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Topological Mapping of the Asymmetric Drug Binding to the Human *Ether-à-go-go*–Related Gene Product (HERG) Potassium Channel by Use of Tandem Dimers[S]

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ABSTRACT

The human *ether-à-go-go* related gene product (HERG) channel is essential for electrical activity of heart cells, and block of this channel by many drugs leads to lethal arrhythmias. Tyr_{652} and Phe_{656} of the sixth transmembrane helix are candidates for the drug binding site. In the tetrameric HERG channel, a drug with asymmetric structure should interact unevenly with multiple residues from different subunits. To elucidate the topology of the drug-binding site, we constructed tandem dimers of HERG channels and the aromatic Tyr_{652} and Phe_{656} residues were replaced by alanine singly or doubly. Eight types of HERG channels, including homotetrameric mutants, having different numbers and arrangements of aromatic residues at the blocking site, were studied. Effects of cisapride on channels expressed in *Xenopus laevis* oocytes were examined electrophysiologically. The inhibition constants (K_i) were increased significantly as the diagonal Tyr_{652} were deleted, whereas those for the diagonal Phe_{656}-deleted mutant were not changed. These results suggest that Tyr_{652} residues from adjacent subunits contributed to the binding. Two types of double mutants of tandem dimers showed significantly distinct affinities, suggesting that the coexistence of Tyr_{652} and Phe_{656} on a subunit in diagonal position is crucial to having a high affinity. Thermodynamic double-mutant cycle analyses revealed interactions between Tyr_{652} and Phe_{656} upon binding. The kinetics and voltage-dependence of blocking suggested transitions of the binding site from low to high affinity. These approaches using a set of mutant HERG channels gave a dynamic picture of the spatial arrangements of residues that contribute to the drug-channel interaction.

Malfunction of the human *ether-à-go-go* related gene product (HERG) potassium channels by either hereditary disorders or drug block leads to the long QT syndrome, which increases the risk of lethal arrhythmias (Ashcroft, 2000; Haverkamp et al., 2000; Sanguinetti and Tristani-Firouzi, 2006). A unique property of the HERG channel is that it is blocked by a variety of drugs (Haverkamp et al., 2000). Understanding the mechanism of the susceptibility to the wide spectra of molecular species is of primary importance in developing drugs without side effects to HERG channels (Mitcheson et al., 2000). Experimental data have accumulated about the residues that contribute to the drug binding, and the most important residues are Tyr_{652} and Phe_{656} in the sixth transmembrane helix (Mitcheson et al., 2000, 2005). The HERG channel is composed of four identical subunits that are assembled into a 4-fold symmetric structure (Morais Cabral et al., 1998); however, the blocking drugs are asymmetric in their structure and large enough to span multiple subunits. Thus, aromatic residues (Tyr_{652} and Phe_{656}) from different subunits could contribute unequally to the binding during drug-channel interaction. To identify the contributing residues and their positions in a tetrameric channel, a logical and powerful strategy is to reduce the symmetry of the HERG channel from 4-fold into 2-fold. We constructed, for the first time, a tandem dimer of the HERG channel and introduced aromatic ring-deleting mutations (F656A and Y652A). Here, we focused on a typical HERG channel blocker, cisapride (Mohammad et al., 1997; Walker et al., 1999; Mitcheson et al., 2000), as a probe for the drug-binding sites (Mitcheson et al., 2000). In tandem dimers, double mutations generated different positional arrangements of mutated sites in a channel. A set of mutants, including homotetrameric mutants, permitted a systematic approach to elucidate the topological map of the cisapride binding in HERG channel.

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ABBREVIATIONS: HERG, human *ether-à-go-go* related gene product; $V_{1/2}$, the half activation voltage; K_i, the blocking inhibition constant.

Materials and Methods

Construction of Tandem Dimers. The wild-type HERG cDNA was cloned at Takeda Pharmaceutical Company Limited (Osaka, Japan), and subcloned into the NheI and EcoRI sites in pCDNA3.1(+) plasmid expression vector (Invitrogen, Carlsbad, CA). Single or double mutations (Y652A and/or F656A) were generated by the overlap extension polymerase chain reaction using Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) and confirmed by sequencing the mutated region and by restriction enzyme analysis. The homotetrameric mutant harboring Y652A or F656A was made by introducing the mutated fragment into the HERG channel gene in the pcDNA3.1(+) vector. The HERG tandem dimers were constructed by linking two monomers that were prepared separately (Christie et al., 1990; Isacoff et al., 1990; Ruppersberg et al., 1990; Liman, 1992). For the N-terminal side monomer, the wild-type or mutant (Y652A) HERG channel gene in pcDNA3.1(+) was modified by deleting its stop codon. For the C-terminal side monomer, the HERG channel gene (wild type, harboring single or double mutations) was subcloned downstream of the in-frame EcoRI site (note that the original NheI site in front of the first methionine of the channel gene was modified to a unique EcoRI site when subcloned) in the pKF3 vector (Takara Bio, Kyoto, Japan). To generate tandem dimers, the entire channel coding region in the pKF3 vector was digested with EcoRI and ligated into the pcDNA3.1(+) vector harboring the stop codon-deleted channel gene. The two monomers were linked by the nucleotide sequence of GAATTC. For expression in *Xenopus laevis* oocytes, a stretch of 30 polyA (dA:dT) residues was inserted behind the stop codon.

Western Blot Analysis. HEK293 cells stably expressing HERG channels were homogenized at 4°C in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM EDTA), containing a protease inhibitor mix (Roche Applied Science, Indianapolis, IN) and spun at 500*g* for 10 min. Pellets of the membrane fractions were produced from the low-speed supernatants by centrifugation at 150,000 rpm for 30 min. For Western blots, membrane proteins were separated on 7% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Membranes were probed with polyclonal rabbit anti-HERG antibody (Millipore Bioscience Research Reagents, Temecula, CA) and the antibody was detected with an ECL detection kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Electrophysiological Recordings. Two-electrode voltage-clamp experiments were performed. Procedures for the isolation of *X. laevis* oocytes, injection of cRNA and electrophysiological experiments have been described in detail previously (Shimizu et al., 2003) and were approved by the Animal Research Committee of University of Fukui. The bath solution contained 4 mM KCl, 96 mM *N*-methyl-D-glucamine Cl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.40. Cisapride (Accurate Chemicals, Westbury, NY) was dissolved into dimethyl sulfoxide to make 10 mM stock solutions and kept at −20°C until use. Voltage clamp experiments were performed on the oocytes 2 to 6 days after injection using a Dagan CA1 amplifier (Dagan Corporation, Minneapolis, IL) at 22–24°C. For the recordings from oocytes expressing the F656A mutant, which has been known as poor expression of channels (Mitcheson et al., 2000; Milnes et al., 2003), electrophysiological experiments were performed within 3 days after the injection until the oocyte became damaged. The oocytes were perfused continually with the perfusates at the rate of 1 ml/min using an infusion pump (TE-331; TERUMO, Tokyo, Japan). Oocytes exhibiting endogenous currents were excluded from analyses.

