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Research Paper

Mutational Analysis of Block and Facilitation of HERG Current by A Class III Anti-Arrhythmic Agent, Nifekalant

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KEY WORDS

anti-arrhythmic agent, HERG, potassium channel, nifekalant, facilitation, docking simulation

ABSTRACT

Chemicals and toxins are useful tools to elucidate the structure-function relationship of various proteins including ion channels. The HERG channel is blocked by many compounds and this may cause life-threatening cardiac arrhythmia. Besides block, some chemicals such as the class III anti-arrhythmic agent nifekalant stimulate HERG at low potentials by shifting its activation curve towards hyperpolarizing voltages. This is called "facilitation". Here, we report mutations and simulations analyzing the association between nifekalant and channel pore residues for block and facilitation. Alanine-scanning mutagenesis was performed in the pore region of HERG. The mutations at the base of the pore helix (T623A), the selectivity filter (V625A) and the S6 helix (G648A, Y652A and F656A) abolished and S624A attenuated both block and facilitation induced by the drug. On the other hand, the mutation of other residues caused either an increase or a decrease in nifekalant-induced facilitation without affecting block. An open-state homology model of the HERG pore suggested that T623, S624, Y652 and F656 faced the central cavity, and were positioned within geometrical range for the drug to be able to interact with all of them at the same time. Of these, S649 was the only polar residue located within possible interaction distance from the drug held in its blocking position. Further mutations and flexible-docking simulations suggest that the size, but not the polarity, of the side chain at S649 is critical for drug induced facilitation.

ABBREVIATIONS

Nifekalant, (6-[2-[(N-2-hydroxyethyl)-3-(4-nitrophenyl)propylamino]ethylamino]-1,3-dimethyl-1H,3H-pyrimidine-2,4-dione monohydrochloride; APD, action potential duration; HERG; human *ether-a-go-go*-related gene; I_{Kr} , the rapidly activating component of the cardiac delayed rectifier K⁺ current; I_{Ks} , the slowly activating component of the cardiac delayed rectifier K⁺ current; I-V, current-voltage; Kv channel, voltage-dependent K⁺ channel; Mes, 2-(N-morpholino) ethanesulfonic acid

INTRODUCTION

Numerous chemical compounds and toxins interact with and modify the function of ion channels and other proteins. Most of these effects rely on the direct association between the compounds and the proteins, which provides important information concerning the relationship between the structure and the function. A number of class Ia and III anti-arrhythmic agents, anti-histamine derivatives and other compounds which include antibiotics and tranquilizers, are known to block the rapidly activating component of the cardiac delayed rectifier K⁺ current (I_{Kr}), whose pore forming subunit is encoded by a human *ether-a-go-go*-related gene (HERG).^{1,2} This block causes an acquired form of QT interval prolongation which can result in fatal polymorphic ventricular tachycardias including the *torsade de pointes*.^{3,4} Because of the clinical importance of this phenomenon numerous studies have attempted to clarify the mechanism of drug-induced block of HERG and thus incidentally the structure-function relationship of the K⁺ channel.^{4,5}

Many class III anti-arrhythmic drugs such as dofetilide and E-4031, are specific blockers of HERG.⁴ Although nifekalant belongs to the same drug class, it has been reported to not only block HERG but also increase the channel current at low voltages.⁶⁻⁸ This "facilitation" effect has also been seen with other HERG blockers such as almokalant⁹ and azimilide.¹⁰ Details of the mechanisms underlying the facilitation of HERG have not been clarified.

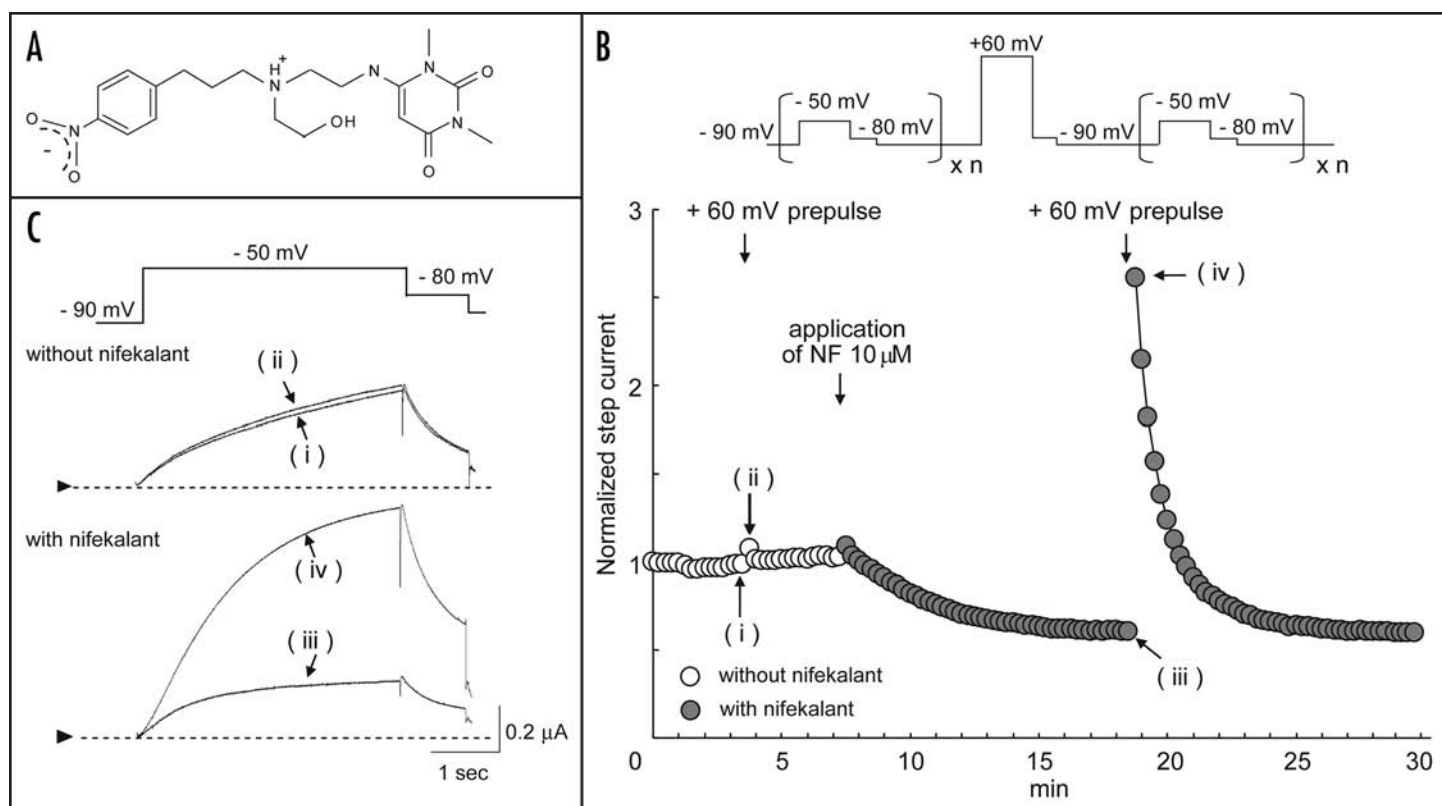


Figure 1. Facilitation of HERG currents by nifekalant. (A) Chemical structure of nifekalant. (B) The time course of HERG current inhibition by nifekalant and voltage-step induced facilitation. The voltage-clamp protocol is inset: membrane potential was held at -90 mV and stepped to -50 mV for 4 sec and to -80 mV for 1 sec. This combination was repeated every 15 sec. After allowing the current amplitude to reach a steady-state level, a depolarizing pulse to +60 mV for 4 sec and to -80 mV for 1 sec was applied (arrows) in the presence (filled symbols) or absence (open symbols) of 10 μ M nifekalant (NF). HERG currents were measured at the end of the depolarizing pulse at -50 mV and normalized to the current amplitude recorded before the first application of the +60 mV prepulse in the absence of nifekalant. (C) Representative current traces elicited by the voltage steps to -50 and -80 mV (inset) before (i, iii) and after the +60 mV prepulse (ii, iv) in the absence (i, ii) or presence of nifekalant (iii, iv).

