) and after treatment with $3 \mu\text{M}$ Rg3. Small horizontal arrows indicate zero current level. Upper panel shows voltage pulse protocol. (B) Effects of Rg3 on normalized $I_{test-max}$ relationships determined before (control) and after achieving steady-state conditions in the presence of indicated concentrations of Rg3 ($n = 7$). Test pulse duration was 4 seconds. Currents were normalized relative to $I_{test-max}$ at $–10 \text{mV}$ under control conditions. (C) Effects of Rg3 on voltage dependence of hERG1 channel activation using 4-second test pulses. $I_{tail-peak}$ in the presence of Rg3 was normalized relative to the maximum $I_{tail-peak}$ measured under control conditions ($n = 7$). (D) Plots of [Rg3]-dependent effects on maximum $I_{tail-peak}$ and shifts in $V_{0.5}$ for activation determined using 4-second test pulses. Data were fitted with logistic equation (smooth curves). $EC_{50} = 255 \pm 34 \text{nM}$, $n_{H} = 1.11 \pm 0.06$ ($n = 7$) for change in maximum $I_{tail-peak}$, $EC_{50} = 414 \pm 49 \text{nM}$, $n_{H} = 1.67 \pm 0.12$ ($n = 7$) for shift in $V_{0.5}$.

Table 1

<table>
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<th>[Rg3]</th>
<th>V_{0.5}</th>
<th>k</th>
<th>n</th>
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<td>0 (control)</td>
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<td>8.7 ± 0.2</td>
<td>7</td>
<td>–34.2 ± 0.3</td>
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<td>6.2 ± 0.4</td>
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<tr>
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<td>–44.4 ± 1.7</td>
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</table>
Fig. 2. Rg3 slows the rate of hERG1 channel deactivation. (A) Representative currents recorded in an oocyte under control conditions and after treatment with 3 μM Rg3. Small horizontal arrows indicate zero current level. Upper panel shows voltage pulse protocol. (B) Rg3 (3 μM) slows the time constants for fast (τf) and slow (τs) components of current deactivation (n = 7; two-way ANOVA, p < 0.0001). (C) Rg3 (3 μM) increases the relative amplitude of the slow component of deactivation, A_s/(A_s + A_f) (n = 7; two-way ANOVA, p < 0.0001). (D) Concentration-dependent slowing of current deactivation by Rg3 in a single oocyte. Currents were activated with a 4-second pulse to 20 mV. Superimposed tail currents were measured for 40 seconds at a V_rest of −70 mV. Black arrow indicates zero current level. (E) [Rg3]-response relationships for fold increase in I_{tail-max} V_rest relationship, V_{50} for inactivation or the time constants for the recovery of channels from inactivation measured over a large range of voltage (Supplemental Fig. 1). Thus, Rg3 modifies both activation and deactivation gating, but it has no effect on inactivation of hERG1 channels.

Slow deactivation of hERG1 channels is dependent on a direct interaction between the N-terminus of one subunit and the C-terminus of an adjacent subunit (Gustina and Trudeau, 2011; Gianulis et al., 2013; Li et al., 2014). Thus, the profound slowing of deactivation by Rg3 could result from stabilization of these intersubunit interactions. To examine this possibility, we determined if truncation of either one or both of the N- and the C-termini of hERG1 subunits altered the ability of Rg3 to slow deactivation. As reported previously (Spector et al., 1996), N-del hERG1 channels deactivate much faster than WT channels. Rg3 at 3 μM increased I_{test-max} and I_{tail-peak} by about 2-fold and greatly slowed the rate of deactivation of N-del hERG1 channel currents (Fig. 3A). Similar to WT hERG1, Rg3 shifted the voltage-dependence of N-del hERG1 channel activation to more negative voltages in a concentration-dependent manner (Fig. 3B). The Rg3-induced shift in V_{50} had an EC_{50} of 458 ± 25 nM (n = 6), and the maximum shift was approximately −22 mV in response to 3 and 10 μM Rg3 (Fig. 3C). The rate of tail current deactivation was fitted well by a single exponential and was slowed by Rg3 over a wide range of V_rest (Fig. 3D), resulting in a −40 mV shift in the voltage dependence of τ_deact (Fig. 3E). The [Rg3]-response relationships for the fold increase in τ_deact at −70 mV is plotted in Fig. 3F (EC_{50} = 1.83 ± 0.23 μM, n = 6) and had a maximum of 13, similar to that observed for WT hERG1 channels (Fig. 2F). C-del hERG1 channels activated and deactivated much faster than WT channels, and Rg3 (3 μM) increased the magnitude and slowed the deactivation of these currents (Fig. 4A). Under control conditions the G-V relationship for 1-second test pulses was best fitted with a single Boltzmann function and had a V_{50} of −24.6 ± 1.1 mV and k of 11.1 ± 0.25 (Fig. 4B). The G-V relationship measured after application of 3 μM Rg3 was not well described by a single Boltzmann function, but was fitted well by the sum of two Boltzmann functions. Under the assumption that one component represents the voltage dependence for activation of unaffected channels, we fixed component “1” of the two Boltzmann functions to the value of V_{50} and k measured under control conditions. Component “2,” presumably representing the Rg3-bound fraction of channels, had a voltage dependence of activation (V_{50:2} = −64.7 ± 0.6 mV, k_2 = 6.9 ± 0.3) that was shifted by −40 mV compared to component 1 and represented 0.74 of the total current (Fig. 4B). Rg3 at 3 μM slowed the rate of deactivation over a wide range of V_rest (Fig. 4C), and increased the value for τ_deact (control: 12.0 ± 0.3 ms; 3 μM Rg3: 172 ± 8.5 ms, n = 7) at −70 mV by 14-fold (Fig. 4D). Rg3 had similar effects on N/C-del channels (Supplemental Fig. 2). Together these findings indicate that Rg3 does not slow the rate of hERG1 deactivation by stabilizing interactions between the N- and C-termini of channel subunits.

