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Pore Opening, Not Voltage Sensor Movement, Underpins the Voltage-Dependence of Facilitation by a hERG Blocker[S]

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ABSTRACT

A drug that blocks the cardiac myocyte voltage-gated K^+ channels encoded by the human ether-à-go-go–related gene (hERG) carries a potential risk of long QT syndrome and life-threatening cardiac arrhythmia, including *Torsade de Pointes*. Interestingly, certain hERG blockers can also facilitate hERG activation to increase hERG currents, which may reduce proarrhythmic potential. However, the molecular mechanism involved in the facilitation effect of hERG blockers remains unclear. The hallmark feature of the facilitation effect by hERG blockers is that a depolarizing preconditioning pulse shifts voltage-dependence of hERG activation to more negative voltages. Here we use a D540K hERG mutant to study the mechanism of the facilitation effect. D540K hERG is activated by not only depolarization but also hyperpolarization. This unusual gating property enables tests of the mechanism by which voltage induces facilitation of hERG by blockers. With D540K hERG, we find that nifekalant, a hERG blocker and class III antiarrhythmic agent, blocks and facilitates not only current activation by depolarization but also current activation by hyperpolarization, suggesting a shared gating process upon depolarization and hyperpolarization. Moreover, in response to hyperpolarizing conditioning pulses, nifekalant facilitates D540K hERG currents but not wild-type currents. Our results indicate that induction of facilitation is coupled to pore opening, not voltage per se. We propose that gated access to the hERG central cavity underlies the voltage-dependence of induction of facilitation. This study identifies hERG channel pore gate opening as the conformational change facilitated by nifekalant, a clinically important antiarrhythmic agent.

SIGNIFICANCE STATEMENT

Nifekalant is a clinically important antiarrhythmic agent and a human ether-à-go-go–related gene (hERG) blocker that can also facilitate voltage-dependent activation of hERG channels after a preconditioning pulse. Here we show that the mechanism of action of the preconditioning pulse is to open a conductance gate to enable drug access to a facilitation site. Moreover, we find that facilitation increases hERG currents by altering pore dynamics rather than acting through voltage sensors.

Introduction

The human ether-à-go-go–related gene (hERG) encodes a voltage-gated potassium channel Kv11.1 subunit. hERG channels underlie the rapid component of the cardiac delayed-rectifier potassium current (I_{Kr}) that mediates the rapid repolarization phase during the ventricular action potential (Sanguinetti et al., 1995; Trudeau et al., 1995; Sanguinetti and Tristani-Firouzi, 2006; Vandenberg et al., 2012). Drugs that decrease hERG current delay cardiac action potential repolarization and can result in long-QT syndrome, potentially leading to fatal arrhythmias such as *Torsade de Pointes* (TdP) (Surawicz, 1989; Sanguinetti et al., 1995; Roden, 2000, 2008; Sanguinetti and Tristani-Firouzi, 2006; Kannankeril et al., 2010; Vandenberg et al., 2012). hERG channels are unusually promiscuous targets for structurally diverse drugs, and designing drugs that do not inhibit hERG channels is a major challenge (Sanguinetti and Tristani-Firouzi, 2006; Vandenberg et al., 2012). Curiously, certain hERG blockers exhibit secondary agonistic effects on hERG current called "facilitation" that increase channel current at potentials close to the threshold for channel activation (Carmeliet, 1993; Jiang et al., 1999; Hosaka et al., 2007; Perry et al., 2010; Furutani et al., 2011, 2019; Yamakawa et al., 2012). This facilitation is proposed to increase I_{Kr} in ventricular myocytes during the repolarization phase of action potentials and lower the risk that a hERG blocker will cause arrhythmia (Furutani et al., 2019).

Nifekalant, a class III antiarrhythmic agent, blocks and facilitates hERG channels. Nifekalant facilitation of hERG channels shifts the conductance-voltage relation negative by

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ABBREVIATIONS: G-V, Conductance-Voltage; HEK, human embryonic kidney; hERG, human ether-à-go-go–related gene; I_{Kr}, rapid component of the delayed-rectifier potassium current; $V_{1/2}$, half-activation voltage; WT, wild-type.

~20 mV (Hosaka et al., 2007; Furutani et al., 2011, 2019). The hallmark feature of hERG facilitation by drugs is that this drug effect is induced by a depolarizing preconditioning pulse. However, how drugs affect the voltage-dependence of hERG activation and why preconditioning is required remains unclear. Here, we consider three molecular mechanisms that might produce the voltage dependence of preconditioning for facilitation: 1) the charged moiety of nifekalant, 2) movement of hERG voltage sensors, or 3) channel opening.

We distinguish between these mechanisms with a hERG mutant that alters the coupling between voltage sensor movement and pore opening. Sanguinetti et al. (1999, 2000) characterized an aspartate 540 to lysine (D540K) mutant located on the S4-S5 linker that can be activated unusually by hyperpolarization, in addition to relatively normal activation in response to depolarization (Sanguinetti and Xu, 1999; Mitcheson et al., 2000). The mechanism of hyperpolarization-induced opening of D540K hERG has identified open channel block and trapping properties of hERG blockers (Mitcheson et al., 2000; Perry et al., 2004; Witchel et al., 2004; Kamiya et al., 2006). Critical residues for nifekalant block and facilitation are distributed in the pore and S6 helix (Kamiya et al., 2006; Hosaka et al., 2007), far from hERG residue 540.

We first show that hyperpolarization-activated channel openings of D540K hERG enable nifekalant to access the binding site for nifekalant inhibition. We then demonstrate that strong hyperpolarization of D540K hERG, but not wild-type (WT) channels, promotes nifekalant's facilitation effect. These studies indicate that opening of the intracellular activation gate favors the binding of nifekalant to facilitate hERG channels.

Materials and Methods

Cells. A human embryonic kidney (HEK) 293 cell line stably expressing hERG (hERG-HEK) was kindly provided by Dr. Craig T. January and maintained in Dulbecco's Modified Eagle Medium (DMEM; Fujifilm Wako Pure Chemical Corp., Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France) and 400 μg/ml G418 (InvivoGen, CA) as previously described (Zhou et al., 1998). An inducible D540K hERG HEK293 cell line (T-REx D540K hERG HEK) was generated using the Flp-In T-REx system (Thermo Fisher Scientific, MA). The D540K hERG mutant was made by site-directed mutagenesis from the wild-type (WT) (GeneBank accession number U04270, WT hERG pCEP4), sequenced, and subcloned into the expression vector (pcDNA5/FRT/TO; Thermo Fisher Scientific). Cell lines were maintained in cell-culture treated polystyrene dishes at 37°C in a 5% CO_2 atmosphere in growth media composed of DMEM media containing 10% FBS, and 1% penicillin–streptomycin solution (Fujifilm Wako Pure Chemical Corp., Osaka, Japan). Cell lines were tested for mycoplasma contamination by DNA staining method with 4′,6-diamidino-2-phenylindole (DAPI) (BioGenex Laboratories, CA). Twenty-four hours before experiments, 1 μg/ml doxycycline HCl (Sigma-Aldrich, MO) was added to media to induce D540K hERG channel expression.