Activation and Inactivation Gate. To characterize the activation gate for mutant channels, the peak amplitudes for the tail currents at −50 mV were plotted as a function of the preceding activation voltages. This tail I-V curve was fitted with the Boltzmann function.

$$I_{tail} = \frac{A_1 - A_2}{1 + \mathrm{Exp}[(V - V_{1/2})/dx]} + A_2 \quad (1)$$

where V is the membrane potential, $V_{1/2}$ is the half activation voltage, dx is the slope factor, and A_1 and A_2 are the amplitude factors. For the inactivation gate, the current amplitudes at the end of the 3-s depolarization pulses (isochronal I-V curves) were drawn, which were superimposed on the tail I-V curves for each channel species (Fig. S1). The isochronal I-V curves show a typical bell shape, indicating the characteristic inactivation of HERG channel. The double-Boltzmann function was used for the fitting.

$$y = \frac{A_2}{1 + \mathrm{Exp}[(V - V_{1/2})/dx] + \mathrm{Exp}[(V - V_{inac})/dx_{inac}]} + A_1 \quad (2)$$

where V_{inac} and dx_{inac} values were the half inactivation voltage and the slope factor. A_1 and A_2 are the amplitude factors. In this fitting, the fitted parameters from the activation data ($V_{1/2}$ and dx) were used.

Blocking Inhibition Constants. Block of the HERG currents at each cisapride concentration was evaluated after the steady state of blocking was reached. This was performed by eliciting repeatedly 3.5-s depolarization pulses to +20 mV followed by repolarization at −50 mV an interval of 20 s (the holding potential was −80 mV) and checking the current amplitudes reached at the steady state. At low concentrations, it took 10 to 20 minutes. Inhibition constants (K_i) were obtained from the peak amplitudes of the tail currents at −50 mV preceded by the depolarizing pulse to +20 mV for 3 s. From concentration dependence of current amplitude, K_i values and the Hill coefficients (n) were obtained from the concentration dependence of current amplitude by fits to the equation:

$$I/I_0 = \frac{1}{1 + ([\mathrm{cisapride}]/K_i)^{n_H}} \quad (3)$$

Voltage-dependence of the binding affinity was obtained by fitting the K_i values with the function.

$$K_i = K_0 \,\mathrm{Exp}[-ezV/k\,T] \quad (4)$$

where K_0 represents the inhibition constant at 0 mV, z is the gating charge, and e, k, and T are the elementary charge, the Boltzmann constant, and the absolute temperature, respectively.

Thermodynamic Double-Mutant Cycle. To estimate the interaction free energy upon binding, thermodynamic double-mutant cycle analysis was applied (Schreiber and Fersht, 1995; Ranganathan et al., 1996; Fersht, 1999; Yan et al., 2006). First, the binding free energy (G) was evaluated from the inhibition constant.

$$G = k\,T \log K_i \quad (5)$$

The differences in the affinities between two channel species were obtained from the differences in G. For example, the change in the binding free energy by a single mutation relative to the wild type (WT) is

$$\Delta G_{singleM-WT} = G_{singleM} - G_{WT} \quad (6)$$

A double mutant can be regarded as a combination of two single mutations. Therefore, ΔG for the double mutant compared with WT is:

$$\Delta G_{doubleM-WT} = G_{singleM-WT} + G_{doubleM-singleM} \quad (7)$$

The ΔG for mutation of Phe_{656} site introduced into either WT or Y652A is $\Delta G_{singleM-WT}$ or $\Delta G_{doubleM-singleM}$. The difference in these energies defines the coupling energy, $\Delta\Delta G_{int}$, which represents interaction between two sites.

$$\Delta\Delta G_{int} = \Delta G_{singleM-WT} - \Delta G_{doubleM-singleM} \quad (8)$$

The values of $\Delta\Delta G_{int}$ indicate cooperativity between residues.

Blocking Kinetics. The apparent blocking rate (equal to the reciprocal of the time constant) for a simple three-state model can be expressed as the following equation, if the binding and unbinding

kinetics are fast relative to the subsequent transition represented by the rate constants of k_3 and k_{off}.

$$blocking\ rate = \frac{[B]k_3}{[B] + K_f} + k_{off} \quad (9)$$

K_f (= k_2/k_1) is the dissociation constant for the initial binding site (see Supplemental Data).

Data Analysis. Experimental data were analyzed with Origin software (OriginLab Corp., Northampton, MA) and Mathematica (Wolfram Research Inc., Champaign, IL). Data are presented as mean ± S.E.M.

Results

Expression of Tandem Dimers. We constructed seven mutants (Table 1), which were expressed in *X. laevis* oocytes and human embryonic kidney 293 cells (Ando et al., 2005). Western blot was performed on human embryonic kidney 293 cells to verify the expression of the mutants (Fig. 1B). Tandem dimers showed protein bands at the expected locations. For monomeric channels, double protein bands were observed at around 135/155 kDa, as has been reported (Zhou et al., 1998; Ficker et al., 2004). For tandem dimers, double bands were seen at 270/310 kDa.