In Kv channels, the VSD has been proposed to be electromechanically coupled to the pore domain by the S4-S5 linkers in each subunit (Long et al., 2005). According to this structural model of channel gating, intramembrane displacement of the S4 segments in response to changes in transmembrane voltage is mechanically linked to the opening and closing of the activation gate (the S6 bundle crossing) via the S4-S5 linkers. However, a recent study found that severing the covalent link between the VSD and the pore domain of hERG1 did not prevent gating. Subunits were split apart at the S4-S5
linked, it is not unexpected that its voltage dependence of channel gating differs from WT channels. In the absence of Rg3, the G-V relationship for these channels was not a simple sigmoidal relationship and resembled more some other Kv channels whose gating is well described by a model that assumes four independent and identical voltage-dependent closed state transitions followed by a final concerted transition to the open state (Koren et al., 1990, Zagotta and Aldrich, 1990). Thus, as described in Materials and Methods, we used this model to analyze the G-V relationship for split channels under both control conditions and in the presence of Rg3 (smooth curves of Fig. 5D). This analysis provided a better fit than a standard Boltzmann function (dashed curves in Fig. 5D), but regardless of the method used the shift in $V_{0.5}$ of the relationship induced by Rg3 was $–14$ mV, similar to that observed for WT hERG1 channels (Fig. 1D). Thus, the effects of Rg3 on hERG1 channel gating are not dependent on a covalent linkage between the VSD and pore domain.

**Effects of Rg3 on rERG3 Channel Gating.** We next examined the effect of Rg3 on another member of the ERG channel subfamily, rERG3. The concentration and voltage-dependent effects of Rg3 on partially C-deleted hERG1 channel currents. (A) Representative C-del hERG1 channel currents recorded from a single oocyte before (control) and after treatment with 3 μM Rg3. Small horizontal arrows indicate zero current level. Upper panel shows voltage pulse protocol. (B) Effects of 0.1–10 μM Rg3 on voltage dependence of N-del hERG1 channel activation using 4-second test pulses. $I_{\text{tail-peak}}$ in the presence of Rg3 was normalized relative to the maximum $I_{\text{tail-peak}}$ measured under control condition ($n = 6$). (C) Plot of [Rg3]-dependent shift in $V_{0.5}$ for activation ($n = 6$). Data were fitted with logistic equation (smooth curve); $EC_{50} = 455 ± 25$ nM, $n_H = 1.48 ± 0.10$. (D) Representative tail currents recorded in an oocyte under control conditions and after treatment with 3 μM Rg3. Small horizontal arrows indicate zero current level. Upper panel shows voltage pulse protocol. (E) Effect of 3 μM Rg3 on the time constants for current deactivation ($\tau_{\text{deact}}$) ($n = 6$; two-way ANOVA, $p < 0.0001$). (F) [Rg3]-response relationships for the fold increase in $\tau_{\text{deact}}$ at $–70$ mV. $EC_{50} = 1.83 ± 0.25$ μM ($n_H = 1.03 ± 0.05$, $n = 6$).

**Fig. 4.** Concentration and voltage-dependent effects of Rg3 on partially C-deleted hERG1 channel currents. (A) Representative C-del hERG1 channel currents recorded from a single oocyte before (control) and after treatment with 3 μM Rg3. Small horizontal arrows indicate zero current level. Upper panel shows voltage pulse protocol. (B) Effects of 3 μM Rg3 on voltage dependence of C-del hERG1 channel activation using 1-second test pulses. $I_{\text{tail-peak}}$ in the presence of Rg3 was normalized relative to the maximum $I_{\text{tail-peak}}$ measured under control condition ($n = 5$). Control data were fitted with a single Boltzmann function ($V_{0.5} = –24.6 ± 1.1$ mV, $k = 11.1 ± 0.25$). G-V relationships measured after application of Rg3 were fitted with the sum of two Boltzmann functions, with component “1” assumed to represent unbound channels having $V_{0.5}$ and $k$ fixed at control values and component “2” assumed to represent the Rg3-bound fraction of channels. For 3 μM Rg3: $A_{\text{max1}} = 0.26$, $A_{\text{max2}} = 0.74$, $V_{0.5(1)} = –64.7 ± 0.6$ mV, $k_2 = 6.9 ± 0.3$. Rg3 data were poorly fit using a single Boltzmann function (dashed smooth curve). (C) Representative tail currents recorded in an oocyte under control conditions and after treatment with 3 μM Rg3. Small horizontal arrows indicate zero current level. Upper panel shows voltage pulse protocol. (D) Effect of 3 μM Rg3 on the time constants for current deactivation ($\tau_{\text{deact}}$) ($n = 7$; two-way ANOVA, $p < 0.0001$).