In the present study, we attempted to identify residues in the channel pore of HERG that are involved in both the blocking and the facilitation effects of nifekalant. The residues T623, S624, Y652 and F656 were involved in nifekalant block of HERG. Similar results had been obtained for other HERG-blockers.¹¹⁻¹⁵ Mutation of a number of other residues either attenuated or enhanced facilitation without affecting block. Among them, S649 was analyzed in detail because this was the only polar residue, which was located within range for interaction with nifekalant when the drug was bound to T623, S624, Y652 and F656. Mutagenesis of S649 to bulky residues caused a significant attenuation of facilitation and a modest reduction of block, which suggested that the size of side chain rather than the hydroxyl group of the amino acid at S649 was important for the association between nifekalant and HERG. Simulations supported the notion that the drug could interact with S649 while bound to T623, S624, Y652 and F656. These results suggest that a strong membrane depolarization induces a change in the association between nifekalant and HERG around the S649 side chain resulting in facilitation.

MATERIALS AND METHODS

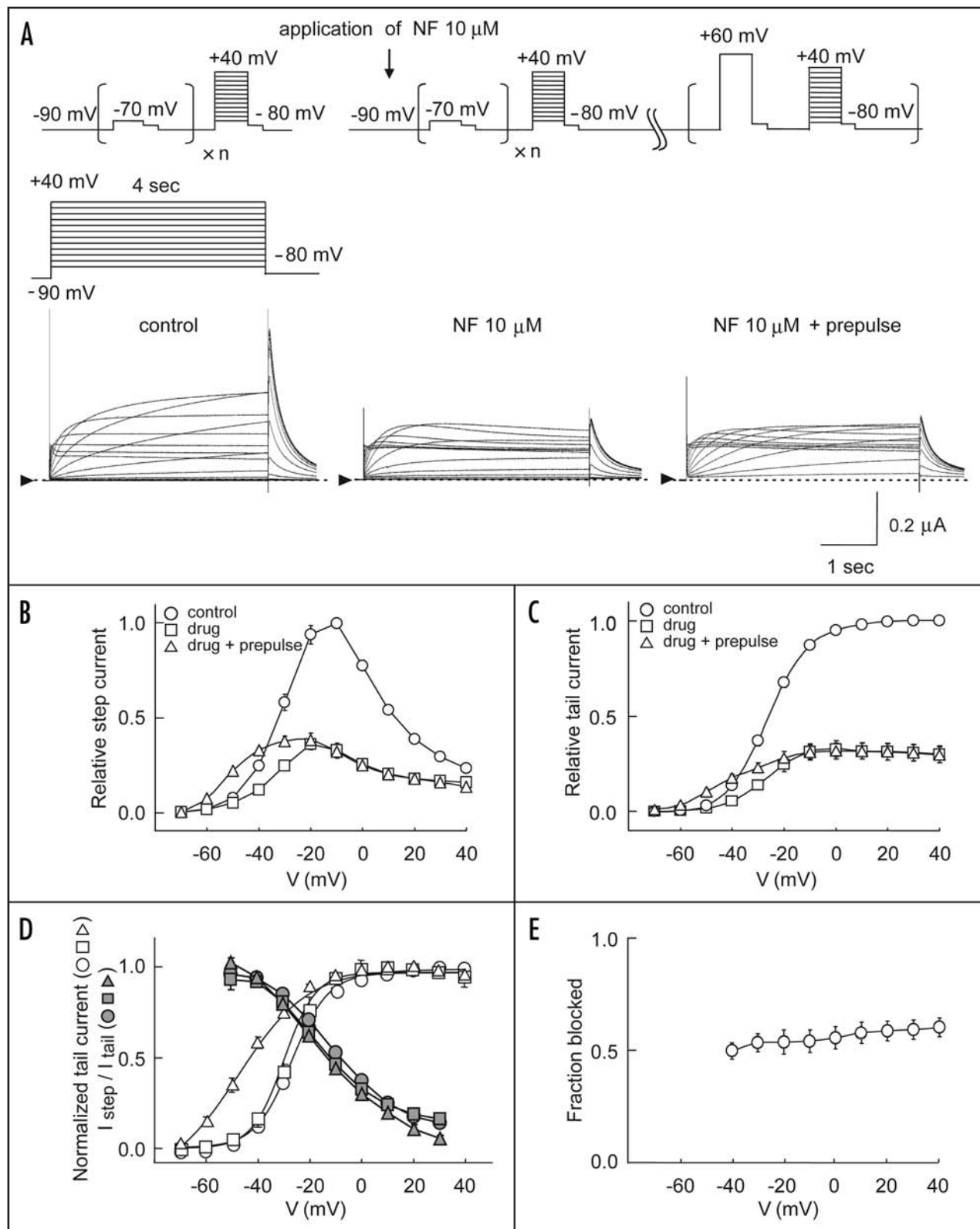
Drug. Nifekalant (Nihon Schering) stock solutions (10 mM) were prepared daily in distilled water. This stock was diluted to the

required concentration in bath solution and the pH was adjusted to 7.6 when necessary.

Site-directed mutagenesis and transcription of cRNA. HERG cDNA in pSP64 vector was kindly provided by Drs. M.T. Keating and M.C. Sanguinetti (University of Utah, Salt Lake City, UT). Point mutations were introduced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Mutation constructs were confirmed with direct DNA sequencing. The pSP64 vectors of the wild-type (WT) and mutants of HERG were linearized with *Eco*RI and transcribed in vitro with SP6 RNA polymerase in the presence of m⁷G(5')ppp(5')G RNA capping analog (Invitrogen).

Isolation of oocytes and injection of cRNA. Frogs (*Xenopus laevis*) were treated in accordance with the guidelines for the use of laboratory animals of Osaka University Graduate School of Medicine. Isolation and maintenance of the oocytes and injection with cRNA were performed as described previously.¹⁶ The cells were injected with 5 ng of cRNA of WT HERG or each mutant, and incubated at 18°C in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH 7.6 with NaOH) supplemented with 50 μ g/ml gentamicin.