Electrophysiology. HEK cells used for electrophysiological study were adhered to the poly-L-lysine-coated (Sigma-Aldrich; molecular weight 30,000–70,000; 0.1 mg/ml for 2 hours at 37°C) coverslips (CS-5R; Warner Instruments Corp., MA) in 12-well plates and transferred to a small recording chamber mounted on the stage of an inverted microscope (IX71; Olympus Corp., Tokyo, Japan) and were continuously superfused with HEPES-buffered Tyrode's solution containing (in mM) 137 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.4 with NaOH). Membrane currents were recorded in a whole-cell configuration established using pipette suction (Hamill et al., 1981). The borosilicate micropipette had a resistance of 2 to 3 MΩ when filled with the internal pipette solution containing (in mM) 120 KCl, 5.374 $CaCl_2$, 1.75 $MgCl_2$, 10 EGTA, and 10 HEPES (pH 7.2 with KOH). Bath and internal solution osmolarity were adjusted to ~300 mOsm/kg with sucrose in some experiments. Liquid junction potential with this internal solution was calculated as less than −4 mV (Liquid Junction Potential Calculator; https://swharden.com/software/LJPcalc/app/), and the off-set was not corrected. Series resistance was typically under 5 MΩ. Series resistance compensation was used when needed to constrain voltage error to <10 mV. Leak compensation was not used. Whole-cell recordings were performed using an Axopatch 200B patch-clamp amplifier, Digidata 1322A interface, and pClamp 9 software (Molecular Devices, Sunnyvale, CA). The data were stored on a computer hard disk and analyzed using Clampfit 9 (Molecular Devices) and Igor Pro 7 and 9 (WaveMetrics, OR). Experiments to characterize hERG facilitation were performed at room temperature (22–25°C).

Nifekalant (CAS number: 130636-43-0) was obtained from Cayman Chemical (Ann Arbor, MI). Nifekalant stock solutions (30 mM) were prepared with distilled water, stored at −20°C until the day of the experiment, and diluted in the bathing solution before the experiments.

Monitoring Current Facilitation. The voltage protocols are indicated in each figure as insets. For determining the voltage dependence of hERG activation and drug actions, the voltage protocols consisted of 20-second cycles containing two voltage steps from the holding potential of −80 mV. The first voltage step is a 4-second "test pulse" to a voltage between +80 mV and +60 mV with 10-mV increments to activate hERG channels. Afterward, the cell is returned to −60 mV for 2 seconds to record tail currents. To test the induction of the facilitation effect by nifekalant, the preconditioning pulse to +60 mV or −160 mV for 4 seconds was applied 20 seconds before each test pulse.

To show the Current-Voltage (I-V) relationships, outward currents induced by a test pulse of −60 to +60 mV were normalized to the currents evoked by voltage steps to +10 mV without nifekalant. Inward currents induced by a test pulse of −160 mV to −60 mV were normalized to the currents evoked by voltage steps to −160 mV without nifekalant. To show the Conductance-Voltage (G-V) relationships (hERG activation curves), depolarization-activated tail currents recorded at −60 mV were normalized to the currents evoked by voltage steps to +10 mV without nifekalant. Hyperpolarization-activated tail currents recorded at −60 mV were normalized to the currents evoked by voltage steps to −160 mV without nifekalant. Fitting was carried out using Igor Pro 9 (WaveMetrics), which employs nonlinear least-squares curve fitting via the Levenberg-Marquardt algorithm. G–V relations were fit with a first power Boltzmann:

$$f(V)=A\left(1+e^{\frac{-\left(V-V_{\frac{1}{2}}\right)zF}{RT}}\right)^{-1}, \quad (1)$$

where $f(V)$ is normalized conductance (G), A is maximum amplitude, $V_{1/2}$ is activation midpoint, z is the valence in units of elementary charge (e_0), F is the Faraday constant, R is the ideal gas constant, and T is absolute temperature.

The minimum Gibbs free energy (ΔG_{Nif}) that nifekalant imparts to conductance was calculated as follows:

$$\Delta G_{Nif} = -R \times T \times \ln\left(fold\ change\ in\ K_{eq}\right). \quad (2)$$

Here, R = 0.00199 kcal/(K·mol) and T = 298 K. K_{eq}, or the equilibrium constant of channel opening, was approximated by the relative conductance of hERG either before or after induction of facilitation by nifekalant at $V_{1/2}$ of block (Table 1).

Data Analyses. Electrophysiological data were acquired and analyzed using commercial software [pClamp 9 software (Molecular Devices); Igor Pro 7 and 9 (WaveMetrics)]. All values are expressed as mean ± standard deviation (S.D.). Drug potencies were averaged using $-\mathrm{Log}IC_{50}$ (pIC_{50}). All statistical tests were performed using R (https://www.r-project.org/). We evaluated differences between multiple groups by one-way analysis of variance with Dunnett post-tests to compare drug-treatment conditions with the control conditions for WT and

TABLE 1
First-order Boltzmann parameter for G-V relationships

Channels	Treatment		G-V Fit Parameters			
			$V_{1/2}$ (mV)[a]	$\Delta V_{1/2}$ (mV) from Control[a]	z_i (e_0)	ΔG_{nif} (kcal/mol)[b]
WT	Control	Outward	−11.5 ± 0.4[A]		3.0 ± 0.2[I]	
	0.1 μM Nif, depo	Outward	−32.4 ± 0.7[B]	−20.9	2.8 ± 0.3[J]	−0.36
D540K	Control	Outward	−6.0 ± 0.4[C]		2.9 ± 0.3[K]	
		Inward	−102.3 ± 2.0[D]		−2.3 ± 0.2[L]	
	0.1 μM Nif, depolarization	Outward	−23.9 ± 0.7[E]	−17.9	2.9 ± 0.2[M]	−0.34
		Inward	−82.4 ± 2.4[F]	19.9	−2.1 ± 0.4[N]	−0.31
	0.1 μM Nif, hyperpolarization	Outward	−29.4 ± 0.9[G]	−23.4	3.0 ± 0.1[O]	−0.37
		Inward	−80.2 ± 1.1[H]	22.1	−2.4 ± 0.3[P]	−0.34

[a]Average $V_{1/2}$ and z values were derived from a first-order Boltzmann fit (eq. 1). All values are given as mean ± S.D.
[b]ΔG_{nif} from eq. 2 at $V_{1/2}$ for control.
Statistical analyses of variance test with Dunnett's multiple comparisons P values: IJ, 0.043; KM, 0.881; KO, 0.974; LN, 0.957; LP, 0.557; AB, CE, CG, DF, DH: <1e-10.

D540K channel. Data were considered statistically significant with P values lower than 0.05. Calculated P values should not be interpreted as hypothesis testing but only as descriptive. The experiments were designed to test the biologic hypotheses about the role of membrane depolarization required for the development of facilitation effect by nifekalant. As this study is exploratory, sample sizes were not predetermined. Neither blinding nor randomization were employed.

Results

The Facilitation-Voltage Relationship for Nifekalant Is Similar to the hERG Activation-Voltage Relationship. In *Xenopus* oocytes, the facilitation-voltage relationship for nifekalant is similar to the hERG activation-voltage relationship (Furutani et al., 2019). We assessed whether this was also the case in the HEK293 cells used in this study. We applied nifekalant at 0.1 μM, a concentration that enables facilitation of hERG in HEK293 cells (Furutani et al., 2019). Figure 1 shows the voltage-dependence of induction of facilitation effect by 0.1 μM nifekalant in WT hERG channels ($V_{1/2}$ = −11.4 ± 0.3 mV, z = 2.8 ± 0.1 e_0). This voltage dependence is within error of the voltage-dependence of hERG activation ($V_{1/2}$ = −11.5 ± 0.2 mV, z = 3.0 ± 0.1 e_0), suggesting that a conformational change involved in hERG activation is required for facilitation. Two types of hERG conformational changes might produce this voltage-dependence: outward voltage sensor movement or conformational change in the intracellular gate upon channel activation (Hille, 1977, 2001). To discriminate