**Fig. 3.** Concentration and voltage-dependent effects of Rg3 on N-terminal deleted hERG1 channel currents. (A) Representative currents recorded from a single oocyte before (control) and after treatment with 3 μM Rg3. Small horizontal arrows indicate zero current level. Upper panel shows voltage pulse protocol. (B) Effects of 0.1–10 μM Rg3 on voltage dependence of N-del hERG1 channel activation using 4-second test pulses. $I_{\text{tail-peak}}$ in the presence of Rg3 was normalized relative to the maximum $I_{\text{tail-peak}}$ measured under control condition ($n = 6$). (C) Plot of [Rg3]-dependent shift in $V_{0.5}$ for activation ($n = 6$). Data were fitted with logistic equation (smooth curve); $EC_{50} = 455 ± 25$ nM, $n_H = 1.48 ± 0.10$. (D) Representative tail currents recorded in an oocyte under control conditions and after treatment with 3 μM Rg3. Small horizontal arrows indicate zero current level. Upper panel shows voltage pulse protocol. (E) Effect of 3 μM Rg3 on the time constants for current deactivation ($\tau_{\text{deact}}$) ($n = 6$; two-way ANOVA, $p < 0.0001$). (F) [Rg3]-response relationships for the fold increase in $\tau_{\text{deact}}$ at $–70$ mV. $EC_{50} = 1.83 ± 0.25$ μM ($n_H = 1.03 ± 0.05$, $n = 6$).
Dependence of $I_{\text{test-max}}$ on voltages negative to $-20$ mV and shifted the peak of the $I_{\text{test-max}}$-$V$ relationship to a more negative potential. Consistent with these effects, Rg3 caused a negative shift in the voltage-dependence of channel activation (Fig. 6B, Table 1). The shift in $V_{0.5}$ for activation by Rg3 achieved a maximum of $-20.5$ mV at $3 \mu M$ with an $EC_{50}$ of $374 \pm 7$ nM ($n = 8-12$; Fig. 6C).

Rg3 increased the maximum $I_{\text{tail-peak}}$ by 44% at $3 \mu M$. The effect of $3 \mu M$ Rg3 on deactivation of rERG3 channels was determined over a voltage range from $-30$ to $-120$ mV (Fig. 6D). Rg3 increased $\tau_a$ and $\tau_d$ for deactivation (Fig. 6E) and the relative amplitude of the slowly deactivating component of $I_{\text{tail}}$ (Fig. 6F) at all but the most negative $V_{\text{ret}}$. In summary, Rg3 shifted the voltage dependence of activation and slowed the rate of deactivation of rERG3 channel currents in a manner and potency similar to that observed for hERG1 channels.

**Effects of Rg3 on oERG1 Channel Gating.** Rg3 increased both the rate of activation and the magnitude of outward oERG1 currents elicited with 1-second depolarizing pulses (Fig. 7A). Note that tail currents were not evident under control conditions or after treatment of cells with Rg3 when the cell was repolarized to a $V_{\text{rest}}$ of $-120$ mV, indicating that the rate of channel deactivation was faster than the duration of the repolarization-induced whole cell capacitance current.

In the absence of quantifiable $I_{\text{tail}}$, the voltage dependence of hEAG1 channel activation was calculated by dividing $I_{\text{test-max}}$ by the electrical driving force ($V_t - E_{\text{rev}}$). As did its effects on ERG channels, Rg3 shifted the voltage-dependence of hEAG1 current activation to more negative potentials in a concentration-dependent manner (Fig. 7, B and C, Table 2). However, the potency of Rg3 on hEAG1 was lower ($EC_{50} = 1.31 \mu M$) and the maximum shift in $V_{0.5}$ for activation was greater ($-28$ mV) compared with ERG channels. In response to membrane depolarization from a holding potential of $-120$ mV, the onset of hEAG1 channel currents was characterized by an initial sigmoidal component, followed by a rapid time course of activation. This current was well described by a single exponential function with a $\tau_a$ that varied from 360 milliseconds at $-40$ mV to 110 milliseconds at $+40$ mV (Fig. 7A). After treatment of the cell with $3 \mu M$ Rg3, the rate of current activation was accelerated, the initial sigmoidal onset was no longer evident (Fig. 7A), and the time dependence of current activation was best described by a biexponential function with a $\tau_a$ that varied from 16 to 11 milliseconds and a $\tau_d$ that varied from about 240 to 175 milliseconds over the 90 mV range of $V_t$ (Fig. 7D). In summary, Rg3 increased the magnitude and greatly accelerated the rate of activation of currents and induced a negative shift in the voltage dependence of activation of hEAG1 channels.