Electrophysiological Characterization of Mutant Channels. The electrophysiological properties of mutant and WT HERG channels expressed in *X. laevis* oocytes were examined. Gating properties for WT and all the mutants, including the tandem WT (td[wt:wt]), were evaluated from the current traces elicited by depolarizing pulses (Fig. 2A). The activation and inactivation gating were measured by plotting the tail I-V curves and the isochronal I-V curves at the end of the depolarization pulses (Fig. 2A, inset, and Supplemental Fig. S1). The voltage dependence of activation and inactivation gating for WT and td[wt:wt] were basically similar, suggesting that concatenation did not affect the steady-state gating. In all mutants, the activation curves were shifted slightly in the negative direction (Table 2), although the slope factor was not changed significantly. Deactivation for td[wt:wt] was faster compared with the WT. This tendency seemed to hold for all the tandem dimers. A contribution of the N-terminal domain to the deactivation gating of HERG channel has been reported (Morais Cabral et al., 1998; Wang et al., 2000). Then a cytoplasmic domain between subunits in the tandem dimers might be restricted by the short linker, which may accelerate the deactivation rate.

The Steady-State Blocking of Cisapride. To examine the concentration dependence of current blocking to cisapride, an oocyte was perfused continually throughout the experiment, and HERG currents were evaluated after the block reached the steady-level. The current traces showed (Fig. 2B) a slow decay during depolarizing pulses for WT and some of the other mutants and mostly reached the steady state at the end of the depolarization pulses. This decay was not observed for Y652A, F656A, and td[Y652A:F656A]. K_i values were obtained from the concentration dependence of the peak amplitudes of the tail currents at −50 mV (Table 3). The Hill coefficients were nearly one, suggesting the binding stoichiometry of the drug-HERG channel as one to one.

The free energy of the binding of cisapride to HERG channels, which was calculated from the K_i values as $G = k\ T \log K_i$, is shown in Fig. 3. It is clearly seen that not only the number of the aromatic residues but also the spatial location of each residue is crucial for the binding affinity. The affinities were reduced dramatically by complete deletion of either of the aromatic residues (F656A and Y652A), confirming previous reports (Mitcheson et al., 2000). The levels of the binding energy for WT and td[wt:wt] were similar, indicating that the concatenation did not affect the binding of the blocker. The affinity for td[wt:F656A] was similar to that of WT, which indicates that, although Phe_{656} contributed to the binding significantly, at most two Phe_{656} residues from diagonal subunits were required for binding. On the other hand, the affinity of td[wt:Y652A] was significantly reduced, suggesting that Tyr_{652} residues from adjacent subunits contributed to the binding. This is the first experimental observation that cisapride may interact with multiple Tyr_{652} residues.

The sequence of the binding energies among mutants with the same number of aromatic residues was most informative (see the rightmost column of Fig. 3). First, to have both Phe_{656} and Tyr_{652} aromatic residues on a channel is more important for high-affinity binding than to have single-species aromatic rings. Second, the 4-fold higher affinity (the difference of the binding free energy: ΔG = 3.5 kJ/mol) of td[wt:Y652AF656A] than td[Y652A:F656A] indicates that having both Tyr_{652} and Phe_{656} on the same subunit is crucial

TABLE 1
Mutant constructs of HERG channels
In this study, the mutants were named in the conventional manner for homotetrameric channels (such as F656A). For tandem dimers, single or double mutations introduced into the N- or C-terminal subunits separated by a colon for the linker.

HERG Channel	Mutations	N-Terminal Side	C-Terminal Side
Homotetramer			
WT	Wild type		
Y652A	Y652A		
F656A	F656A		
Tandem dimer			
td[wt:wt]		Wild type	Wild type
td[wt:Y652A]		Wild type	Y652A
td[wt:F656A]		Wild type	F656A
td[wt:Y652AF656A]		Wild type	Y652A + F656A
td[Y652A:F656A]		Y652A	F656A

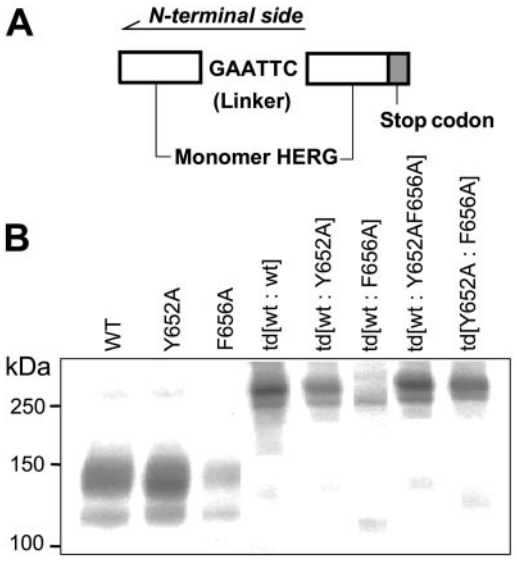

Fig. 1. Mutants of HERG channels. A, a schematic diagram of the tandem dimer construct of the HERG gene. B, Western blot analyses of homotetrameric and tandem dimeric HERG gene products.

for a channel to show a high affinity. Energetic considerations provided further clues on the contributions of residues to the binding and interactions between residues upon binding, which will be discussed below.

Voltage-Dependent Blocking. Voltage-dependent blocking was evaluated from current traces elicited by depolarizing pulses (Fig. 2B). In WT and some mutants, the slow decay of the currents seen at depolarized potentials was accelerated as the membrane potential was depolarized more. In the presence of cisapride, the tail I-V curves (the peak amplitudes of the tail currents as a function of the preceding depolarizing voltages) showed a maximum at around 0 mV for WT (Fig. 4A). The current amplitudes were depressed slightly as the membrane potential was depolarized further. This pattern is an indication of the presence of voltage-dependent block (Walker et al., 1999; Sánchez-Chapula et al., 2003). At each voltage, the K_i values were calculated from the concentration-dependence curves (Fig. 4B).

Likewise, the K_i values for mutants at different membrane potentials were obtained (see Supplemental Data) and are also shown in Fig. 4B. K_i values were voltage-dependent for WT, td[wt:wt], td[wt:F656A], td[wt:Y652AF656A], and F656A. td[Y652A:F656A], td[wt:Y652A], and Y652A showed no voltage dependence. It should be noted that voltage-dependence was almost abolished even when only two diagonal Tyr_{652} residues, not four of them, were deleted.