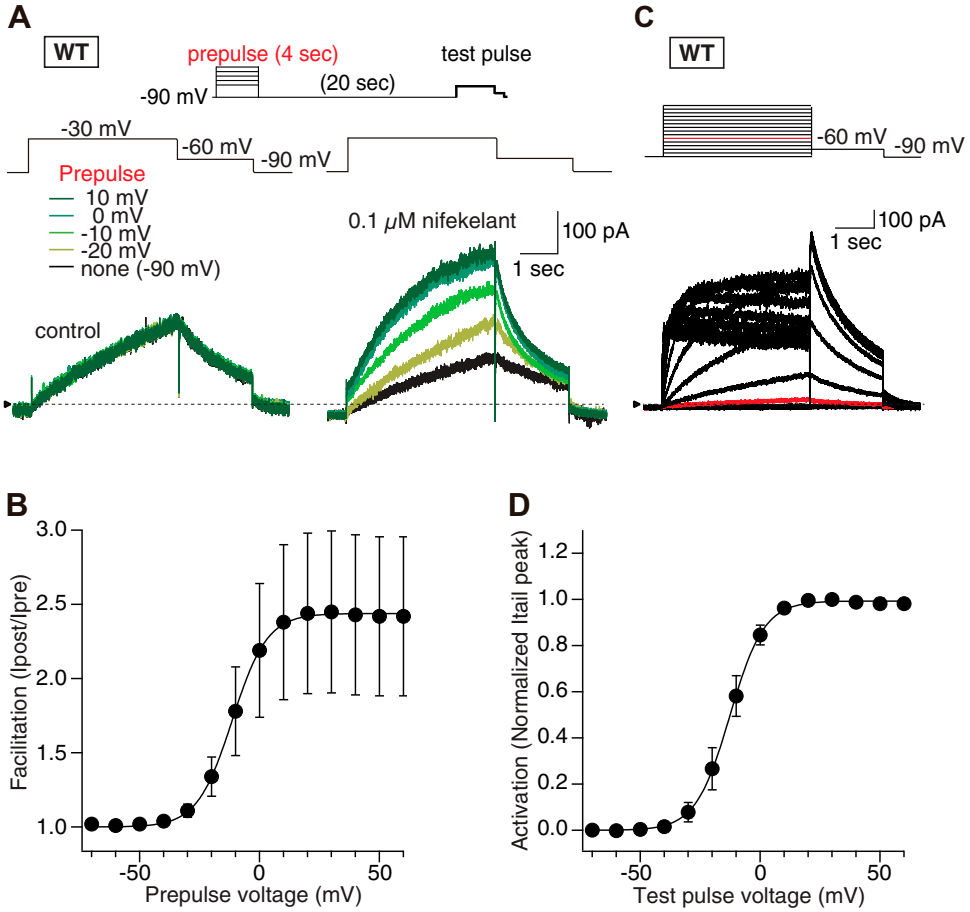

Fig. 1. Effects of a preconditioning step depolarization on the induction of facilitation in WT hERG channels. (A and B) Effect of a preconditioning step depolarization on the induction of facilitation in WT hERG channels. hERG currents were recorded from HEK293 cells stably expressing hERG at room temperature. 0.1 μM nifekalant was treated. (A) The amplitudes of a 4-second preconditioning depolarization (prepulse) were changed from −70 mV to +60 mV by 10-mV increments, and the effects on the currents upon the test pulse (−30 mV for 4 s, then −60 mV for 2 s) were assayed. Representative traces in the absence (left) and the presence (right) of 0.1 μM nifekalant upon test pulse are shown. (B) The relationship between membrane voltage of preconditioning pulse and the steady-state current amplitude. Data are mean ± S.D. (n = 4 cells). The curves were fitted with the Boltzmann function (eq. 1). (C and D) Voltage-dependent activation of WT hERG channels. (C) The macroscopic hERG currents in response to test pulses step (from −70 to +60 mV by 10 mV increments for 4 s, then −60 mV for 2 s) at room temperature; representative traces. (D) The relationship between membrane voltage of step pulse and the peak tail current amplitude. Data are mean ± S.D. (n = 6 cells). The curves were fitted with the Boltzmann function (eq. 1).

between these mechanisms, we altered the coupling between voltage sensor movement and pore opening.

Nifekalant Is an Open-Channel Blocker of hERG Channels and Inhibits D540K hERG Channels at Hyperpolarized Potentials. We used D540K hERG to explore the facilitation mechanism because of its unusual activation by hyperpolarization (Sanguinetti and Xu, 1999; Mitcheson et al., 2000). We confirmed the unique gating properties of this D540K hERG. Consistent with the prior reports, current elicited by depolarization from a holding potential of −80 mV appeared to activate instantaneously, followed by rapid inactivation. In the same cell, hyperpolarization from the holding potential of −80 mV activated a small instantaneous current followed by a much slower inward current (Supplemental Fig. 1, A and B). The instantaneous component has been proposed to represent channels open at −80 mV D540K hERG (Sanguinetti and Xu, 1999; Mitcheson et al., 2000). We found $V_{1/2}$ for depolarization-induced activation (−6.0 ± 0.2 mV, z = 2.9 ± 0.1 e_0) and $V_{1/2}$ for hyperpolarization-induced activation (−102.3 ± 1.0 mV, z = −2.3 ± 0.1 e_0) (Supplemental Fig. 1, C and D).

Before studying the facilitation induction, nifekalant block of D540K hERG channels was evaluated. Nifekalant inhibits WT and D540K hERG channels in a concentration-dependent manner (Fig. 2). The IC_{50} of nifekalant inhibition of outward WT hERG current at + 30 mV was 150 nM (mean of pIC_{50} ± error is 6.823 ± 0.062, n = 8 cells). The IC_{50} of D540K hERG at +30 mV was 663 nM (mean of pIC_{50} ± error is 6.178 ± 0.064, n = 5 cells), indicating a lower inhibitory potency (Fig. 2B). In addition, nifekalant is an even less potent blocker of D540K hERG inward currents, with an IC_{50} at −160 mV of 2923 nM (mean of pIC_{50} ± error is 5.534 ± 0.012, n = 5 cells) (Fig. 2, B and C). The difference in IC_{50} between +30 mV and −160 mV did not appear to be due a charge on nifekalant, as neither the inward nor outward current block had substantial voltage dependence.

Some hERG blockers have gated access to their blocking site, where onset of block requires opening of a path to the central cavity. To characterize how inhibition by nifekalant responds to voltage activation of WT and D540K hERG, we designed a 2-pulse protocol containing a brief test-pulse to +10 mV followed by repeating hyperpolarizing pulses to −120 mV that open only D540K but not WT hERG channels (Supplemental Fig. 1). In 1 μM nifekalant, WT hERG current amplitudes in response to this 2-pulse protocol decay over time (Fig. 3C). Furthermore, no inhibition occurs when cells are held at −80 mV or during repeating hyperpolarizing step-pulses to −120 mV (Fig. 3D), suggesting that hyperpolarization does not gate the path for nifekalant inhibition in WT hERG. In contrast, in 1 μM nifekalant, D540K hERG current progressively declines during repeated hyperpolarizing voltage steps to –120 mV (Fig. 3, A and B). As hyperpolarizing pulses to –120 mV open D540K hERG, these results suggest that opening of the conductance gate allows nifekalant to access its blocking site and that outward movement of voltage sensors is not required for access.

Hyperpolarization-Induced Currents of D540K hERG Can Be Facilitated by Nifekalant. Next, we asked if a depolarizing preconditioning pulse that induces facilitation in WT hERG channels (Hosaka et al., 2007; Furutani et al., 2011; Yamakawa et al., 2012) could induce facilitation in D540K hERG channels. As mentioned above, 0.1 μM nifekalant suppressed the outward currents of D540K hERG activated at depolarized potentials to 88% of control but not the inward currents activated at depolarized potentials (Fig. 2). In the