Electrophysiological recording and data analysis. Membrane currents were recorded with a conventional two-microelectrode voltage-clamp technique¹⁷ with a GeneClamp 500 amplifier (Axon



Instruments) 2–4 days after cRNA injection. All experiments were conducted at ambient temperature (22–24°C). The glass microelectrodes had a resistance of 0.6–1.5 M Ω when filled with 3 M KCl. Oocytes were bathed in a low-Cl⁻ solution (96 mM Na-2-(*N*-morpholino) ethanesulfonic acid (NaMes), 2 mM KMes, 2 mM CaCl₂, 1 mM MgCl₂ and 5 mM HEPES, pH 7.6 with methane sulfonic acid) to minimize interference from endogenous

Cl⁻ currents. In some experiments KMes was increased to 96 mM by replacing NaMes. Voltage clamp protocols are described in each figure legend.

During recording, 20 ms prepulses from -80 to -100 mV were applied to estimate the leak current level. Data analysis was mainly carried out using Clampfit software (Axon Instruments). Data are presented as mean \pm s.e.m. (n = number of observations). Statistical

Figure 2 (page e3). The effects of nifekalant on HERG currents. (A) The effect of nifekalant on the current-voltage relationship of HERG. The voltage protocol is shown in the top panel. Membrane potential was held at -90 mV. The preconditioning prepulse was to -70 mV for 4 sec and to -80 mV for 1 sec and repeated with an interval of 15 sec until the steady-state was achieved. To elicit the current-voltage (*I-V*) relationship the membrane potential was depolarized from -70 to +40 mV in 10 mV increments for 4 sec each and followed by 1 sec at -80 mV. Facilitation was elicited by a preconditioning prepulse to +60 mV rather than to -70 mV before each *I-V* test pulse. Typical HERG current traces evoked by the *I-V* protocol are shown recorded in the absence (left panel), and the presence of 10 μ M nifekalant (NF) without (middle panel) or with the prepulse (right panel). (B) *I-V* relationship for steady-state currents. HERG currents were measured at the end of the 4 sec test pulse. Current amplitudes were normalized to that recorded at -10 mV in the absence of nifekalant. (C) *I-V* relationship for tail currents. HERG channel currents were measured at the peak during the repolarizing pulse to -80 mV. Currents amplitudes were normalized to that recorded following a voltage step to +40 mV in the absence of nifekalant. (D) Activation curves of HERG tail currents and the ratio of steady-state current to tail current. Tail current amplitude was normalized to the maximum current recorded under each condition, and the values were fit with the Boltzmann function. The $V_{1/2}$ values were: control, -26.0 ± 0.8 mV (open circles); 11 μ M nifekalant, -28.1 ± 1.5 mV (open squares); 11 μ M nifekalant with the strong depolarizing prepulse, -44.3 ± 2.3 mV (open triangles). The conventional analysis for C-type inactivation was carried out by dividing step pulse end currents (B) by tail peak currents (C) in the absence of nifekalant (gray circles), and in the presence of drug with or without the strong depolarizing prepulse (gray squares and triangles, respectively). (E) Voltage-dependent block of HERG channel current by nifekalant. Fractional block of tail currents measured without the strong depolarizing prepulse was plotted against the membrane potentials of the test pulse. Symbols with error bars represent mean \pm s.e.m. ($n = 5$).

analyses were performed using paired or unpaired *t*-tests, with $p < 0.05$ being considered significant.

Molecular modeling and drug-docking simulations. An open channel conformation of a HERG pore region was generated with the homology modeling technique MODELER⁶¹⁸ using the crystal structure of the bacterial voltage-dependent K⁺ channel KvAP as a template.¹⁹ The HERG pore region (between positions 548 and 673) had 24% amino acid identity and 36% homology with the corresponding region of KvAP. The sequence alignment of KcsA, KvAP and HERG was used as the initial condition to increase the accuracy of homology modeling. The ligand molecule was prepared in Sybyl mol2 file format. The atomic charges of the ligand were determined by the restricted electrostatic point charge procedure using HF/6-31G*-level quantum chemical calculations.²⁰ GAMESS²¹ and Gaussian98 (revision A.9; Gaussian) were used to perform the quantum chemical calculations. The atomic charges for the proteins were the same as the atomic charges in AMBER parm99.²⁰ Protein-compound docking simulations were performed by SievGene, which is a protein-ligand flexible docking program for in silico drug screening.²²

RESULTS

Facilitation of HERG channel currents by nifekalant. Nifekalant is a class III anti-arrhythmic agent which blocks and facilitates HERG current (Fig. 1A).⁶⁻⁸ A strong depolarizing voltage step is needed to induce drug-associated facilitation of HERG current.^{9,10} In Figures 1B and C, the effect of a depolarizing prepulse to +60 mV on the HERG current was compared in the absence and presence of nifekalant. The holding potential was -90 mV. A test pulse to -50 mV for 4 sec followed by a voltage step to -80 mV for 1 sec was applied every 15 sec (top panels in Fig. 1B and C). The amplitude of the HERG current was measured at the end of the test pulse. In the absence of the drug, the HERG current was not significantly affected by a prepulse (4 sec in duration) to +60 mV (Fig. 1B and C, i and ii). When 10 μ M nifekalant was added to the bath solution, the amplitude of HERG current gradually declined to $56.1 \pm 8.7\%$ ($n = 4$) of the control. When the current reached a steady-state level, the depolarizing prepulse was applied again and this caused a clear increase in the current evoked by subsequent test pulses (Fig. 1B and C, iii and iv). This is the phenomenon which has been named "facilitation" by Carmeliet⁹ and Jiang et al.¹⁰ In the presence of nifekalant the amplitude of the current elicited by the first test voltage step after the depolarizing prepulse was $261.4 \pm 19.4\%$

($n = 4$) of that recorded in control conditions (Fig. 1B and C). The facilitated current then gradually declined to a steady-state level over 5 min (Fig. 1B). The depolarizing prepulse-evoked facilitation of HERG current was not observed in the presence of either E-4031 (0.3-3 μ M) or dofetilide (0.1-0.3 μ M) (data not shown). Thus, facilitation appeared to be an action that is unique for the subset of HERG blockers which includes nifekalant.

Current-voltage relationships of blocked and facilitated HERG currents. To further characterize facilitation, we analyzed the effect of a depolarizing prepulse on the current-voltage (*I-V*) relationship of the HERG channel in the presence of nifekalant. The voltage clamp protocols are shown at the top of Figure 2A. The *I-V* relationship corresponding to facilitation was obtained by applying a prepulse to +60 mV (4 sec in duration) before each test pulse (Fig. 2A, right panel).

The *I-V* relationships of HERG current recorded at the end of 4 sec test pulses are shown in Figure 2B. Under control conditions, the current was activated at potentials more positive than -60 mV and increased until -10 mV. The current amplitude then decreased with the development of C-type inactivation.²³ In the presence of nifekalant, HERG current amplitude was reduced at every potential with a slightly negative shift of the peak. The prepulse to +60 mV was associated with an increase in the HERG current amplitude at potentials more negative than -20 mV. At potentials between -60 and -40 mV, the facilitated current in the presence of nifekalant was not only larger than that recorded in nifekalant without a prepulse but it was also larger than that recorded under control conditions. In the presence of the drug, the amplitudes of peak tail currents were also increased by the prepulse (Fig. 2C).

Figure 2D shows normalized tail current amplitude recorded in three different conditions, i.e., control, and in the presence of nifekalant with or without a depolarizing prepulse. While without the prepulse the activation curve of nifekalant blocked current was almost the same as that of control, the curve with the prepulse was significantly shifted to the left ($p < 0.001$). Figure 2E shows the fraction of HERG current blocked by 10 μ M nifekalant at each potential without facilitation. Nifekalant blocked the channel in an apparently voltage-independent manner.