Kinetics of Blocking. Current traces of some of the mutants as well as WT exhibited slow decays at depolarized potentials, whereas others did not. To examine the mechanisms underlying the different blocking kinetics, we focused on F656A. F656A showed voltage-dependent block, but the currents did not show a decay at depolarized potentials. We found that the shapes of the tail currents were changed in the presence of cisapride (Fig. 5). Expansion of the tail current traces showed that the currents reached their peak with a slower rising phase in the presence of cisapride (Fig. 5, right),

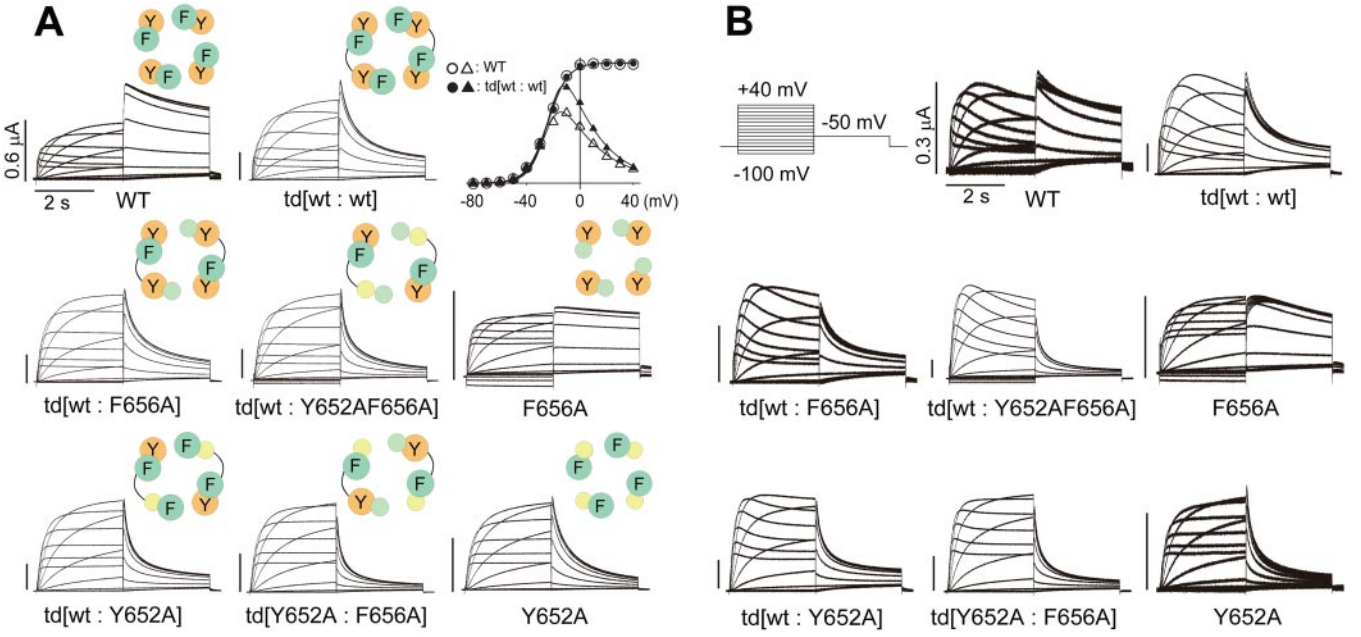

Fig. 2. Representative current traces for WT and mutant HERG channels. Currents were elicited by 3-s step pulses from −100 to +40 mV with 10-mV increments followed by 3-s pulses to −50 mV (the holding potential was −80 mV). In this figure, the current amplitudes were normalized to emphasize the differences among the various channels. A, current traces for HERG channels in the absence of cisapride. Cartoons represent the arrangements of Tyr_{652} and Phe_{656} residues around the symmetrical axis of the channel. The partial overlap of the symbols for Tyr_{652} and Phe_{656} show that they are on the same subunit. Small circles represent mutations to alanine. Schemes with linkers represent tandem dimers. For tandem dimers, N- and C-terminal subunits were arranged counter-clockwise. Inset, the tail I-V curves (open symbols) and the isochronal I-V curves (closed symbols) at the end of the depolarizing pulses for WT (black) and td[wt:wt] (red). B, current traces in the presence of cisapride. Cisapride concentrations (micromolar) were 0.63 for WT, 0.25 for td[wt:wt], 0.63 for td[wt:F656A], 0.63 for td[wt:Y652AF656A], 1.6 for F656A, 0.63 for td[wt:Y652A], 1.6 for td[Y652A:F656A], and 10 for Y652A. Inset, the voltage command.

TABLE 2

Gating properties of HERG channels

The half activation and inactivation voltages ($V_{1/2}$) and their slopes are shown. *N* represents the number of observations.

HERG Channels	Activation		Inactivation		*N*
	$V_{1/2}$	Slope	$V_{1/2}$	Slope	
			mV		
WT	−21.0 ± 0.7	7.2 ± 0.2	−61.6 ± 4.5	−26.7 ± 1.1	8
td[wt:wt]	−22.2 ± 0.8	7.0 ± 0.2	−61.4 ± 3.5	−26.2 ± 0.9	9
td[wt:F656A]	−25.1 ± 0.8	7.3 ± 0.1	−60.2 ± 4.0	−30.2 ± 1.0	10
td[wt:Y652A]	−24.3 ± 1.1	7.6 ± 0.1	−51.4 ± 4.6	−32.1 ± 1.1	10
td[wt:Y652AF656A]	−24.2 ± 0.4	7.7 ± 0.2	−56.7 ± 4.9	−28.3 ± 1.9	9
td[Y652A: F656A]	−25.7 ± 0.7	7.5 ± 0.2	−57.0 ± 4.9	−36.6 ± 1.2	10
F656A	−27.3 ± 0.7	6.7 ± 0.1	−87.1 ± 4.6	−41.7 ± 3.0	12
Y652A	−22.4 ± 0.6	7.6 ± 0.1	−50.0 ± 3.7	−35.5 ± 1.1	10

which was not observed in the absence of cisapride (Fig. 5, left). The slowly rising phases became more prominent as the preceding voltages were more depolarized. The tail currents of F656A were fitted with a double-exponential function in the absence of cisapride (the recovery from the inactivation and deactivation components) and with a triple-exponential function in the presence of cisapride. The novel kinetic component having an intermediate time constant (~100 ms) between those of recovery from the inactivation and deactivation seems to be related to blocking. In fact, the time constant of the intermediate component was reduced as the cisapride concentration was increased (see Supplemental Data). This is in contrast to the time constant for recovery from the inactivation being unaltered. These results suggest that the intermediate component represented unblocking kinetics. The amplitudes of the unblocking component increased as the prepotential was more depolarized (see Supplemental Data). This is because more channels had been blocked at more depolarized potentials, and those blocked channels released more drug upon repolarization. These are typical voltage-dependent unblocking kinetics (Hille, 2001). The plot of the apparent rate of block (equal to the reciprocal of the unblocking time constant) as a function of cisapride concentration showed a nearly linear relationship (Fig. 7A, open diamonds). This kinetic feature will be discussed with respect to the blocking mechanism below.