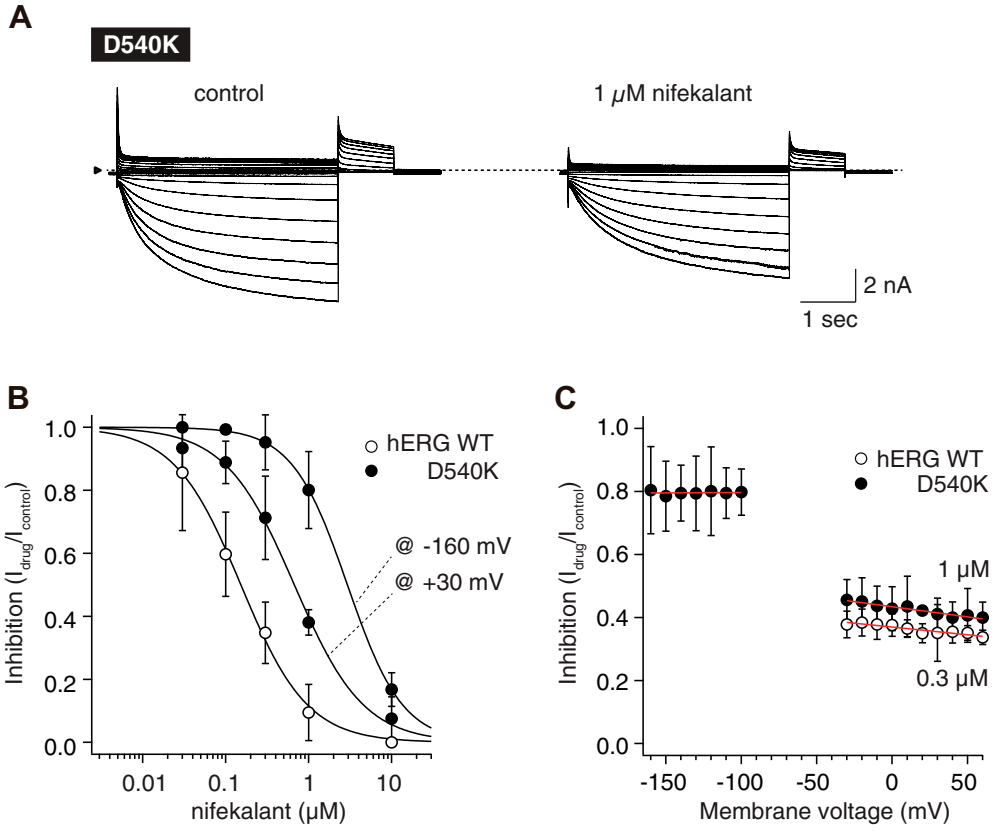

Fig. 2. Concentration-dependence block of WT and D540K hERG channels by nifekalant. Effects of nifekalant on WT and D540K hERG channels in HEK293 cells. hERG currents were recorded at room temperature. (A) Representative macroscopic D540K hERG currents in response to test pulses step (from −160 to +60 mV by 10 mV increments for 4 s, then −60 mV for 1 s). (B) Concentration-dependence block of WT (n = 8 cells) and D540K hERG channels (n = 6 cells) by nifekalant. Data are mean ± S.D. The curves were fitted with Hill equation. IC_{50} and Hill coefficient (h) are 152 ± 8 nM and 1.1 for nifekalant against WT hERG; 671 ± 47 nM and 1.0 against outward D540K hERG currents in HEK293 cells; and 2924 ± 36 nM and 1.3 against inward D540K hERG currents in HEK293 cells, respectively. (C) Voltage-dependence block of WT and D540K hERG channels by nifekalant. Concentrations of nifekalant are 0.3 μM and 1 μM against WT (n = 4 cells) and D540K hERG channels (n = 5 cells), respectively. Data are mean ± S.D. The plots were fitted by a linear.

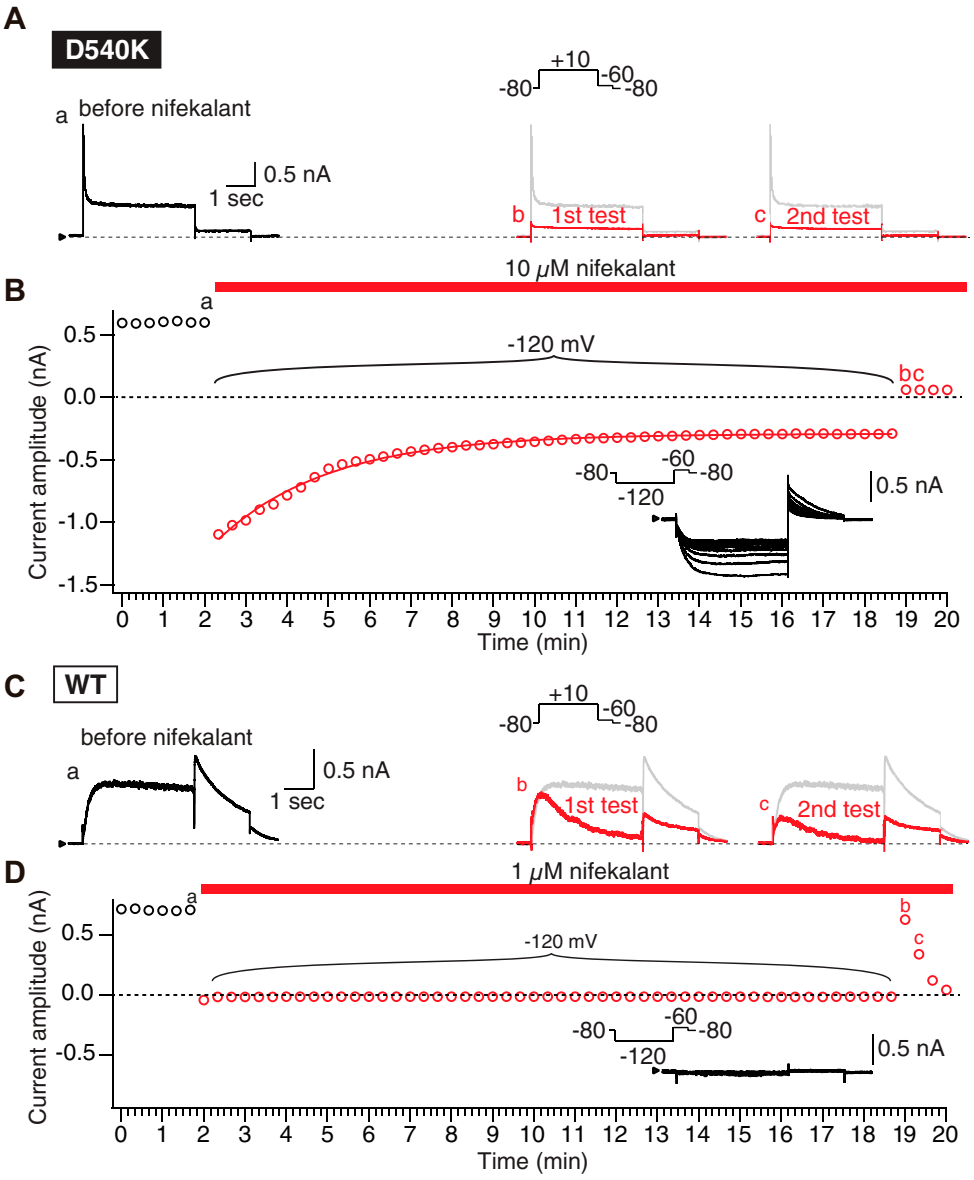

Fig. 3. Voltage stimuli are required for nifekalant to inhibit hERG channels. (A and B) Effect of hyperpolarizing voltage stimuli for nifekalant to inhibit D540K hERG channels. (A) Current traces from a representative cell expressing D540K hERG in vehicle (black and gray) or 10 μM nifekalant (red). Voltage protocol from a holding potential of −80 mV is a 4-second step to +10 mV followed by a 2-second step to −60 mV. Red A–C indicate time points labeled in (B). (B) Peak currents during step pulse (circles). Red bar indicates application of 10 μM nifekalant. Insets are representative of D540K hERG current traces in response to hyperpolarizing test pulses (−120 mV for 4 s, then −60 mV for 2 s) after the treatment of 10 μM nifekalant. Currents evoked by hyperpolarization step to −120 mV in nifekalant are fitted with an exponential function (red curve). Dotted black line indicates Zero current level. (C and D) Effect of hyperpolarizing voltage stimuli for nifekalant to inhibit WT hERG channels. (C) Current traces from a representative cell expressing WT hERG in vehicle (black and gray) or 1 μM nifekalant (red). Voltage protocol is the same as (A). Red A–C indicate time points labeled in (D). (D) Peak currents during step pulse (circles). Red bar indicates application of 1 μM nifekalant. Insets are representative WT hERG current traces in response to hyperpolarizing test pulses (−120 mV for 4 s, then −60 mV for 2 s) after the treatment of 1 μM nifekalant. Dotted black line indicates Zero current level.

presence of 0.1 μM nifekalant, we applied a 4-second prepulse to +60 mV 20 seconds before each test pulse since this voltage protocol is used to monitor the hERG facilitation in WT hERG channels (see Fig. 1). We found that the outward currents of D540K activated at depolarized potentials, especially at potentials close to the threshold, increased after prepulse to +60 mV in 0.1 μM nifekalant (Fig. 4). The $V_{1/2}$ of activation of the facilitated fraction was −23.9 ± 0.3 mV, shifted negative from the control ($\Delta V_{1/2}$ = −17.9 mV) by roughly the same degree as WT ($\Delta V_{1/2}$ = −20.9 mV) (Furutani et al., 2019). Such a shift is a hallmark feature of facilitation by nifekalant and other facilitating blockers (CarBeliet, 1993; Jiang et al., 1999; Hosaka et al., 2007; Perry et al., 2010; Furutani et al., 2011; Yamakawa et al., 2012; Furutani et al., 2019).