Two possible explanations for nifekalant inducing facilitation of HERG current are the reduction of C-type inactivation and the modulation of the activation gating process. The conventional analysis for C-type inactivation consists of dividing currents recorded at the end of a voltage step by the peak of the tail currents. Figure 2D shows that there was no difference in the voltage dependency of

Table 1 **Biophysical parameters of WT and mutant HERG channels for their activation**

HERG Channel	$V_{1/2} \pm \text{s.e.m. (mV)}$	$k \pm \text{s.e.m. (mV)}$	n
WT	-25.5 ± 0.6	7.2 ± 0.6	6
I647A	-27.1 ± 0.7	7.7 ± 0.3	4
Y652A	-27.3 ± 1.0	6.8 ± 0.4	4
S654A	-27.6 ± 2.3	7.8 ± 0.2	4
F656A	-36.4 ± 1.0	6.6 ± 0.4	3
S660A	-27.9 ± 0.3	7.5 ± 0.1	6
I663A	-20.7 ± 1.7	6.8 ± 0.5	4
Y667A	-27.5 ± 0.8	8.0 ± 0.5	4
S624A	-30.5 ± 1.0	7.4 ± 0.1	6
S624T	-23.2 ± 0.6	7.3 ± 0.2	3
S624N	-16.0 ± 1.3	6.4 ± 0.1	3
S649A	-29.7 ± 0.6	7.3 ± 0.1	5
S649T	-20.3 ± 2.2	5.7 ± 0.2	4
S649V *	-21.2 ± 1.6	6.4 ± 0.4	3
S649I *	-25.6 ± 2.0	5.8 ± 0.1	3
S649L *	-23.0 ± 0.3	5.7 ± 0.2	3

$V_{1/2}$, potential of half-maximal activation; k , slope of the activation curve; n , number of oocytes. Tail peak currents were recorded after repolarization to -80 mV followed by the voltage steps (4 sec) between -90 to $+40$ mV. *The current response of three mutants was measured at the peak during the repolarizing pulse to -50 mV.

C-type inactivation of blocked HERG currents in the absence or the presence of facilitation. To confirm this, C-type inactivation using the fully activated I - V relationship of HERG was examined (data not shown, ref. 45). The voltage dependence of C-type inactivation in the presence of nifekalant was shifted by ~ 5 mV compared to control, which is consistent with a previous report.⁸ This change in kinetics could enhance current block, but does not increase the current at low voltages. Therefore, the reduction of C-type inactivation seems unlikely and there remains the possibility that nifekalant causes facilitation by affecting the activation gating process of HERG.

Effects of alanine-scanning mutagenesis on nifekalant-HERG interaction. To determine which residues in the HERG channel were involved in block and facilitation by nifekalant, we replaced each of the residues from the pore helix to the selectivity filter (L622-V625) and in the S6 helix (L646-Y667) with alanine, and examined the effect of nifekalant on the mutants. Biophysical parameters of the activation of the important mutants are summarized in Table 1. Most of the mutants retained properties comparable to those of the wild-type (WT). The half-activation voltage ($V_{1/2}$) remained within 10 mV of the WT value, and the slope factor (k) value was largely not altered. The concentration of nifekalant required for half-maximal inhibition of WT-HERG at 0 mV was $1.7 \mu\text{M}$ with a Hill coefficient of 0.99. We therefore used $11 \mu\text{M}$ nifekalant, which caused 85% inhibition of the WT-HERG current to characterize the reactions of the mutants to the drug. Because T623A-, V625A- and G648A-HERG mutants showed low expression, we measured their currents with 96 mM potassium extracellular solution.¹¹

Inhibition of the HERG current was assessed by the voltage clamp protocol illustrated at the top of Figure 3A. The current amplitude

was measured at the end of the test pulse to 0 mV (Fig. 3A, left panel). Nifekalant reduced the magnitude of most mutant channels to a similar extent as WT-HERG (Fig. 3Ba, left panel). A mutation located at the end of the pore helix (T623A), a mutation in the selectivity filter (V625A) and three in the S6 helix (G648A, Y652A, and F656A) almost abolished the blocking effect of nifekalant. These mutations also reduce the block of HERG by MK-499, dofetilide, E-4031, clofilium and ibutilide, as well as analogs of clofilium and ibutilide.^{11-13,15} The mutant V625A reduced K^+ selectivity.^{11,12} Thus, the side chain of V625 may be crucial for maintaining the structure of the selectivity filter and V625A might alter the inner surface of the central cavity which would indirectly reduce the affinity for nifekalant. G648 is conserved in all voltage-dependent K^+ (Kv) channels and it is thought to function as a hinge for bending of the S6 helix and channel gating.¹⁹ The homology model of HERG (Fig. 4A) suggests that G648 is not exposed to the pore and therefore the effect of mutation of G648 on the affinity to nifekalant is also due to an indirect action. It is reported that polar or electronegative *para*-substituents on the phenyl ring of clofilium and its analogs associate with the polar groups of S624 and T623.¹³ Nifekalant possesses a *para*-nitrophenyl group at one end of the molecule (Fig. 1A) and the S624A mutant showed less block (Fig. 3B), indicating that S624 contributes to nifekalant block of HERG. Taken together, four residues T623, S624, Y652 and F656, seemed to be involved in the binding of nifekalant to induce channel current block, as proposed for other HERG-blockers.^{11-13,15}

Next, the facilitation effect of nifekalant was examined in these mutants. After having reached a stable state in the presence of nifekalant, one prepulse to $+60$ mV was applied. The amplitudes of HERG current at the end of the step to -50 mV just before (Pre) and after (Post) the prepulse (I_{pre} and I_{post} , respectively) were compared (Fig. 3A, right panel). Facilitation was assessed as the ratio of $I_{\text{post}}/I_{\text{pre}}$ (Fig. 3Ba, right panel). The $I_{\text{post}}/I_{\text{pre}}$ value for WT-HERG in the presence of nifekalant ($10 \mu\text{M}$) was 4.4 ± 0.37 ($n = 4$). The mutants (T623A, V625A, G648A, Y652A, and F656A), which were not blocked by the drug, did not show any facilitation. The mutant S624A, which showed attenuated block, exhibited less facilitation than WT. Of the other mutants which were blocked more or less than WT-HERG, some of them showed greater facilitation than WT, while others showed less (Fig. 3Ba). The $V_{1/2}$ values for the activation curve measured in each mutant suggested that these alterations in facilitation were not due to the shift of the activation curve in the absence of the drug.