For other types of channels, the kinetics of tail current were examined, but kinetic components relevant to the unblocking of cisapride were not found. However, the blocking kinetics was evaluated on a longer time scale from a pulse protocol shown in Fig. 6. Depolarizing pulses to +20 mV were prolonged progressively from 50 to 7000 ms, and the tail currents at −50 mV were recorded. The peaks of the tail currents were plotted as a function of the duration of the depolarization (the envelope current; Fig. 6B, inset). In the absence of cisapride, the current envelopes reached saturation as the depolarization pulses were prolonged (inset, filled squares). These represent the time courses of the activation gating. In the presence of cisapride the envelope currents were depressed as the duration of depolarization was prolonged. This depression was seen for WT, td[wt:wt], td[wt:F656A], td[wt:Y652A], and td[wt:Y652AF656A] (these chan-

TABLE 3

Blocking parameters of cisapride to HERG channels at +20 mV

These K_i values represent drug binding at depolarized potential of +20 mV, since unblocking was slow and could not be detected at the time of peak-current measurements for most of the channel species except for F656A. The K_i value for F656A at +20 mV was obtained by correcting for the unblocking component at the tail potential (see Supplemental Data). *N* represents the number of observations

HERG Channel	K_i	Hill Coefficient	*N*
	μM		
WT	0.20 ± 0.02	0.96 ± 0.05	4
td[wt:wt]	0.25 ± 0.01	1.36 ± 0.07	4
td[wt:F656A]	0.17 ± 0.01	1.04 ± 0.05	5
td[wt:Y652A]	0.85 ± 0.06	1.15 ± 0.09	5
td[wt:Y652AF656A]	0.50 ± 0.05	1.10 ± 0.05	5
td[Y652A: F656A]	2.05 ± 0.04	1.19 ± 0.01	5
F656A	3.63 ± 0.44	0.99 ± 0.09	6
Y652A	9.15 ± 0.67	1.23 ± 0.11	5

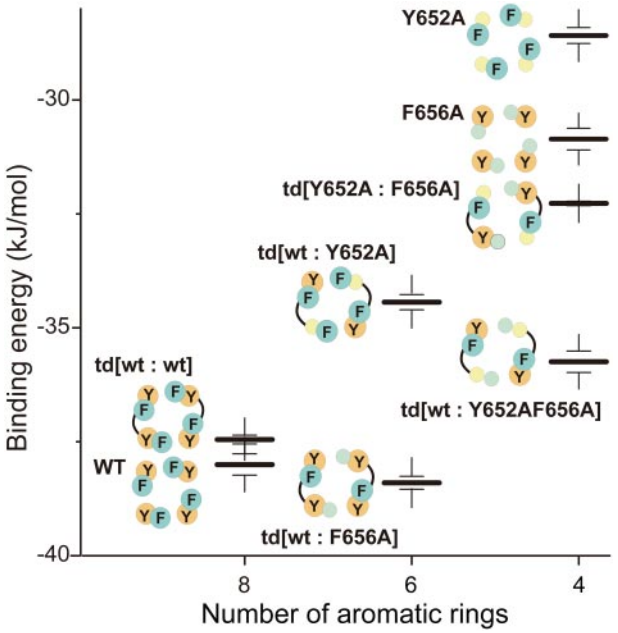

Fig. 3. The binding energetics. The vertical axis represents the free energy of binding. The binding energy for each mutant is shown as a level bar. Each column represents channel species having the same numbers of aromatic residues.

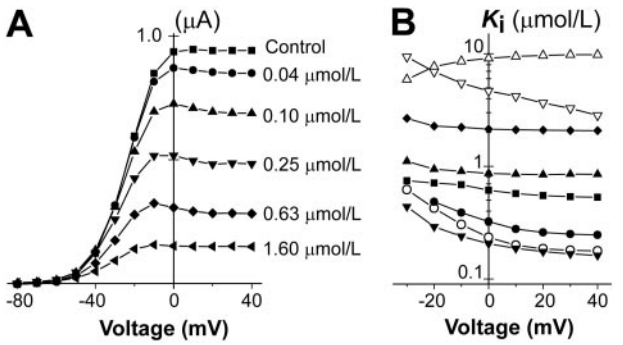

Fig. 4. Voltage-dependent block. A, the tail I-V curves of WT in the absence and in the presence of increased concentrations of cisapride. B, voltage-dependence of K_i values for WT and mutant HERG channels. The scale of the vertical axis is logarithmic. Symbols indicate: ○, WT; ▼, td[wt:F656A]; ●, td[wt:wt]; ■, td[wt:Y652AF656A]; ▲, td[wt:Y652A]; ◆, td[Y652A:F656A]; ▽, F656A; △, Y652A. Voltage dependence of the block was expressed by the gating charge (see Supplemental Data): 0.40 *e* for WT, 0.48 *e* for td[wt:wt], 0.37 *e* for td[wt:F656A], 0.03 *e* for td[wt:Y652A], 0.01 *e* for td[Y652A:F656A], 0.21 *e* for td[wt:Y652AF656A], 0.33 *e* for F656A, and −0.10 *e* for Y652A (*e* is the elementary charge). The value for F656A was corrected for the unblocking (see Supplemental Data).