Interestingly, prepulse to +60 mV in 0.1 μM nifekalant also increases the inward currents of D540K (Fig. 4). The voltage-midpoint of the hyperpolarization-activated D540K currents was −82.4 ± 1.2 mV, shifted to more positive voltages by 19.9 mV (Fig. 4D). This interesting result reveals that nifekalant can facilitate both the usual depolarization activation mechanism and the unusual hyperpolarization activation mechanism of D540K hERG.

Hyperpolarization Can Induce hERG Facilitation in D540K but Not WT hERG. The hypothesis that facilitation is induced by stimuli that activate the hERG conductance predicts that facilitation will be also induced when the D540K hERG conductance is activated by hyperpolarization. To examine this possibility, we subjected D540K cells to a hyperpolarizing prepulse in the presence of nifekalant. First, we experimented with 0.1 μM nifekalant, the concentration in Fig. 4. We found that a 4-second hyperpolarizing prepulse to −160 mV could not induce facilitation (Fig. 5). We reasoned that this could be because the 0.1-μM concentration of nifekalant is insufficient to interact with D540 hERG channels at −160 mV, as 0.1 μM does not appreciably block inward currents activated at hyperpolarized potentials (Fig. 2B) and that facilitation occurs only with concentrations that also block channels (Furutani et al., 2011). To block D540K hERG at –160 mV to a similar degree as 0.1 μM nifekalant blocked at +20 mV (19%, see Fig. 4, C and D), we increased nifekalant to 1 μM to block

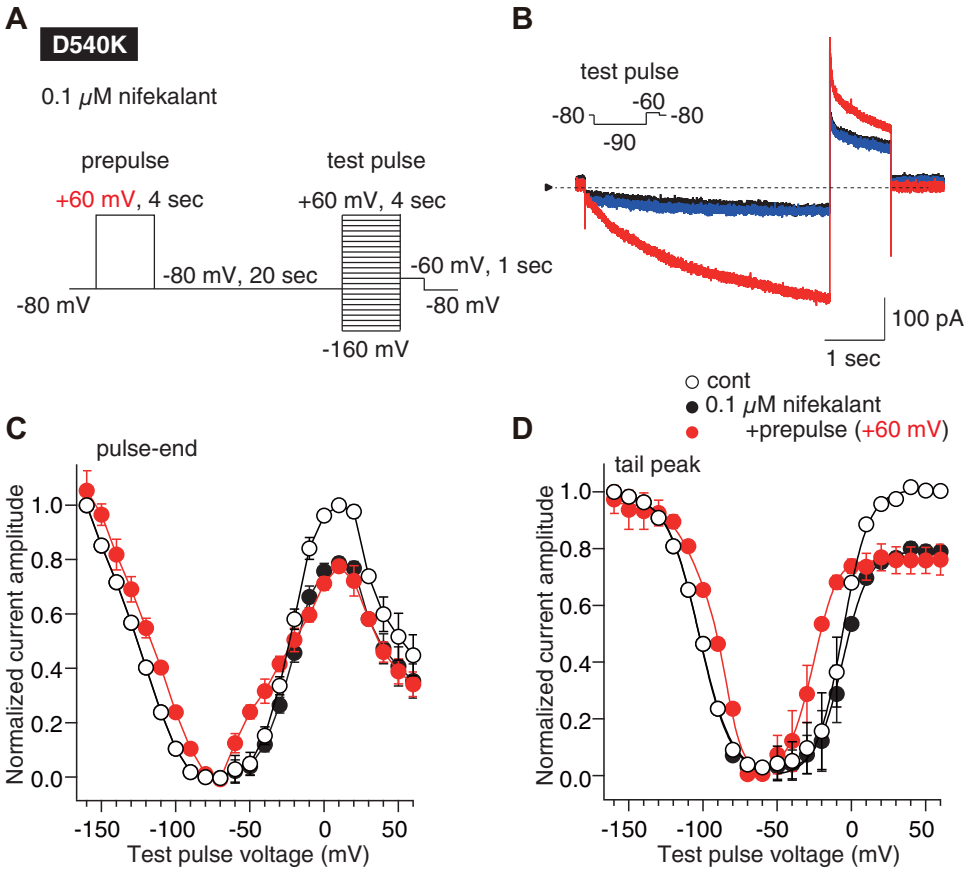

Fig. 4. Nifekalant facilitates D540K hERG activation at both depolarized and hyperpolarized potentials. (A) Pulse protocol of this experiment. The amplitudes of a 4-second preconditioning depolarization (prepulse) were +60 mV, and the effects on the currents from HEK cells expressing D540K hERG upon the test pulse (from −160 mV to +60 mV by 10 mV increments for 4 s, then −60 mV for 2 s) were assayed. (B) Superimposed D540K hERG currents recorded in the same cell before (cont., black) and after perfusion of 0.1 μM nifekalant with (+prepulse, red) or without (blue) the preconditioning pulse of +60 mV. (C and D) The relationship between membrane voltage of test pulse and the steady-state current amplitude (C) and the tail current amplitude (activation curve) (D). (C) The amplitudes of hERG step currents in the absence (open circles) and in the presence of 0.1 μM nifekalant with (filled black circles) or without (filled red circles), the preconditioning pulses were measured at the pulse-end during the test pulse to indicated voltages. Outward currents recorded at −50 mV to +60 mV were normalized to the currents evoked by voltage steps to +10 mV in the absence of nifeka lant. Inward currents recorded at −160 mV to −60 mV were normalized to the currents evoked by voltage steps to −160 mV in the absence of nifekalant. (D) Voltage-dependent hERG activation. The amplitudes of hERG tail currents in the absence (open circles) and in the presence of 0.1 μM nifekalant with (filled black circles) or without (filled red circles), the preconditioning pulses were measured at the peak after the test pulse to indicated voltages. Depolarization-activated tail currents recorded at −60 mV was normalized to the currents evoked by voltage steps to +10 mV in the absence of nifekalant. Hyperpolarization-activated tail currents recorded at −60 mV were normalized to the currents evoked by voltage steps to −160 mV in the absence of nifekalant. Data are mean ± S.D. (n = 5 cells). In activation curve (D), the curves were fitted with the single (for control and nifekalant without prepulse) or double Boltzmann equation (for nifekalant with prepulse).

inward current of D540K hERG channels at −160 mV by 20% (Fig. 6). In 1 μM nifekalant, a –160-mV prepulse induced facilitation (Fig. 6). The facilitated $V_{1/2}$ for the currents activated at depolarized potentials and hyperpolarized potentials were −29.4 ± 0.5 mV ($\Delta V_{1/2}$ from the unfacilitated $V_{1/2}$ = −23.4 mV) and −80.2 ± 0.6 mV ($\Delta V_{1/2}$ = 22.1 mV), respectively (Fig. 6D). Thus, in 1 μM nifekalant, a –160-mV prepulse induced facilitation similar to a +60-mV prepulse in 0.1 μM.