Rg3 **Produces a Large Negative Shift in the Voltage Dependence of hELK1 Channel Activation.** hELK1 channel currents are activated at more negative potentials than other members of the EAG subfamily of channels (Engeland et al., 1998) and also inactivate slightly at voltages less negative than $-100$ mV. Therefore, under control conditions, hELK1 channel currents were elicited from a holding potential of $-100$ or $-110$ mV and preceded by a 1-second prepulse to $-150$ mV to ensure that all channels were in a closed state before activation with depolarizing test pulses. As illustrated in Fig. 8A, time-dependent currents were activated in response to a depolarizing pulse to $-80$ mV or higher potential and a slight time-dependent decay (inactivation) was observed at $V_t > 0$ mV. After application of $3 \mu M$ Rg3, currents were instantaneous in onset at any $V_t$ examined, and the magnitude of $I_{\text{test-max}}$ was decreased and did not inactivate at positive potentials (Fig. 8A). The average $I_{\text{test-max}}$-$V$ relationships for hELK1 before and after treatment of oocytes with $3 \mu M$ Rg3 ($n = 12$) are plotted in Fig. 8B. Note that $I_{\text{test-max}}$ was increased modestly by Rg3 when $V_t$ was between $-120$ and $-40$ mV but was decreased by Rg3 when $V_t$ was $> -20$ mV.

The kinetics for the onset of, and recovery from, the effects of Rg3 on hELK1 channels was measured by superfusing the small oocyte chamber (volume = $0.2 \text{ ml}$) with solution at a rate of $6.5 \text{ ml/min}$. The rate of Rg3 onset was monitored by measuring the amplitude of initial current ($I_{\text{init}}$) in response to a train of 0.6-second pulses to a $V_t$ of $0$ mV applied once every 4 seconds from a holding potential of $-100$ mV. The time constant for the onset ($\tau_{\text{init}}$) of $0.3 \mu M$ Rg3 effects on $I_{\text{init}}$ was $6.1 \pm 0.8$ seconds and the time constant for recovery ($\tau_{\text{rec}}$) was $19.3 \pm 1.8$ seconds ($n = 10$, Supplemental Fig. 3). In the presence of Rg3, the voltage dependence of activation of hELK1 channels was dramatically shifted to more negative potentials. At the very negative $V_{\text{rest}}$ that was required to elicit...
measurable $I_{\text{tail}}$, it is difficult to distinguish between currents produced by deactivating hELK1 channels from time-dependent endogenous Cl current. Thus, the voltage dependence of hELK1 channel activation was estimated by measuring $I_{\text{init}}$, the initial current activated at $+20\,\text{mV}$ immediately after a 2-second prepulse to a variable $V_{\text{pre}}$ (Fig. 8C). The rates of current activation during the pulse to $+20\,\text{mV}$ are more easily visualized in Fig. 8D, where currents are depicted on an expanded time-scale. The ratio $I_{\text{init}}/I_{\text{test-max}}$ before and after treatment of an oocyte with Rg3 were normalized to their peak value determined under control conditions to obtain a measure of the relative conductance ($g/g_{\text{max}}$) that was plotted as a function of $V_{\text{pre}}$. The resulting conductance-voltage ($G$-$V$) relationships for multiple cells and concentrations of Rg3 are presented in Fig. 8E. Under control conditions, the $G$-$V$ relationship was well described by a single Boltzmann function with a $V_{0.5}$ of $-68.9 \pm 0.4\,\text{mV}$ and a slope factor ($k$) of $11.8 \pm 0.3\,\text{mV}$ ($n = 30$; Fig. 8E, black circles). However, the $G$-$V$ relationships for currents measured in the presence of 0.1 or 0.3 mM Rg3 were not described by a simple sigmoidal function, perhaps because the data reflect the voltage dependence of activation for two populations of channels (Rg3-bound and Rg3-free). Thus, the $G$-$V$ relationships for $0.1, 0.3$, and $1\,\text{mM}$ Rg3 were fitted by the sum of two Boltzmann functions, with the $V_{0.5}$ and $k$ for one component fixed at the values determined from the single Boltzmann fit under control conditions. Rg3 induced a concentration-dependent negative shift in the more negative $V_{0.5}$ values of the $G$-$V$ relationship (representing the presumed Rg3-bound population of channels) with an $EC_{50}$ of $197 \pm 35\,\text{nM}$ (Fig. 8F).