To confirm this, we further analyzed the facilitation effect of nifekalant in all of the mutants listed in Table 1 with a voltage ramp protocol (data not shown, but see Fig. 4B). The membrane potential was held at -90 mV and a voltage ramp from -70 to 0 mV (10 sec in duration) was applied every 35 sec. To induce facilitation, a voltage pulse to $+60$ mV for 4 sec was applied 10 sec prior to the ramp. Thus, we could distinguish whether the facilitation was due to the drug effect or the alteration of an intrinsic biophysical property of the HERG mutants. With the ramp protocol, the facilitation of HERG currents could be clearly measured at potentials between -60 and -30 mV (WT in Fig. 4B). All of the mutants that showed a facilitation response to the step-pulse protocol (Fig. 3Ba) clearly exhibited a left-ward shift of activation during the ramp which resulted in an increase in the current amplitude at -50 mV. Therefore, drug-induced facilitation was due to the consequence of drug-channel interaction

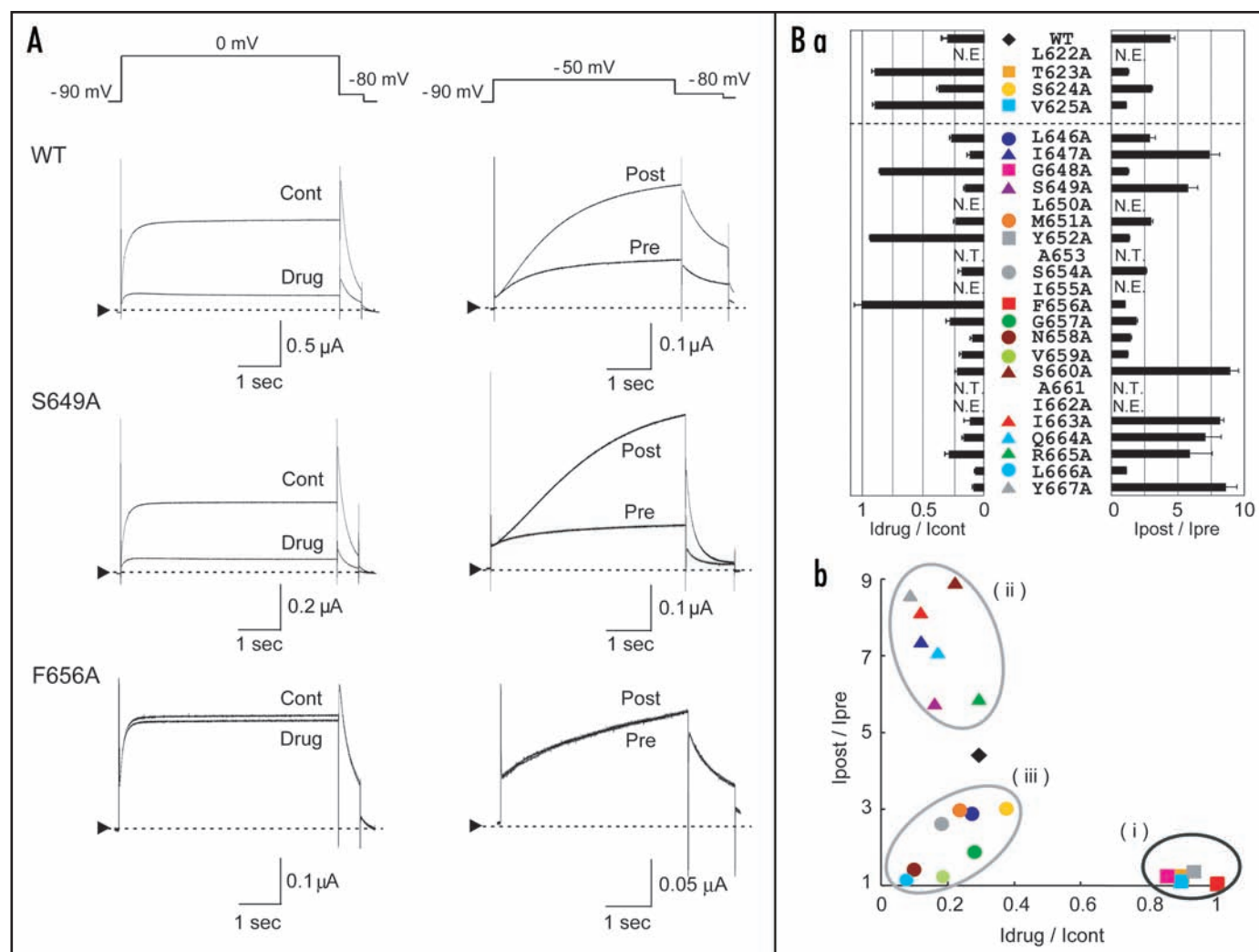


Figure 3. Effects of nifekalant on mutant HERG channel currents. (A) Block (left column) and facilitation (right column) in current traces obtained from wild type (WT), S649A and F656A mutants. Left column: the currents were elicited by test pulses to 0 mV for 5 sec and then to -80 mV for 0.5 sec from a holding potential of -90 mV in either the absence (Cont) or the presence of nifekalant (Drug). Right column: in the presence of nifekalant, the currents were elicited by test pulses to -50 mV for 4 sec and then to -80 mV for 1 sec from a holding potential of -90 mV, before (Pre) and after a single depolarizing pulse to +60 mV (Post). (Ba) The effects of alanine substitution screening upon block and facilitation of HERG currents by nifekalant. Normalized block ($I_{\text{drug}}/I_{\text{cont}}$) was calculated by dividing the current amplitude recorded after the application of nifekalant by the current recorded under control conditions. Normalized facilitation ($I_{\text{post}}/I_{\text{pre}}$) was calculated by dividing the current amplitude recorded following the strongly depolarizing prepulse by the current recorded before the prepulse ($n = 4-6$ for each, error bars; \pm s.e.m.). N.T.; not tested, N.E.; channels that lacked functional expression. (Bb) The relationship between block and facilitation by nifekalant in HERG channel mutants. The normalized facilitation is plotted against the normalized block for each mutant. Mutants could be classified into three groups as follows: (i), mutants showing neither block nor facilitation (squares); (ii), mutants showing the same degree of block as WT but stronger facilitation than WT (triangles); (iii), mutants showing the same degree of block as WT but weaker facilitation than WT (circles). Symbols as in Ba.

and not due to an alteration of the intrinsic biophysical properties of the mutants. Because facilitation of current is due to a leftward shift of the HERG activation curve, any step between drug-channel interaction and activation gating can influence the phenomenon. Therefore it is not unexpected that most of the amino acids in the HERG pore are somehow involved in the induction of facilitation.

Figure 3Bb summarizes the relationship between block and facilitation by nifekalant for WT and the mutants. The mutants could be classified into three groups: (i) a group showing neither block nor facilitation; (ii) a group showing block equivalent to WT-HERG but larger facilitation than WT; and (iii) a group showing block equivalent to WT-HERG but less facilitation than WT. These results suggest that the extent of facilitation did not correlate with that of

channel block but because drug binding leads to block, binding would seem to be a prerequisite for facilitation.