To eliminate the possibility that hyperpolarizing prepulses induce facilitation of WT hERG, we conducted controls with the same hyperpolarizing prepulse protocol on WT hERG. Facilitation was not induced by a –160-mV prepulse with either 0.1 μM or 1 μM nifekalant (Fig. 7).

These results indicate that to induce facilitation by nifekalant, 1) a preconditioning pulse that activates hERG is required and 2) nifekalant must enter a facilitation site in the pore, resulting in block, during the preconditioning pulse.

Discussion

These results with D540K hERG advance our understanding of the mechanism of hERG channel facilitation by blocking agents. Specifically, these results clarify that facilitation is a stabilization of the open conformation of the pore rather than a negative shift of voltage sensitivity and that the prepulse that induces facilitation acts by opening a gate within the channel.

What Is the Mechanism Underlying the Induction of Facilitation by a Prepulse? A preconditioning pulse to a depolarized or positive potential is required to induce facilitation by nifekalant (Hosaka et al., 2007; Furutani et al., 2011). This depolarization-dependence of the induction of facilitation is a common characteristic of facilitation induction for other hERG channel inhibitors (Carmeliet, 1993; Jiang et al., 1999; Perry et al., 2010; Furutani et al., 2011; Yamakawa et al., 2012). In this study, we find that activation of the hERG channel conductance, not depolarization per se, is

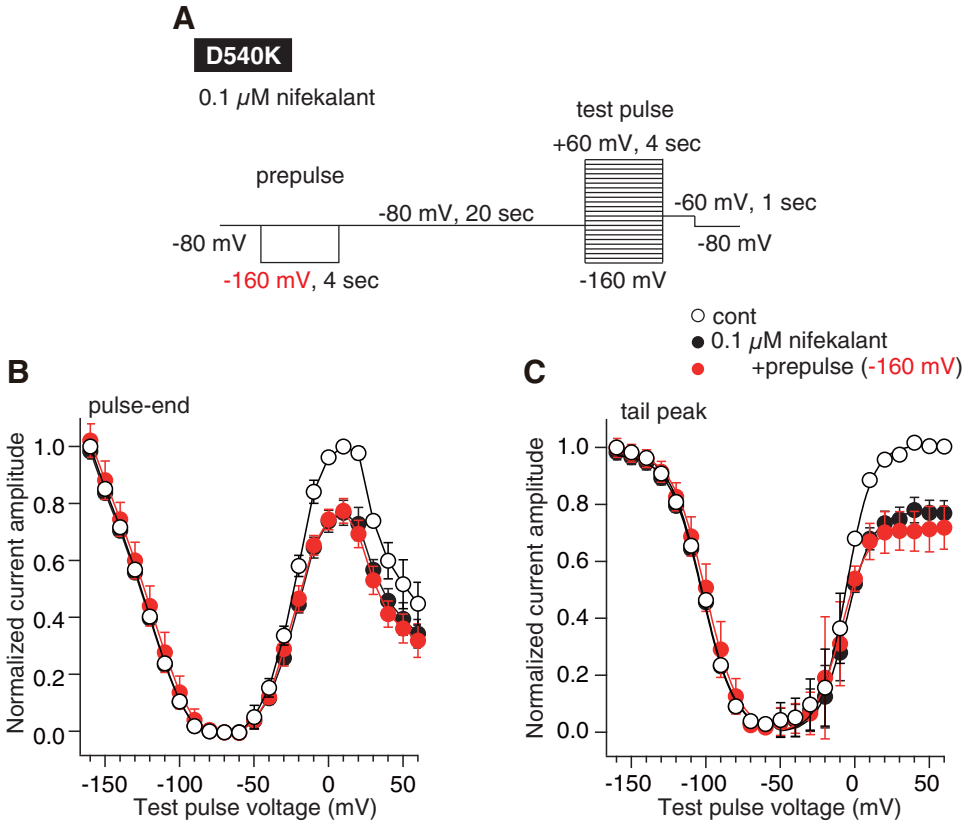

Fig. 5. Hyperpolarizing prepulse does not induce facilitation effect on D540K hERG activation in the presence of 0.1 μM of nifekalant. (A) Pulse protocol of this experiment. The amplitudes of a 4-second preconditioning hyperpolarization (prepulse) were −160 mV, and the effects on the currents from HEK cells expressing D540K hERG upon the test pulse (from −160 mV to +60 mV by 10 mV increments for 4 s, then −60 mV for 2 s) were assayed. (B and C) The relationship between membrane voltage of test pulse and the steady-state current amplitude (B) and the tail current amplitude (activation curve) (C). (B) The amplitudes of hERG step currents in the absence (open circles) and in the presence of 0.1 μM nifekalant with (filled black circles) or without (filled red circles), the preconditioning pulses were measured at the pulse-end during the test pulse to indicated voltages. Outward currents recorded at −50 mV to +60 mV were normalized to the currents evoked by voltage steps to +10 mV in the absence of nifekalant. Inward currents recorded at −160 mV to −60 mV were normalized to the currents evoked by voltage steps to −160 mV in the absence of nifekalant. (C) Voltage-dependent hERG activation. The amplitudes of hERG tail currents in the absence (open circles) and in the presence of 0.1 μM nifekalant with (filled black circles) or without (filled red circles), the preconditioning pulses were measured at the peak after the test pulse to indicated voltages. Depolarization-activated tail currents recorded at −60 mV were normalized to the currents evoked by voltage steps to +10 mV in the absence of nifekalant. Hyperpolarization-activated tail currents recorded at −60 mV were normalized to the currents evoked by voltage steps to −160 mV in the absence of nifekalant. Data are mean ± S.D. (n = 5 cells). In activation curve (C), the curves were fitted with the single Boltzmann function (eq. 1).

required to induce facilitation, as activating the D540K conductance by hyperpolarization also induces facilitation. Why is activation of the hERG conductance required to induce facilitation? We propose that activation of the hERG conductance allows the hERG blocker to access a facilitation site. Voltage-gated ion channel activation involves opening an S6 intracellular gate that can allow channel blockers to access a binding site in the central cavity of channel (del Camino and Yellen, 2001; Sanguinetti and Tristani-Firouzi, 2006; Hosaka et al., 2007; Vandenberg et al., 2012; Asai et al., 2021). Alanine-scanning mutagenesis has suggested that amino acid residues of the central cavity around the pore helix such as T623, S624, V625 and on S6 helix, such as G648, Y652, and F656 are important for facilitation (Hosaka et al., 2007). Thus, it is likely that facilitating hERG blockers enter the central cavity when the intracellular gate is opened. This idea is supported by the finding that nifekalant must block during a prepulse to induce facilitation, whether the prepulse activates D540K by depolarization or hyperpolarization (Figs. 4–6). This suggests that when facilitation is induced, the membrane potential must open the intracellular gate such that the drug can enter a site in the central cavity. It is unclear if the drug binds similarly to block and facilitate. A previous mutagenesis study showing different impacts of single amino substitutions on block and facilitation suggests that the molecular interactions for facilitation are different than for block (Hosaka et al., 2007). Still, both interactions occur in the central cavity. These actions occur either by outward movement of the voltage-sensor domain (WT channels) or in response to inward movement of the voltage-sensor domain (for D540K channels). Thus, distinct stimuli that open the path to the central cavity enable both block and facilitation (Fig. 8).