The large negative shift in the $G$-$V$ relationship for hELK1 channels induced by Rg3 and the presence of time-dependent endogenous currents made it difficult to accurately measure rates of current deactivation at very negative potentials in the presence of Rg3. Therefore, we limited our analysis of deactivation to $0.3\,\text{mM}$ Rg3 over a limited range on $V_{\text{ret}}$. In the example shown in Fig. 9A, $0.3\,\text{mM}$ Rg3 caused a modest slowing of $I_{\text{tail}}$ decay at $-130\,\text{mV}$. The time-course of $I_{\text{tail}}$ was best fit with a double exponential function, and Rg3 increased the time constants for both components $\tau_1$ and $\tau_2$ by 2- to 3-fold (Fig. 9B). The relative amplitude of the slow component of deactivation was unaffected ($p = 0.18$; Fig. 9C). Thus, in contrast to hERG1 and rERG3 channels, Rg3 induces a much larger negative shift in the $G$-$V$ for hELK1 channel activation, but a more modest effect on the rate of deactivation.

hERG1 and rERG3 channels exhibit strong voltage-dependent inactivation, greatly reducing the magnitude of outward currents at positive potentials. In contrast, the voltage-dependent inactivation of hELK1 channels is barely detectable, with a maximum decrease in current magnitude of about 5% at positive potentials. The voltage pulse protocol used to determine the $G$-$V$ relationship for activation was used to estimate the voltage dependence of inactivation for hELK1 channel currents (Fig. 10A). The peak outward currents activated during the second pulse to $+20\,\text{mV}$ (highlighted by box in Fig. 10A) were normalized to the maximum current elicited after a prepulse to $-140\,\text{mV}$ and plotted as a function of $V_{\text{ret}}$.
multiple oocytes. The normalized G-V was fitted with a Boltzmann function (Fig. 10B) to determine the cumulative effects of 0.1 and 0.3 mM Rg3 (Fig. 7), or 3 μM Rg3 (Fig. 6). Rg3 at 3 μM caused an apparent decrease in 60 mV. Data were fitted with a logistic equation (smooth curve). \( k_{\text{max}} = 1.31 \pm 0.16 \mu M \) (\( n_{\mu M} = 0.70 \pm 0.05 \)). (D) Plot of time constants for current activation. Control currents were best fit with a single exponential, whereas currents in the presence of Rg3 were best fit with two exponentials \( n_{\mu M} = 3 \). (E) Fraction of the fast component of deactivation, \( k_{\text{A}} / k_{\text{A}} + k_{\text{A}} \) for currents measured in the presence of 3 μM Rg3 \( n_{\mu M} = 18 \).

**Fig. 7.** Rg3 accelerates the rate and shifts the voltage dependence of activation for hEAG1 channels. (A) Representative hEAG1 currents recorded from a single oocyte before (control) and after treatment with 3 μM Rg3. Upper panel shows voltage pulse protocol. (B) Effects of Rg3 on voltage dependence of hEAG1 channel activation using 2-second test pulses. Rg3 data were normalized relative to \( \gamma_{\text{max}} \) under control conditions \( \gamma_{0.5} \). (C) \([\text{Rg3}]\)-response relationship for the shift in \( \gamma_{0.5} \) for hEAG1 activation. Data were fitted with a logistic equation. \( E_{50} = 1.31 \pm 0.16 \mu M \) (\( n_{\mu M} = 0.70 \pm 0.05 \)). (D) Plot of time constants for current activation. Control currents were best fit with a single exponential, whereas currents in the presence of Rg3 were best fit with two exponentials \( n_{\mu M} = 18 \). (E) Fraction of the slow component of deactivation, \( k_{\text{A}} / k_{\text{A}} + k_{\text{A}} \) for currents measured in the presence of 3 μM Rg3 \( n_{\mu M} = 18 \).

...suitable parameters for reconstruction of hELK1 currents, and the fit errors were considerably larger than for the six-state model. Further studies with more complex models, for instance, the 10-state and 12-state model previously applied by us (Garg et al., 2012) for modeling of EAG1 channels, did not lead to smaller fit errors using the same protocols for parameter optimization as for a six-state model. Additionally, we explored a model with voltage-independent inactivation; however, this model was not further investigated because parameterization led to large fit errors with experimental data. We also explored models with inactivation coupled to closed states. Here the parameterization did not improve fit errors versus the presented six-state model. Preliminary studies also suggested that the current equation implemented on the basis of the \( K^+ \) reversal potential is superior to using the Goldman-Hodgkin-Katz current equation.

Simulated currents produced by the six-state model are presented in Fig. 11. The rate constant parameters \( \alpha_{\text{a}}, \alpha_{\text{b}}, \kappa_{\text{a}}, \kappa_{\text{b}} \) for the model are presented in Supplemental Table 3. Modeled currents are presented in Fig. 11, B–D, and predicted and experimental measures of \( I_{\text{test-max}} / \gamma_{\text{v}} \) relationships and voltage dependence of inactivation of control currents are presented in Figs. 11, E and F, respectively. Note that simulated currents shown in Figs. 11, B and D, compare well with the experimental currents elicited with the same voltage pulse protocol presented in Figs. 8, A and C. G-V relationships for hELK1 simulated with the two-compartment model under control conditions and after treatment with 0.1–3 μM Rg3 are plotted in Fig. 11G.