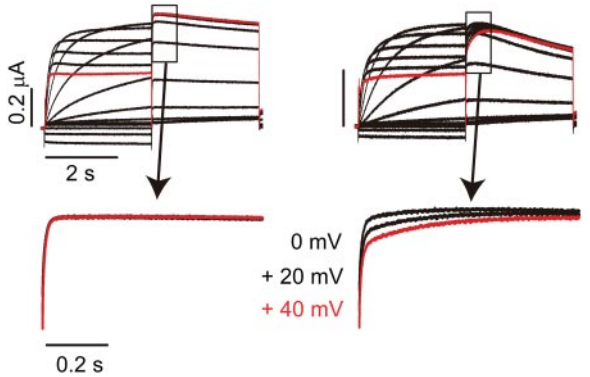

Fig. 5. Unblocking of cisapride from F656A. Left, current traces at different membrane potentials (top) along with those of tail currents on an expanded time scale in the absence of cisapride (bottom). The right panel shows current traces at 1.6 μM cisapride. A slow rising phase appeared in the presence of cisapride. Kinetic analyses were performed on the tail current traces (see detail in Supplemental Data).

nels are named here as the high-affinity channels) but not for td[Y652A:F656A], F656A, and Y652A. The envelope currents were fitted with a double-exponential function (Fig. 6B, inset). The fast and slow components represent the time course of activation and block. The envelope experiments were performed at different cisapride concentrations. The time constants of activation for all the channel species were similar and did not change significantly with cisapride concentration. On the other hand, the time constants for the blocking changed significantly. For channel species exhibiting depression of the current envelopes, plots of the apparent rates of block (equal to reciprocal of the blocking time constants) as a function of cisapride concentration showed nearly linear relationships (Fig. 7A).

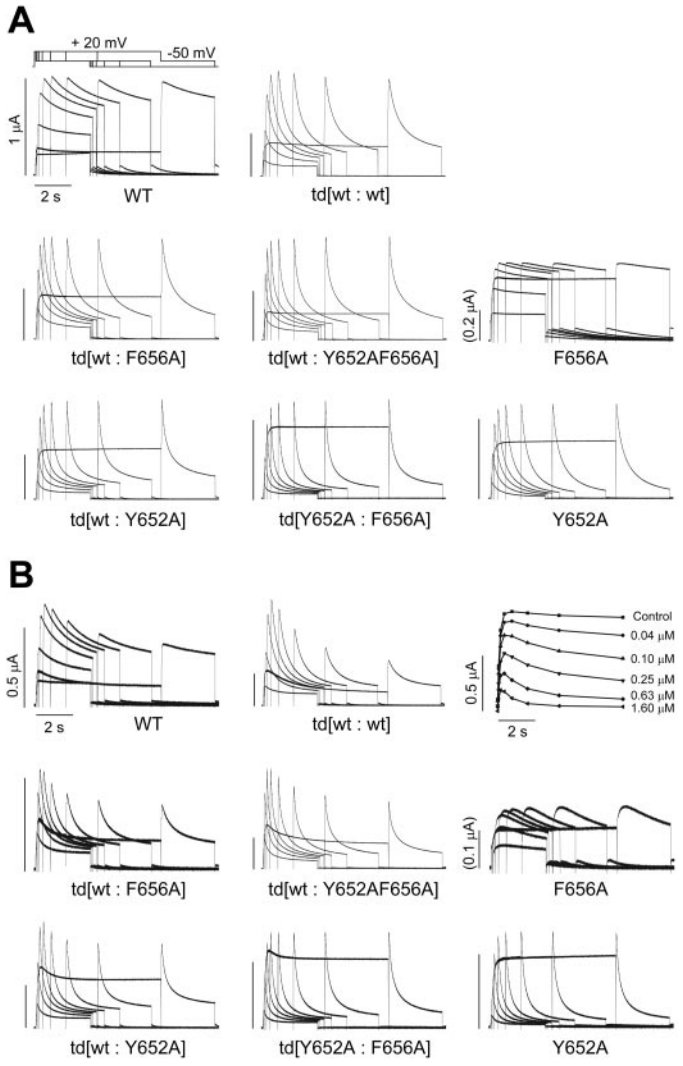

Fig. 6. Envelope analyses of HERG channels without (A) and with cisapride (B). A, representative traces of envelope current for WT and mutants. Inset, the command potentials. The longest depolarization pulse was 7 s. B, representative traces of envelope current for WT and mutants at the following cisapride concentrations (micromolar): 0.25 for WT, 0.25 for td[wt:wt], 0.63 for td[wt:F656A], 0.25 for td[wt:Y652AF656A], 4.0 for F656A, 0.63 for td[wt:Y652A], 1.6 for td[Y652A:F656A], and 10.0 for Y652A. It should be noted that current traces early in depolarization pulses for pulses of different durations overlapped completely. This signifies that the currents were recorded in the steady state of blocking. Inset, the time course of block for WT. The peaks of the tail currents were plotted as a function of the duration of depolarizing pulses. The time courses of the currents were fitted with a double-exponential function (curved lines). Current data with a fitted line were shown for different cisapride concentrations. As the cisapride concentrations were increased, current amplitudes were depressed and the decay of current (blocking) was accelerated.

Discussion

In this study, tandem dimers of HERG channels were constructed for the first time to investigate the mechanisms underlying the block of HERG channels. Introducing single or double mutations into tandem dimers, including single-mutation homotetramers, produced channels with variable arrangements of aromatic residues. The tandem dimers exhibited channel activity with the fast deactivation. This might be a signature that both subunits of the concatenated dimers were incorporated into tetrameric channels: a cytoplasmic domain between concatenated subunits may be restricted in the closely packed subunits which affected the deactivation gating (Morais Cabral et al., 1998; Tu and Deutsch, 1999; Wang et al., 2000). Interactions between cisapride and these mutant HERG channels were examined electrophysiologically, and the steady-state binding, its voltage dependence, and the kinetics of block were analyzed.

Binding Energetics and Topology. The set of mutants revealed the importance of the spatial arrangement of distinct residues to the binding. In this study, we found that cisapride binding was not confined to a single subunit but spans multiple subunits in a tetrameric channel: Tyr_{652} residues of adjacent subunits contribute to the binding. In contrast, at most two Phe_{656} residues in diagonal subunits, rather than those in adjacent subunits, were enough for binding, although complete loss of Phe_{656} (F656A) destabilized the binding dramatically. For the high-affinity binding, retaining intact subunits seems to be important.