How Does Nifekalant Affect Activation of hERG? How nifekalant facilitates activation remains a question. The present study demonstrates that nifekalant can also facilitate hyperpolarization-induced activation of D540K hERG channels. Thus, the polarity of the facilitation effect is malleable, indicating that facilitation does not simply stabilize the voltage sensors in an up state. This result suggests that nifekalant acts on a shared mechanism underlying D540K hERG openings by depolarization and hyperpolarization. Although the mechanism by which D540K hERG opens at

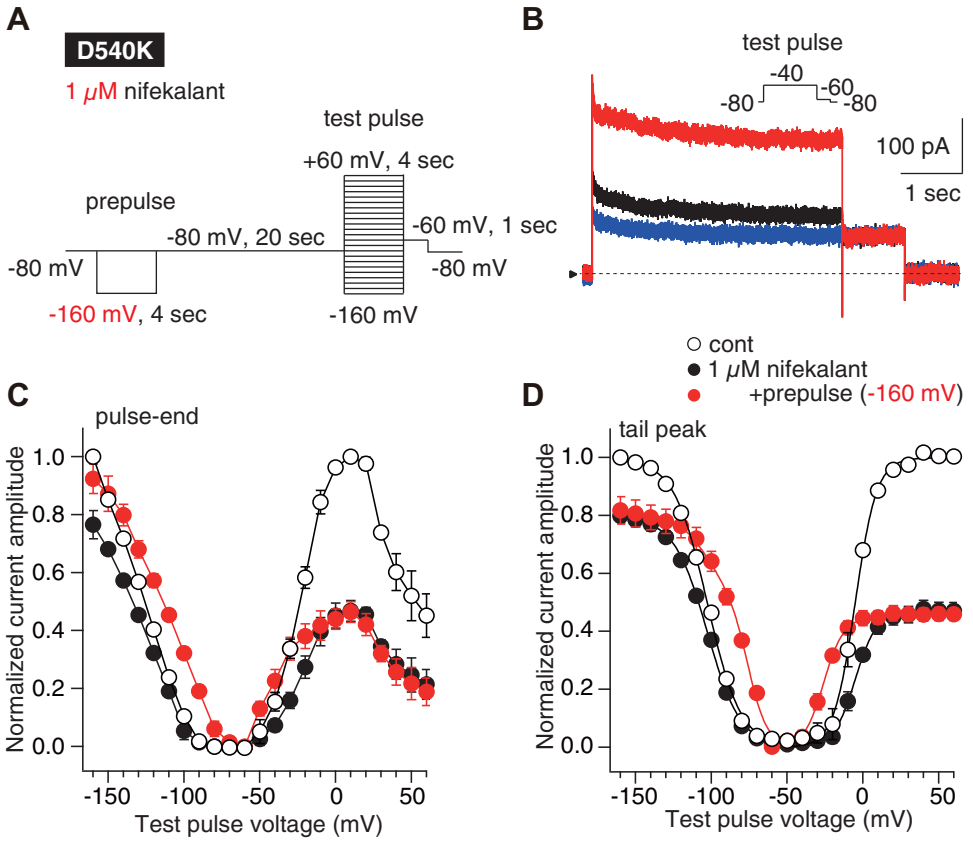

Fig. 6. Hyperpolarizing prepulse induces facilitation effect on D540K hERG activation in the presence of 1 μM of nifekalant. (A) Pulse protocol of this experiment. The amplitudes of 4-second preconditioning hyperpolarization (prepulse) were −160 mV, and the effects on the currents from HEK cells expressing D540K hERG upon the test pulse (from −160 mV to +60 mV by 10 mV increments for 4 s, then −60 mV for 2 s) were assayed. (B) Superimposed hERG currents recorded in the same cell before (cont., black) and after perfusion of 1 μM nifekalant with (+prepulse, red) or without (blue) the preconditioning pulse of −160 mV. (C and D) The relationship between membrane voltage of test pulse and the steady-state current amplitude (C) and the tail current amplitude (activation curve) (D). (C) The amplitudes of hERG step currents in the absence (open circles) and in the presence of 0.1 μM nifekalant with (filled black circles) or without (filled red circles), the preconditioning pulses were measured at the pulse-end during the test pulse to indicated voltages. Outward currents recorded at −50 mV to +60 mV were normalized to the currents evoked by voltage steps to +10 mV in the absence of nifekalant. Inward currents recorded at −160 mV to −60 mV were normalized to the currents evoked by voltage steps to −160 mV in the absence of nifekalant. (D) Voltage-dependent hERG activation. The amplitudes of hERG tail currents in the absence (open circles) and in the presence of 1 μM nifekalant with (filled black circles) or without (filled red squares), the preconditioning pulses were measured at the peak after the test pulse to indicated voltages. Depolarization-activated tail currents recorded at −60 mV were normalized to the currents evoked by voltage steps to +10 mV in the absence of nifekalant. Hyperpolarization-activated tail currents recorded at −60 mV were normalized to the currents evoked by voltage steps to −160 mV in the absence of nifekalant. Data are mean ± S.D. (n = 5 cells). In activation curve (D), the curves were fitted with the single (for control and nifekalant without prepulse) or double Boltzmann equation (for nifekalant with prepulse).

hyperpolarized potentials is not completely clear, it involves a change in coupling between voltage-sensor and pore domains leading to hyperpolarization-triggered opening of the intracellular S6 gate (Tristani-Firouzi et al., 2002). As the facilitation effect tracks conductance, this suggests that facilitation responds to and alters pore dynamics directly rather than acting through voltage sensors.

We reported that drugs that facilitate hERG currents such as nifekalant have no detectable effects on the inactivation properties and have not seen indications that inactivation is required for facilitation (Furutani et al., 2011; Yamakawa et al., 2012). In addition, our previous study showed a negative shift of the voltage dependence of the activation and deactivation time constants (τ_{fast} and τ_{slow} of a Hodgkin–Huxley model of macroscopic hERG current), successfully reproducing the experimentally observed facilitation phenomena (Furutani et al., 2019). These findings indicate that nifekalant accelerates the kinetics of activation and slows the kinetics of deactivation but does not affect the kinetics of inactivation.

Blockers that bind within the central cavity of Kv channels can alter channel gating. For example, the pore blocker 4-aminopyridine requires opening of the S6 gate of Shaker Kv1 channels to block and then stabilizes pores in closed conformations (Armstrong and Loboda, 2001). Interestingly, 4-aminopyridine can cause an increase in the conductance of Kv6.4/Kv2.1 heteromeric channels (Stas et al., 2015). The Kv2 blocker RY785 accelerates channel deactivation to trap itself (Marquis and Sack, 2022). On the other hand, quaternary ammonium ions such as tetraethylammonium stabilize open channels, albeit in a blocked conformation, and this can cause a shift of the conductance voltage relation to more negative voltages (Armstrong, 1971; Holmgren et al., 1997; Melishchuk and Armstrong, 2001). We suggest that stabilization of an open S6 gate by blockers could play a role in facilitation.

Nifekalant Block Varies with Current Direction, Not Membrane Voltage. The nifekalant block of D540K hERG inward currents was weaker than block of outward currents (Fig. 2). Nifekalant has pKa of 7.05, suggesting that