We also modeled G-V relationships for hELK1 channels in the presence of multiple concentrations of Rg3 assuming \( K_{\text{d}}, K_{\text{b}}, K_{\text{d}}, K_{\text{b}} \). When the rates were slowed by a factor of 100, the G-V relationships were unchanged compared with the G-V relationship presented in Fig. 11G. In contrast, when the rates increased by a factor of 100, the G-V relationships appeared to be reduced to a single Boltzmann function (Supplemental Fig. 4C). We explored several model variants for reconstructing Rg3 effects. Evaluation of fit errors yielded the presented two-compartment model on the basis of increased forward rates for voltage-dependent activation and opening in one compartment.

**TABLE 2 Effects of Rg3 on the voltage dependence of activation for hEAG1 channels**

<table>
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<th>( n )</th>
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<tr>
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**Markov Model for hELK1 Channels.** Mathematical models of WT hELK1 channel currents were developed to reconstruct experimental data. The best model for hELK1 channel currents in the absence of Rg3 included four closed states, one open state, and one inactivated state (Fig. 11A). Several variants of this model were explored in the initial development phase. Preliminary studies with a less complex model, i.e., four closed states and one open state, did not yield acceptable results for reconstructing experimental data. The best model for hELK1 simulated with the two-compartment model under control conditions and after treatment with 0.1–3 μM Rg3 is plotted in Fig. 11G.

**Discussion**

**Channel Selectivity of Rg3.** Choi et al. (2011a) had previously reported that Rg3 shifts the voltage dependence
of activation and slows the rate of deactivation of hERG1, but our study is the first to investigate effects of this compound on other members of the EAG family of K^+ channels. Rg3 affects activation gating of EAG family K^+ channels with an EC_{50} that varied from approximately 0.2 μM (hELK1) to 1.2 μM (hEAG1), a potency that is 5–500 times greater than what has been reported for Rg3 to activate or inhibit other voltage-gated channels. For example, Rg3 inhibits Kv1.4 channels with an

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Fig. 8. Rg3 induces a large negative shift in the voltage dependence of hELK1 channel activation. (A) Representative example of hELK1 currents recorded from a single Xenopus oocyte before (control) and after application of 3 μM Rg3. The holding potential was −110 mV. A 1-second prepulse to −150 mV was applied before each 1-second test pulse to a V_t that ranged from −140 to +40 mV. Small horizontal arrows indicate zero current level. (B) Normalized I_{test}-V_t relationships for hELK1 channel currents before and after application of 3 μM Rg3 (n = 12). (C) Voltage pulse protocol and corresponding hELK1 currents recorded before (control) and after application of 1 μM Rg3. Prepulses were applied to a V_{pre} that was varied from −150 to −10 mV before application of a test pulse to +20 mV. Small horizontal arrows indicate zero current level. (D) Expanded view of current traces during time highlighted by red boxes in (C). (E) G-V relationships for hELK1 channel currents under control conditions and after application of 0.1–3 μM Rg3. Control data were fitted with a single Boltzmann function (V_{0.5} = −68.9 ± 0.4 mV, k = 11.8 ± 0.3, n = 30). G-V relationships for currents measured after application of Rg3 were fitted with the sum of two Boltzmann functions, with component “1” assumed to represent unbound channels having V_{0.5} and k fixed at −68.9 mV and 11.8, respectively, and component “2” assumed to represent the Rg3-bound fraction of channels. For 0.1 μM Rg3: A_{max1} = 0.6, A_{max2} = 0.4, V_{0.5(2)} = −120 ± 0.4 mV, k_2 = 9.8 ± 0.3 (n = 5). For 0.3 μM Rg3: A_{max1} = 0.12, A_{max2} = 0.67, V_{0.5(2)} = −138 ± 0.4 mV, k_2 = 10.5 ± 0.4 (n = 6). For 1 μM Rg3: A_{max1} = 0.05, A_{max2} = 0.94, V_{0.5(2)} = −170 ± 0.4 mV, k_2 = 12.5 ± 1.4 (n = 7). The range of relative conductance (g_{max}/g_{max}) for 3 μM Rg3 (n = 9) was too narrow for accurate fitting, but V_{0.5(2)} was estimated to be −184 mV under the assumption that A_{max} = 0. (F) Average V_{0.5(1)}−V_{0.5(2)} values plotted as a function of [Rg3]. Data were fitted with the logistic equation (smooth curve); EC_{50} = 197 ± 35 nM; n_H = 0.8 ± 0.1.