For the two double mutants (td[wt:Y652AF656A] and td[Y652A:F656A]), the binding energy differed significantly, which indicates the importance of the arrangements of the aromatic residues in a channel. The contribution of residues to the binding and the interactions between residues upon binding can be evaluated quantitatively by examining the coupling energies ($\Delta\Delta G_{int}$) in the thermodynamic double-mutant cycle analysis (see *Materials and Methods*; Schreiber and Fersht, 1995; Ranganathan et al., 1996; Fersht, 1999; Yan et al., 2006). For each of two double mutants, a cyclic diagram was drawn (Fig. 8A). In these diagrams, a double mutant can be regarded as the consequences of two successive single mutations reached through two different routes. Inspection of a set of cartoons for a cycle gives ideas how the residues interact upon drug binding.

For the upper cycle (toward td[wt:Y652AF656A]), the cartoons for four channel species exhibit a common feature; i.e., a diagonal pair of subunits is intact. This means that the double mutant was generated by introducing two mutations successively into subunits adjacent to the intact subunits. From a thermodynamic relationship (eq. 8), $\Delta\Delta G_{int}$ of 0.3 kJ/mol was obtained. This value of $\Delta\Delta G_{int}$ did not deviate statistically from zero, indicating that no interaction took place between Tyr_{652} and Phe_{656} upon binding. The results indicate that, as far as intact subunits were retained in a channel, Tyr_{652} and Phe_{656} residues on the subunits adjacent to the intact subunits contributed to the binding independently of each other. This additivity is important because it suggests that deleting bulky aromatic rings in two sites did

not significantly change the structures of the binding site (Fersht, 1999). This is an implicit assumption for studies of mutational analysis in general but has not been supported for HERG channels until this study.

Next, look at another cycle with td[Y652A: F656A] as a double mutant (Fig. 8A, lower panel). The $\Delta\Delta G_{int}$ value of −3.1 kJ/mol indicates significant interactions. The negative sign of $\Delta\Delta G_{int}$ represents that Phe_{656} and Tyr_{652} contribute to the binding with negative interactions. What is the origin of this cooperativity? In the double-mutant cycle analysis, significant coupling is explained as being attributable to the proximity of mutated residues (Schreiber and Fersht, 1995). The set of cartoons demonstrates that two mutational sites are proximally positioned on the two adjacent subunits. Then the strong interaction can be interpreted that Phe_{656} and Tyr_{652} residues on two adjacent subunits are closely apposed.

In the thermodynamic double-mutant cycle analysis, two different types of interactions were elucidated. Tyr_{652} and Phe_{656} on the same subunits interact additively (upper cycle) and Tyr_{652} and Phe_{656} on adjacent subunits interact cooperatively (lower cycle). One may imagine that the Tyr_{652} and Phe_{656} residues from adjacent subunits are placed closer than those placed on the same subunits.

Hypothetical Binding Pose of Cisapride in the High-Affinity Site of HERG Channels. Cisapride contains two aromatic moieties and one basic tertiary nitrogen (Fig. 8B) that can interact with the aromatic amino acids by cation-π and π-π interactions or by hydrophobic interactions (Mitcheson et al., 2000; Fernandez et al., 2004). Here we propose a possible binding mode of cisapride. Two Tyr_{652} residues from adjacent subunits and one Phe_{656} form a favorable binding site, whereas Phe_{656} in the adjacent subunit hinders the binding (Fig. 8B).

This mode of interaction was compared with the computational docking model built on a homology model of HERG channel (Aronov and Goldman, 2004; Osterberg and Aqvist, 2005; Farid et al., 2006). It was predicted that multiple simultaneous aromatic ring stacking and/or hydrophobic interactions between Tyr_{652} and Phe_{656} side chains and aromatic/hydrophobic blocker groups are involved in the docking poses (Farid et al., 2006). It was also predicted that Tyr_{652} and Phe_{656} from multiple subunits might be involved in cisapride binding (Farid et al., 2006). This docking pose is in general agreement with our experimental data.

Voltage-Dependent Block Suggested a Sequential Binding Mechanism. The voltage-dependence of cisapride block has been reported (Walker et al., 1999), and in this study, it was shown that the K_i values for WT and some mutants were voltage-dependent (Fig. 4). Not only Y652A but also td[Y652A:F656A] and td[wt:Y652A] exhibited voltage independence. The presence or absence of the voltage dependence for different mutants indicates that voltage-dependent block occurs through voltage-dependent conformational changes of the binding site rather than the binding of a charged drug to the site under the influence of the membrane electric field. To introduce voltage-dependence into the blocking mechanism, a sequential binding model was applied:

$$O \overset{[B]}{\longleftrightarrow} B_1 \longleftrightarrow B_2$$

in which O represents the open state, B_1 represents the first blocked state, and B_2 is the subsequent blocked state. $[B]$ indicates the concentration of blocker. In this model, drug-bound channels undergo conformational changes between the B_1 and B_2 states in a voltage-dependent manner, leading to more stable binding of the drug in the B_2 state. A similar model has been proposed for blocking by chloroquine (Sánchez-Chapula et al., 2002).

The behavior of channels exhibiting voltage-independent blocking (Y652A, td[wt:Y652A] and td[Y652A:F656A]) can be explained by the absence of transitions from B_1 to B_2. In these mutants, some of Tyr_{652} residues were deleted, suggesting that they contribute to the voltage-dependence through changes in their orientation upon depolarization (Sánchez-Chapula et al., 2003). In this study, we found that the voltage dependence was almost abolished even for td[wt:Y652A]. On the other hand, td[wt:Y652AF656A] retained voltage dependence. It is suggested that rearrangements of Tyr_{652} residues seem to be affected by surrounding Phe_{656} residues.