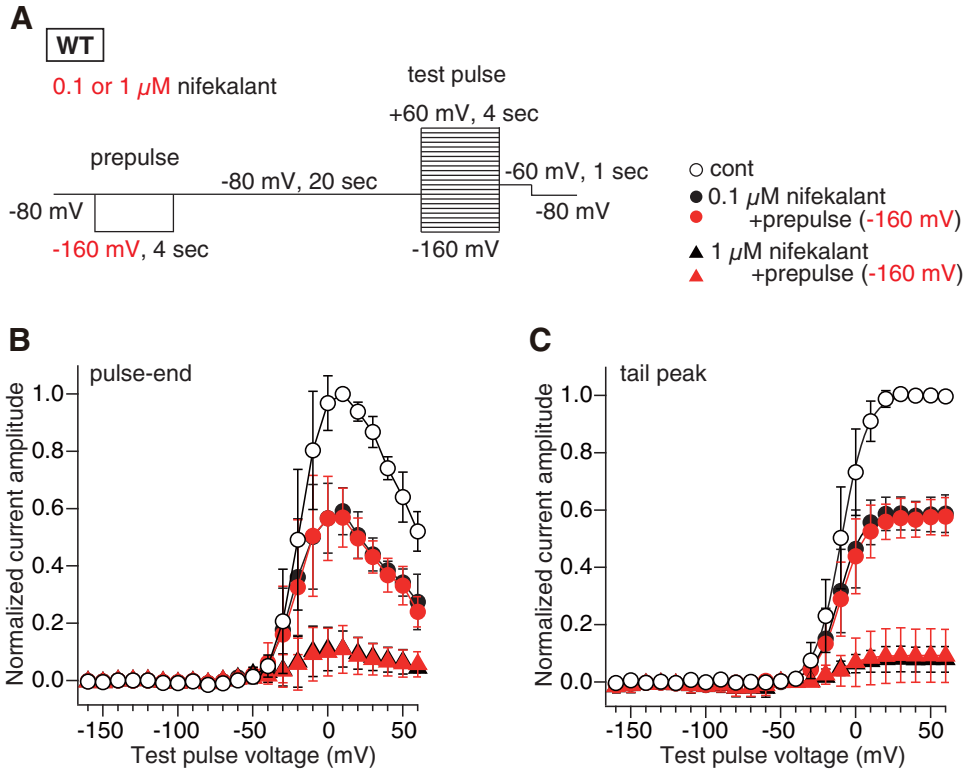

Fig. 7. Hyperpolarizing prepulse does not induce facilitation effect on WT hERG activation in the presence of 0.1 or 1 μM of nifekalant. (A) Pulse protocol of this experiment. The amplitudes of a preceding hyperpolarization (prepulse) were −160 mV, and the effects on the currents from HEK cells expressing WT hERG upon the test pulse (from −160 mV to +60 mV by 10 mV increments for 4 s, then −60 mV for 2 s) were assayed. (B and C) The relationship between membrane voltage of test pulse and the steady-state current amplitude (B) and the tail current amplitude (activation curve) (C). (B) The amplitudes of hERG step currents in the absence (open circles, $n = 8$ cells) and in the presence of 0.1 μM nifekalant with (filled black circles, $n = 5$ cells) or without (filled red circles, $n = 5$ cells) or in the presence of 1 μM nifekalant with (filled black triangles, $n = 5$ cells) or without (filled red triangles, $n = 5$ cells), the preconditioning pulses were measured at the pulse-end during the test pulse to indicated voltages. Outward currents recorded at −50 mV to +60 mV were normalized to the currents evoked by voltage steps to +10 mV in the absence of nifekalant. Inward currents recorded at −160 mV to −60 mV were normalized to the currents evoked by voltage steps to −160 mV in the absence of nifekalant. (C) Voltage-dependent hERG activation. The amplitudes of hERG tail currents in the absence (open circles, $n = 8$ cells) and in the presence of 0.1 μM nifekalant with (filled black circles, $n = 5$ cells) or without (filled red circles, $n = 5$ cells) or in the presence of 1 μM nifekalant with (filled black triangles, $n = 5$ cells) or without (filled red triangles, $n = 5$ cells), the preconditioning pulses were measured at the peak after the test pulse to indicated voltages. Depolarization-activated tail currents recorded at −60 mV were normalized to the currents evoked by voltage steps to +10 mV in the absence of nifekalant. Hyperpolarization-activated tail currents recorded at −60 mV were normalized to the currents evoked by voltage steps to −160 mV in the absence of nifekalant. Data are mean ± S.D. In activation curve (C), the curves were fitted with the single Boltzmann function (eq. 1).

30% of nifekalant is positively charged in the recording solution at pH 7.4. However, little voltage dependence can be attributed to charge on nifekalant, as the fractional block of either inward or outward K^+ currents changed little with voltage (Fig. 2C). Thus, the difference between inward and outward currents could arise from an interaction of permeant

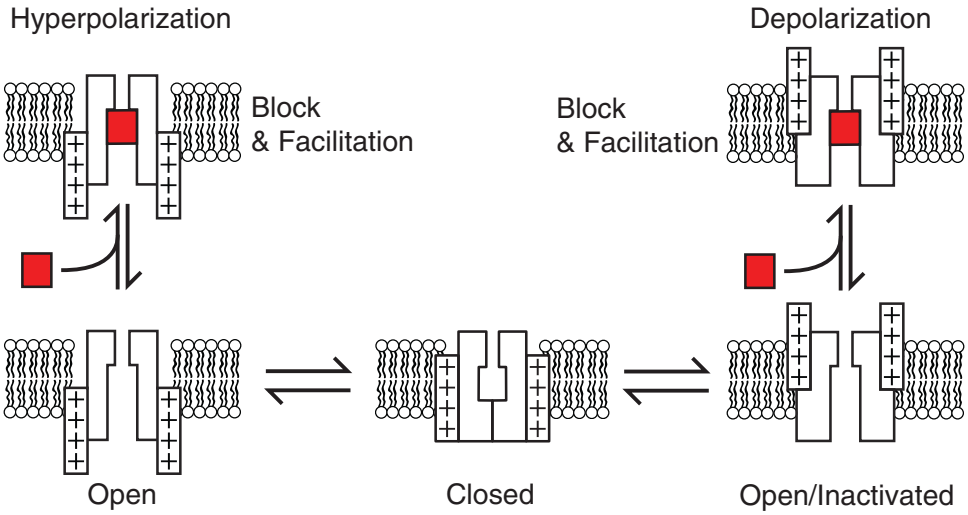

Fig. 8. Scheme summarizing effects of hERG D540K gating on facilitation. When the channel is opened by either depolarization or hyperpolarization, nifekalant (red) can block the channel and induce facilitation.

K^+ ions with a drug trapped within the pore (Pareja et al., 2013), a difference in inactivation, or another conformational change of the channel. Mutation of S4-S5 linker has only minor effects on the rate of hERG channel inactivation, and D540K hERG channels are inactivated at depolarized potentials and less inactivated at hyperpolarized potentials (Sanguinetti and Xu, 1999). Nifekalant may have a higher affinity to the inactivated state than the activated state (Kushida et al., 2002), and this could also possibly account for the weaker affinity, as the inward currents of D540K do not inactivate. In addition, nifekalant has a weaker efficacy against D540K than WT. This is also the case for hERG blockers such as MK-499 (Mitcheson et al., 2000), and the mechanism undergirding this efficacy difference between WT versus D540K is not well understood. Previous studies with D540K hERG do not rule out the possibility that reduced binding affinity to the mutant channels results from an allosteric effect unrelated to channel state.

Insights into the Risk of Cardiac Arrhythmias. hERG blockers carry a risk of lethal arrhythmias. Mechanism-based quantitative prediction of hERG-associated drug-induced arrhythmias has been a challenge (Sager et al., 2014; Gintant et al., 2016; Li et al., 2020). The present finding that channel activation is essential for facilitation suggests a link between blockade and facilitation, which may help assess the arrhythmic risk of drugs. We previously conducted simulations suggesting that facilitation by hERG blockers may decrease incidence of early afterdepolarizations in cardiac myocytes (Furutani et al., 2019). In that study, we used experimental data with nifekalant as the representative drug to block and facilitate hERG channels. Because the exact mechanism of induction of and recovery from facilitation was unknown, we modeled facilitation based on experimentally obtained dose-response curves without a mechanistic underpinning. A Markov chain model with explicit conformational states will be needed to meaningfully incorporate open state-dependence of facilitation. Further clarification of the mechanism of the facilitation effect could enable a more refined theoretical approach for prediction of arrhythmia risk.

Conclusion

In this study, we analyzed the facilitation effect of nifekalant on hyperpolarization-evoked D540K hERG channel activation and demonstrated that nifekalant exerts a facilitation effect on hERG currents at hyperpolarized potentials as well as depolarized potentials. The facilitation effect is induced by the drug interaction with hERG channels while their conductance is activated.

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Authorship Contributions

Participated in research design: Furutani, Sack, Kita.

Conducted experiments: Furutani, Kawano, Ichiwara, Adachi.

Contributed new reagents or analytic tools: Furutani, Adachi, Clancy, Sack.

Performed data analysis: Furutani, Sack.

Wrote or contributed to the writing of the manuscript: Furutani, Sack, Kita.

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