Fig. 9. Rg3 induces a modest slowing of hELK1 channel deactivation. (A) Representative hELK1 tail currents measured before and after treatment of oocyte with 0.3 μM Rg3. Channels were activated by pulsing to 0 mV for 1 second, and tail currents were elicited at −130 mV. Currents were scaled to have equal amplitudes. (B) Fast (τ_f) and slow (τ_s) time constants for hELK1 deactivation under indicated conditions (n = 4; two-way ANOVA, p < 0.0001 compared with control). (C) Relative amplitude of the slow component of deactivation, A_s/(A_s + A_f) is not altered by 0.3 μM Rg3 (two-way ANOVA, p = 0.18).
Rg3 Activates EAG Family Channels

IC$\text{S}_{50}$ of 33 $\mu$M (Lee et al., 2008a). We examined Kv1.5 to confirm the relative lack of effect of Rg3 on Kv1 channel gating. At 3 $\mu$M, Rg3 reduced the magnitude of Kv1.5 channel currents by only 4% and shifted V$\text{S}_{50}$ for activation by only +2 mV (Supplemental Fig. 5). Rg3 also blocks KCNQ1 channels with an IC$\text{S}_{50}$ of 4.8 $\mu$M (Choi et al., 2010) and is a low potency activator (EC$\text{S}_{50}$ 15 $\mu$M) of BKCa channels (Choi et al., 2011b) and KCNQ1/KCNQ1 channels (Choi et al., 2010). Rg3 also suppresses a variety of voltage-gated Na$^+$ and Ca$^{2+}$ channels and ligand-gated channels, including 5-HT$\text{v}$ receptor and a7 nicotinic acetylcholine receptor channels with IC$\text{S}_{50}$ values ranging from 16–100 $\mu$M (Nah, 2014). Thus, although Rg3 is a nonspecific modulator of multiple channels, the potency of Rg3 activation on EAG family K$^+$ channels is 13–280 times greater than its effects on other ion channels. Screening of more channel types will be required before the relative channel selectivity of Rg3 can be fully understood.

Mechanisms of Rg3 Action. The major effects of Rg3 described in this study were a shift in voltage dependence of ERG1, ERG3, EAG1, and ELK1 activation, a slowing of ERG and ELK1 channel deactivation, acceleration of hEAG1 channel activation and enhancement of hELK1 inactivation. Rg3 could shift the voltage dependence of channel activation by 1) binding to the VSD and directly stabilizing the S3b-S4 segments in the activated configuration, 2) promoting electro-mechanical coupling between the VSD and the activation gate, or 3) a combination of both effects.

The relatively slow deactivation of native ERG channels is dependent on an interaction between specific residues in the N-terminal EAG domain [specifically within the Per-Arnt-Sim (PAS) domain or PAS-cap region] and the C-terminus of a neighboring hERG1 subunit (Gustina and Trudeau, 2011; Gianulis et al., 2013; Li et al., 2014), and/or the S4-S5 linker (Li et al., 2010; de la Peña et al., 2011; Ng et al., 2011). It was also recently proposed that specific charge-pair interactions may mediate slow deactivation, including Arg56 in the PAS domain with Asp803 in the CNBHD, and Arg residues in the PAS-cap domain with Glu residues in the C-linker (Ng et al., 2014). Thus, Rg3 could potentially promote a further slowing of deactivation if it stabilized one or more of these proposed N- to C-termini interactions. However, our finding that Rg3 dramatically slowed deactivation of N-del and C-del hERG1 channels, where such interactions are impossible, rules out such mechanisms. Alternatively, Rg3 might promote coupling of the link between the S4-S5 linker and C-terminal region of the S6 segment in hERG1 (Ferrer et al., 2006) and thereby slow the rate of channel closure. While the effects of Rg3 on hERG1 channel gating were independent of a covalent linkage between the VSD and pore domain, the compound may facilitate noncovalent interactions between these two channel regions.

Rg3 activates hERG1 and rERG3 channels by altering three distinct biophysical properties (V$\text{S}_{50}$ for activation, deactivation rate, current magnitude). The potency of Rg3 for altering these different properties of hERG1 channel function were relatively similar, suggesting that the activity of Rg3 is mediated, either directly or by allosteric mechanisms, by binding to a common binding site on the channel. Rg3 did not alter the voltage dependence of hERG1 channel inactivation. Thus, the [Rg3]-dependent increase in $I_{\text{tail-max}}$ is probably caused by an increase in single-channel open probability or an increase in single-channel conductance. Single-channel recordings will be required to further define the mechanism of enhanced current magnitude induced by Rg3.