Modeling and further mutational analysis of nifekalant-induced block and facilitation. Nifekalant is known to associate with the channel when HERG is in the open state conformation (Fig. 2 and ref. 8) and the crystal structure of the bacterial Kv channel, KvAP, possesses an open pore.¹⁹ Because the HERG and KvAP pore regions have a similar amino acid sequence we generated a homology model for the open conformation of the HERG pore based on the KvAP structure (Fig. 4A). But the HERG channel exhibits very rapid and strong C-type inactivation compared with other types of voltage-dependent K⁺ channels and mutation of amino acid residues surrounding the selectivity filter eliminates C-type inactivation.⁴ This

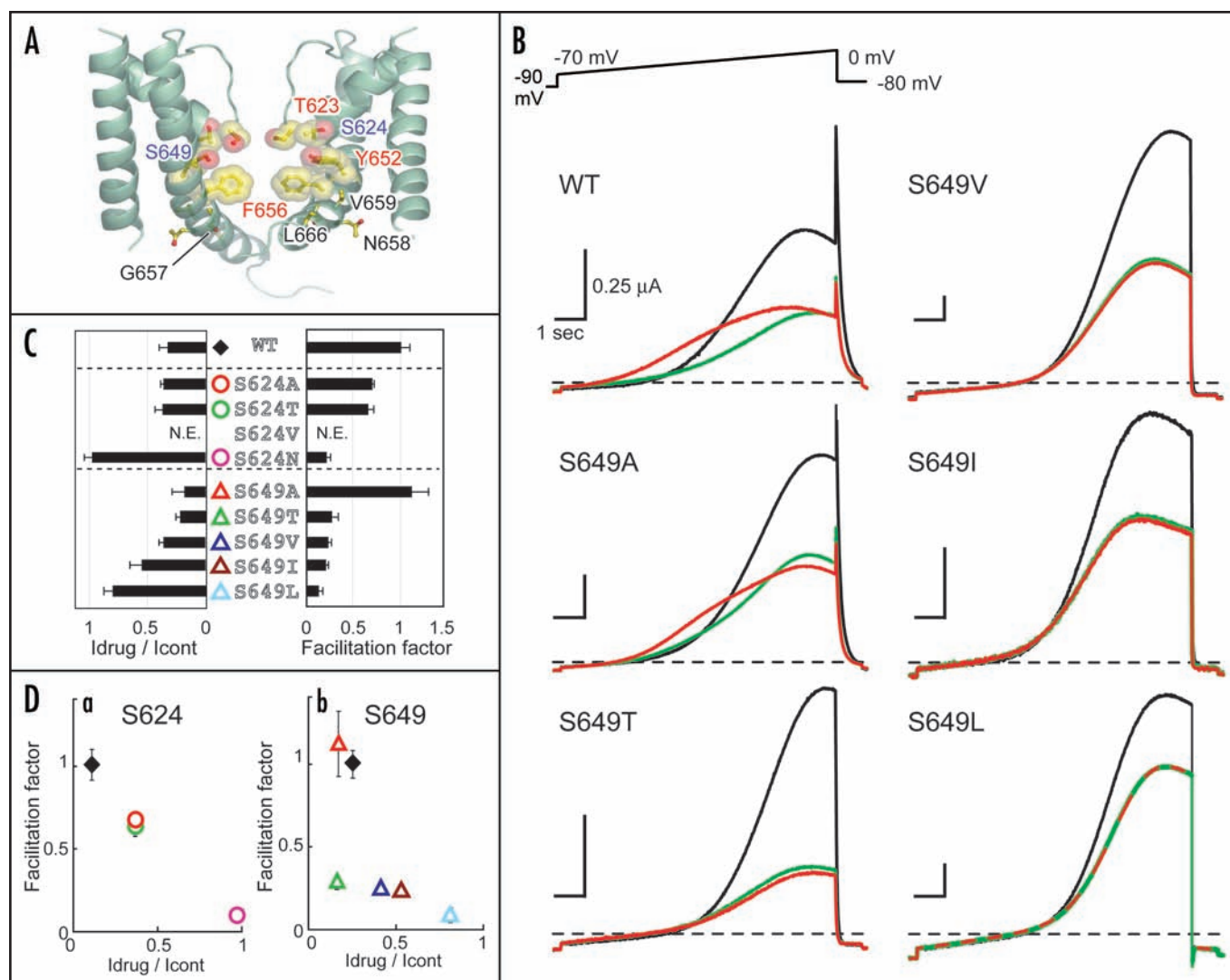


Figure 4. The involvement of S649 in nifekalant and HERG channel facilitation. (A) Homology model of the pore region of the HERG channel. A model of the open channel conformation of HERG was based on the crystal structure of the KvAP channel (PDB ID: 1ORQ). Two subunits have been omitted from the front and the back for clarity. Side chains of amino acids crucial for drug block and facilitation of HERG current are represented as stick models. Side chains of residues presumably involved in the direct association with nifekalant are shown with spheres. Carbon and oxygen atoms are colored yellow and red, respectively. (B) Representative currents from HERG channels mutated at S649. A voltage ramp protocol from -70 to 0 mV was applied to test HERG mutants S649A, S649T, S649V, S649I and S649L for nifekalant-induced block (green) and facilitation (red). These currents were superimposed onto that recorded in the absence of nifekalant (black). Dashed lines indicate zero current level. (C) The effects of nifekalant on block and facilitation of currents from HERG channels mutated at S624 and S649. The effects of nifekalant-induced block (left panel) and facilitation (right panel) in HERG mutants were evaluated as described in (Fig. 3B). (D) The relationship between block and facilitation by nifekalant in HERG mutants. The facilitation factor is plotted against the ratio of current block for each mutation of S624 (a) and S649 (b). Symbols as in C ($n = 3-6$ for each, mean \pm s.e.m.).

places a clear limitation upon the use of an homology model based on KvAP. However, because C-type inactivation was not affected by nifekalant (Fig. 2) the homology model should provide some useful information on the interaction between the HERG channel and the drug. The model showed that the side chains of amino acids T623, S624, Y652 and F656 are exposed to the central cavity. This is in agreement with the mutational analysis that suggests that these residues were responsible for the binding of nifekalant to HERG (Fig. 3). These residues therefore contribute to the direct association between nifekalant and HERG.

At the present time, T623/S624, Y652 and F656 are thought to interact with respectively polar, ionized and hydrophobic regions of HERG blockers.^{4,14,24} According to pharmacophore analysis,^{25,26}

the distance between ionized and hydrophobic regions in different HERG blockers ranges from 5.2 Å to 9.1 Å. On the other hand, the model showed that the center of the aromatic ring of Y652 was about 8 Å distant from that of F656. This may imply that if the drug interacts with Y652, it cannot extend beyond the side chain of F656 to the cytoplasmic side. Therefore, the drug should interact with amino acid residues located around the central cavity of HERG.

Because drug binding is a prerequisite for facilitation, the position of the drug in the channel pore cavity causing facilitation should be confined to within the limits that are required for binding. Mutation of the distal S6 helix near the cytoplasmic side of the channel exhibited different magnitudes of facilitation, while maintaining WT block. The mutants G657A, N658A, V659A, and L666A showed

little facilitation, while S660A, I663A, Q664A, R665A and Y667A exhibited greater facilitation than WT (Fig. 3B). Because these residues are predicted to be located physically too far from the drug which is restricted to its binding site, they may not directly interact with the drug but participate indirectly in drug-induced facilitation.

Because nifekalant binding is a prerequisite for facilitation, there may be overlap between the interactions between the channel pore residues and nifekalant that cause block and facilitation. But there may also be a specific interaction associated with facilitation. This interaction for facilitation requires a strongly depolarizing prepulse in the presence of the drug. Upon strong depolarization, the basic nitrogen at the center of nifekalant may shift in the outward direction. Within the restriction that block requires T623, S624, Y652 and F656, S649 is the only polar amino acid residue within range of the drug size. The hydroxyl group of S649 is 3.9 Å to 6.6 Å distant from the side chains of T623, S624, Y652 and F656.^{25,26} Therefore, while the alanine substitution at S649 did not have a significant effect on facilitation (Fig. 3), from a geometrical standpoint this amino acid might be a key residue for nifekalant's induction of facilitation. To test this hypothesis, we examined the effects of various mutations of S649 on nifekalant-induced block and facilitation, and compared them with mutations of S624, which contributes to the binding of nifekalant for block.