Rg3 induced only a modest negative shift in the voltage dependence of activation for WT hERG1, N-del hERG1, rERG3, and EAG1 channels. Even in the presence of a maximal effective concentration of Rg3, the G-V relationships for these channels resembled a single Boltzmann function. However, Rg3 induced a much greater shift of activation gating for C-del hERG1, N/C-del hERG1, and hELK1 channels, and the resulting G-V relationships were not adequately described by a single Boltzmann function. Instead, a double Boltzmann function was used to describe the G-V relationships of these channels with one component representing Rg3-free channels and a second component representing a population of channels bound by Rg3. If Rg3 only bound to a single site/channel, the value of V$\text{S}_{50(2)}$ would not have varied with [Rg3]. However, we observed instead that the negative shift in V$\text{S}_{50(2)}$ for hELK1- was a function of [Rg3]. This finding suggests that Rg3 binds to more than one receptor site per channel and that the shift in activation gating is in some way proportional to the number of occupied binding sites. Our previous studies of concatenated hERG1 channel tetramers have indicated that other activators such as PD-118057 (Wu et al., 2014), RPR-260243 (Wu et al., 2015), and ICA-105574 (Wu et al., 2014) bind to four identical hydrophobic pockets situated between two adjacent subunits of the tetrameric hERG1 channel complex. A similar concatenated channel approach is required to better understand how Rg3 affects the gating of hELK1 channels.

Modeling of Rg3 Effects on hELK1 Channels. The developed six-state Markov model combined with a current
equation on the basis of the K\(^+\) reversal potential successfully reproduced major features of currents through hELK1 channels. Simulations with the two-compartment model support our hypothesis of multiple channel populations in the presence of Rg3, which are activated by a different range of transmembrane voltages (Fig. 11G). However, it is important to note that the Rg3 compartment illustrated in Supplemental Fig. 4A probably represents a diversity of channel populations, consisting of channels bound by one or more Rg3 molecules. Our model relies on the slow off-rate for unbinding of Rg3 from the hELK1 channel that was estimated by measuring the reversal of 0.3 \(\mu\)M Rg3 effects on channel gating during rapid washout of the compound. The rate of recovery from Rg3 effects (\(\tau_{\text{recovery}}\) of 19.3 seconds) was slow compared with the rate of Rg3 onset (\(\tau_{\text{onset}}\) of 6.1 seconds), indicating that washout was not hindered by a physical barrier such as the vitelline membrane. Simulations on the basis of a much faster off-rate of Rg3 yielded \(G-V\) relationships that are accurately described by single Boltzmann functions. Thus, we suggest that the combination of a slow off-rate for Rg3 and a large negative shift in the voltage dependence of gating by Rg3 result in \(G-V\) relationships that are described by the sum of two Boltzmann functions.

**Model Limitations.** Although our parameterization approach required only small user interaction, a limitation of this approach is the need for specification of weighting factors \(\omega_i\) and \(\omega_{CD}\) (Supplemental Table 1). Weighting factors were specified on the basis of visual inspection of simulations reproducing measured data and assumptions for channel gating. Further limitations are related to the development of the two-compartment model for Rg3 effects. We did not explore models with more compartments, which would have been able to describe channel populations with various Rg3 molecules bound to a hELK1 channel. Also, several approaches for setting microscopic reversibility in this model exist (Colquhoun et al., 2004), and it is difficult to identify the most appropriate approach. Our approach for setting microscopic reversibility led to progressively decreased unbinding rates for the pairs of states \(C_0/C_{d,0}\), \(C_1/C_{d,1}\), ..., \(C_5/C_{d,5}\). We believe that the model is not unique because a preliminary model, which did not account for microscopic reversibility, yielded very similar results (data not shown) as the presented model.

**Rg3 Binding Site.** At high concentrations, Rg3 is a non-specific ion channel modulator; however, it alters the gating of multiple members of the EAG family of Kv channels.

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**Fig. 11.** Markov modeling of hELK1 channel gating. (A) Schematic of six-state Markov model. (B) Simulated currents activated by voltage pulses to a \(V_t\) ranging from \(-100\) to \(40\) mV and applied in \(20\) mV increments. (C) Simulated currents in response to a 1-second \(V_{\text{pre}}\) to \(0\) mV and a 2-second pulse to \(V_{\text{ret}}\) that was varied from \(-140\) to \(-50\) mV in 10-mV increments. (D) Simulated currents in response to a \(V_{\text{pre}}\) that was varied from \(-150\) to \(-10\) mV in 10 mV increments, followed by a \(V_t\) to \(+20\) mV. (E) Comparison of simulated and measured \(I_{\text{unmax}}\) vs. \(V\) relationships. (F) Comparison of simulated and measured voltage dependence of hELK1 channel inactivation. (G) Comparison of simulated and measured \(G-V\) relationships for hELK1 activation in the absence (control) and presence of multiple concentrations of Rg3. Simulations were performed with the two-compartment model. The scaling factors for \(a_0\) of the model are presented in Supplemental Table S4.
at submicromolar concentrations. Rg3 is a large compound (MW = 785 Da), consisting of two sugar moieties attached to a sterol structure and its effects on channel gating are rapid in onset, suggesting an extracellular-accessible binding site. Thus, Rg3 probably alters the voltage dependence of channel activation by direct interaction with the outer region of the VSD. Other effects of Rg3 (e.g., increased current magnitude, slowed deactivation) may be mediated by binding to another site or allosteric modulation of channel function via binding to a single extracellular-accessible site. Finally, structural modifications of Rg3 and related ginsenosides may lead to the discovery of more selective and potent gating modulators of EAG family K⁺ channels.

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

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