Amino acid substitutions at both S649 and S624 did not cause a dramatic alteration in the voltage dependence of HERG activation (Table 1). Nevertheless, we applied ramp pulses to assess drug-induced facilitation (Fig. 4B). Currents were recorded during the continuous change in voltage from -70 to 0 mV in the absence of drug (black lines), and in the presence of the drug with or without a depolarizing prepulse (red or green lines). Nifekalant reduced the WT-HERG current at potentials more positive than -40 mV, while the drug obviously increased the current amplitude at potentials between -60 to -30 mV when a prepulse to +60 mV had been applied. The currents of S649A and S649T mutants were blocked by nifekalant to the same extent as WT-HERG, while the currents of S649V, S649I and S649L were much less reduced. While the drug-induced facilitation was nearly the same as WT in the S649A mutant, it was negligible in the mutants S649T, S649V, S649I and S649L. These results strongly suggested that different mutations at S649 yielded different sensitivities to nifekalant, leading to block and facilitation.

To quantitatively analyze the effect of mutations on nifekalant-induced block and facilitation, voltage steps to 0 mV and -50 mV were applied for the assessment of block and facilitation effects of the drug, respectively (Fig. 4C). For comparison, we analyzed various mutants at S624 and S629.

Substitutions of S624 to alanine or threonine caused moderate reductions in block and facilitation due to nifekalant (Figs. 3 and 4B and 4C). Even though asparagine has a volume similar to threonine, the S624N mutant was completely insensitive to the drug. These results indicate that not only the size of the S624 side chain, but also its hydroxyl group, is crucial for drug-HERG association, supporting the proposal that S624 participates in the binding of nifekalant to the channel pore. Figure 4Da shows that there is a strong correlation between drug-induced block and facilitation for the S624 mutants. This agrees with the idea that the drug binding is a prerequisite for facilitation (Fig. 3).

On the other hand, the alanine replacement at S649 did not significantly affect either block or facilitation (Figs. 3, 4B, 4C

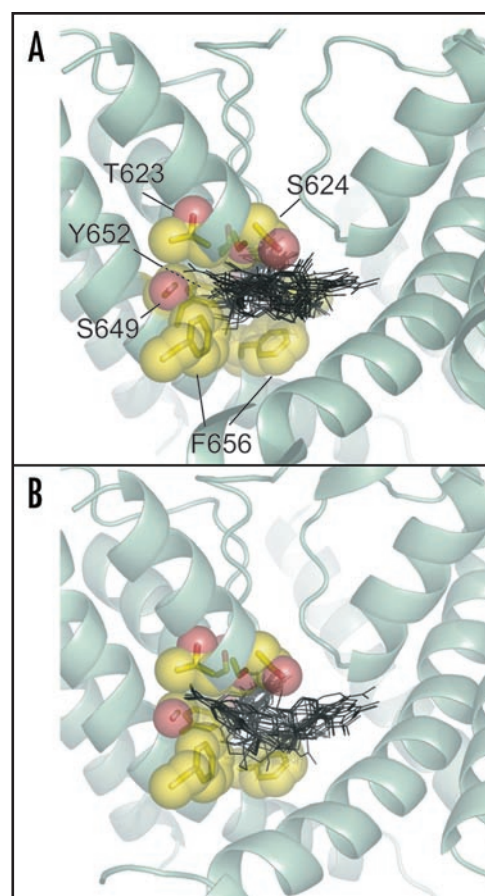


Figure 5. Flexible docking simulation of the association of nifekalant with HERG. The association between nifekalant and HERG was simulated as described in 'Materials and Methods'. The simulations were performed with constraints for the association of nifekalant with T623, S624, Y652 and F656 to mimic the mode of current block (A), and with T623, S624, S649, Y652 and F656 to mimic the mode of current facilitation (B). Forty different solutions developed by each simulation are superimposed in the HERG model. The side chains of residues located in close vicinity to nifekalant are represented with sphere models. Their carbon and oxygen atoms are colored yellow and red, respectively.

and 4Db). However, even though threonine is only 27 Å³ larger than serine, the S649T mutation caused a prominent reduction in drug-induced facilitation without an obvious effect on block. Because both serine and threonine possess a hydroxyl group on their side chains, the contribution of the hydroxyl group of S649 to nifekalant-induced facilitation may be insignificant. Therefore, the reduction of facilitation caused by the threonine substitution at S649 may be due to the increase in volume of the side chain. Further increasing size at S649 by the substitution of bulky amino acids such as valine, isoleucine and leucine caused slight progressive further attenuation of facilitation. It also caused progressive reduction in block, which correlated well with the size of the residues. These results can be interpreted as implying that the size of the side chain at the position of S649 is critical for drug-channel interaction for facilitation and that it is also involved in the interaction for block although in the latter case, the size effect is much less sensitive.

To obtain further insights into the interaction between the drug and the HERG channel pore, we used the flexible docking simulation method.²² The simulation was first performed with constraints based

on the association of the drug only with T623, S624, Y652 and F656. This should simulate the drug-channel interaction for block. In Figure 5A the geometrical arrangement of the drug is shown for the forty successive examples with the highest score (i.e., the lowest value of the sum of electrostatic interaction and van der Waals force for channel amino acid and drug interaction). Nifekalant is situated in a pocket surrounded by T623, S624, Y652 and F656. But even under these conditions it was noticed that S649 was located near the drug and seemed to form a part of the pocket. This is consistent with the mutational analysis that indicated that nifekalant could be positioned in close vicinity to the side chain of S649 during block without facilitation (Fig. 4).

We then added the requirement for an association between S649 and nifekalant in a simulation of drug-channel interaction, which would correspond to their association during facilitation. The forty successive simulation results with the highest score are shown in Figure 5B. The drug approached S649 in ten cases out of the forty, which compared with only one approach out of forty without the constraint of an association with S649. These results indicate that nifekalant can interact with S649 while retaining its association with the four amino acid residues that are important for block, i.e., T623, S624, Y652 and F656. This supports the notion that S649 may play an important role in the depolarization-induced facilitation of HERG current in the presence of nifekalant.

DISCUSSION

Nifekalant-HERG association for current block. The HERG channel allows a spectrum of compounds different in size and shape to interact with it and prevent ion flow. The computational analysis of the three-dimensional stereoelectronic characteristics of HERG blockers have identified at least four pharmacophores distributed in these chemical compounds.^{13,25-27} These studies showed that a tertiary amine in the middle of these compounds is the most crucial feature of HERG blockers. The basic nitrogen center of the compounds is thought to be protonated at physiological pH and involved in the cation- π interaction with Y652 on the S6 helix.¹⁴ The other pharmacophoric features are hydrophobic moieties, mainly consistent with aromatic rings. One of these hydrophobic moieties associates with the benzyl group of F656 on the S6 helix, presumably based on π -stacking interaction.^{11,14,28} The other hydrophobic moiety is predicted to lie adjacent to points sensitive to steric and electrostatic properties.²⁶ From the mutational analysis of the HERG pore using clofilium and ibutilide analogs, Perry et al.¹³ demonstrated that the hydroxyl groups of T623 and S624 function in association with polar or electronegative *para*-substituents with the phenyl ring of the drugs. As shown in Figure 4A, the HERG pore model for the open channel state indicated that all of these residues, T623, S624, Y652 and F656, exposed their side chains to the central cavity. Taking into account the geometrical constraints of the pharmacophore,^{26,27} HERG blockers would interact and be located at the top of the pore cavity during the block of ion flow.

But Cavalli et al.²⁶ reported that one of the hydrophobic pharmacophores permitted structural variation among the blockers, suggesting that the position of drugs was not restricted to within the central cavity. The structure based drug-binding models for terfenadine and cisapride²⁷ and MK-499¹¹ and HERG showed that these blockers occupied an entire region of the HERG pore cavity. Here,

the flexible docking simulation of channel-drug interaction used a homology model of the HERG pore based on KvAP structure. The simulation constrained nifekalant to T623, S624, Y652 and F656 on the same HERG subunit (Fig. 5A) and strongly suggested that the entire drug molecule is positioned in the pocket at the top of the HERG pore cavity and that it does not extend beyond F656 in the cytoplasmic direction. This simulation could also support the mutation results that indicated that the drug may be located in close vicinity to S649 even during block without facilitation (Figs. 4C, 4D and 5A). In addition, the simulation suggested the possibility that F656 on the adjacent HERG subunit may also participate in forming the binding pocket for nifekalant, even though the constraint was confined to four amino acids on the same channel subunit. Thus residues on several subunits may participate in the association between nifekalant and HERG. This result also supports the idea of a heterogeneous nature for the drug-binding site of the HERG channel pore that has been indicated by the effects of different drugs on the mutant Y652A. While the mutant Y652A is not blocked by HERG-blockers such as nifekalant (Fig. 3), MK-499, clofilium, ibutilide, dofetilide and E-4031,^{11-13,15} it is blocked by vesnarinone, propafenone and bepridil.^{15,29,30} Systematic measurements of effects of various HERG blockers on HERG channel mutants would shed light on the divergent mechanisms of drug-HERG interaction.

Nifekalant-HERG association for current facilitation. A strong depolarized voltage step is a prerequisite for nifekalant to evoke facilitation (Fig. 1). This characteristic allowed us to examine the residues responsible for drug-induced facilitation of HERG separately from block (Fig. 4). We have identified the possibility that S649 is involved in nifekalant-induced facilitation. Either the volume or other properties of the side chain of S649 appeared to influence the interaction between HERG and nifekalant for facilitation. The mutational analysis of S649 indicated that in block without facilitation nifekalant seemed to be located close to the S649 side chain, but not to interact directly with it (Fig. 4). This was supported by the flexible docking simulation. The difference between the facilitated and the purely blocked conditions for the association between nifekalant and HERG is caused by a prior strong depolarization. Although at present it is unclear whether the depolarization causes a conformational alteration in nifekalant or in the HERG pore region, the flexible docking simulation with the addition of S649 showed that it was possible for nifekalant to reach the side chain of S649 while retaining its association with other binding residues. This result is consistent with our hypothesis that S649 is a critical residue for the facilitation effect of nifekalant.

The facilitation of HERG by its blocker has been reported with other compounds such as almokalant⁹ and azimilide.¹⁰ It was suggested that azimilide might interact with residues at the outer mouth of the HERG channel to induce facilitation.¹⁰ But we found that mutations in this region lost the channel's selectivity for K⁺ and C-type inactivation. It is therefore difficult to determine whether these residues are involved in the facilitation of HERG current by nifekalant.

Possible gating mechanism underlying nifekalant-induced facilitation of HERG current. There are at least two possible mechanisms for HERG current facilitation induced by nifekalant. The first is that nifekalant may attenuate C-type inactivation of the HERG channel at low voltages and thus apparently shift the activation curve to more negative voltages. Azimilide did not induce detectable alterations

in the inactivation time course.¹⁰ Nifekalant shifted the voltage dependence of C-type inactivation by at most ~5 mV (Fig. 2D and ref. 8). This effect may enhance the drug-induced block, but cannot lead to an increase of current. Thus, it seems unlikely that facilitation of current by nifekalant is caused by attenuation of HERG channel C-type inactivation.

The second possibility is that the drug affects the activation gating process itself. Crystal structures of various K⁺ channels reveal that the inner pore helix corresponding to S6 in HERG alters its position during gating.³¹ Our mutations of residues on the distal S6 helix caused either an increase or a decrease in the facilitation associated with nifekalant (Fig. 3). But these residues are unlikely to directly interact with the drug because of the geometrical constraints of the pharmacophores and the positions of the amino acids involved in drug binding. The distal S6 helix is currently thought to be responsible for channel activation gating. Therefore, the substitution of the side chains along the S6 helix would itself either enhance or attenuate channel gating.

Whereas similar negative shifts of activation curves have been reported during the modulation of other ion channels including β -adrenergic enhancement of I_{Ks} ³² and L-type Ca²⁺ channel currents,³³ the precise mechanisms have not been elucidated so far. The association of nifekalant and S649 led to the alteration of the activation gating mechanism of HERG. Therefore, this type of regulation of activation gating may be one of the control mechanisms of ion channel activation, which can be modified by chemical compounds and protein phosphorylation.

It has been reported that many chemicals can increase ion channel currents by acting on various ion channel properties. Among them, the activation gating mechanism is one of the important targets for chemicals acting on the channel pore. Bay K 8644, a dihydropyridine derivative, enhances L-type voltage-dependent Ca²⁺ channels by altering the channel mode from I to II.^{34,35} In this case, not only is the channel open time increased but also the activation curve of the channel shifts in the negative direction. Similar alterations were reported for the actions of BDF 9198³⁶ and pyrethroid insecticides³⁷ on voltage-dependent Na⁺ channels. In the case of TRPC5 which is inactive in control conditions, S-nitrosylation at the N-terminus of pore helix causes opening of its activation gate.³⁸ Further studies are needed to clarify how the interaction of chemicals with channel pores results in alteration of ion channel activation gating mechanisms. This will be of help to further elucidate the relationship between structure and function in ion channels.

Mechanistic and clinical significance. We confirm that nifekalant is associated with the facilitation of HERG current, and that pure I_{Kr} blockers E-4031 and dofetilide exhibit no such effect. It was reported that nifekalant effectively suppresses ventricular tachyarrhythmias not only in canine models,³⁹⁻⁴² but also in patients with acute myocardial infarction or severe ventricular dysfunction.⁴³ The effectiveness of nifekalant against lethal ventricular arrhythmias might be related to its effect of facilitating HERG channels. In this context it is interesting to note that while the reduction of HERG channel current by E-4031 increased the magnitude of rate-dependent action potential duration (APD) alternans in a computer model of canine endocardial myocytes,⁴⁴ increasing HERG channel current by shifting the activation curve to more negative voltages abolished APD alternans.³⁹ Nifekalant not only blocks HERG current but also induces facilitation via a negative shift of the activation curve. This

drug is therefore likely to have a lower risk for induction of alternans and may thus be more appropriate for the treatment of arrhythmias than pure I_{Kr} blockers. Further studies on the detailed mechanism of facilitation should be useful for the logical design of clinically effective I_{Kr} blockers with reduced proarrhythmic effects.

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