Kinetic Data Suggest a Low-Affinity Binding Site. Blocking kinetics were evaluated by two different methods: unblocking kinetics after repolarization and slow blocking kinetics by the envelope analyses. In either case, the apparent rate constants of block were found to be a nearly linear

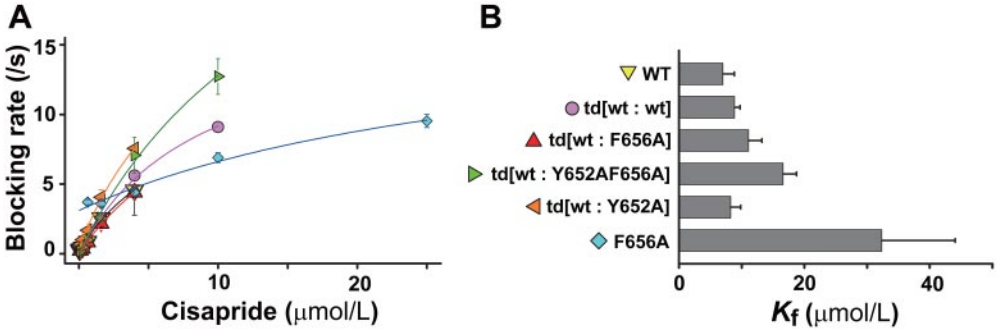

Fig. 7. Blocking kinetics. A, the apparent rate of blocking as a function of cisapride concentration. Curves represent fits with eq. 9 (see *Materials and Methods*). The intercepts of the *y*-axis represent the values of k_{off}. The k_{off} values (per second) for the mutants were as follows: 0.10 ± 0.13 for WT, 0.01 ± 0.15 for td[wt:wt], 0.01 ± 0.08 for td[wt:F656A], 0.03 ± 0.00 for td[wt:Y652AF656A], 0.32 ± 0.16 for td[wt:Y652A], and 3.1 ± 0.4 for F656A. The k_3 values (per second) were 0.015 for WT, 0.022 for td[wt:wt], 0.034 for td[wt:F656A], 0.017 for td[wt:Y652AF656A], 0.017 for td[wt:Y652A], and 0.012 for F656A. The symbols for channel species are shown in A. B, K_f values (micromolar) obtained from fitting the data shown in A. The fit parameters roughly reproduced the K_i values obtained from the concentration dependence of the steady-state block. Nearly linear relationships suggest that experiments were performed in the concentration range below the K_f value for each channel species.

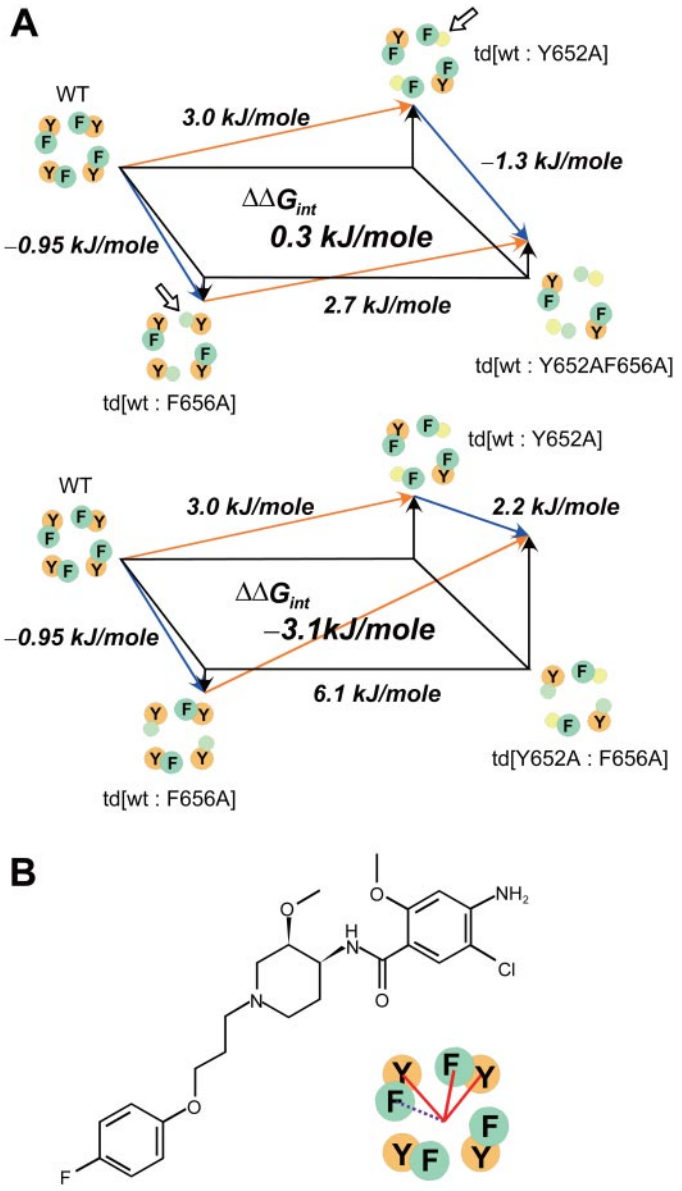

Fig. 8. Energetics and orientation of cisapride binding. A, the thermodynamic double-mutant cycle analysis. Cartoons for mutant channels are presented without the linker to show differences in special arrangements of residues between mutant channels. Orange arrows represent mutations from Tyr_{652} to Ala and blue ones for Phe_{656} → Ala. There are two pathways to get double mutants: first, Tyr_{652} → Ala (the upper orange arrow), then Phe_{656} → A (the right blue arrow). Another route is Phe_{656} → A (the left blue arrow) first and Tyr_{652} → A (the lower orange arrow) later. The upper panel shows the cycle to td[wt:Y652AF656A] and the lower to td[Y652A:F656A]. Numerical values indicate ΔGs (see *Materials and Methods*). The parallelogram indicates the level of the binding free energy for td[wt:wt], from which up and down arrows were drawn for ΔGs. The changes in the binding energy from td[wt:wt] to td[wt:Y652AF656A] were evaluated by the sums of ΔGs through either of the pathways ($\Delta G_{singleM1\text{-}WT} + \Delta G_{doubleM\text{-}singleM1} = \Delta G_{singleM2\text{-}WT} + \Delta G_{doubleM\text{-}singleM2}$). If two residues contribute to the binding independently, ΔG of a mutation at one site should not depend on whether another site is intact or mutated ($\Delta G_{singleM1\text{-}WT} = \Delta G_{doubleM\text{-}singleM2}$). This is seen in the mutations from td[wt:wt] to td[wt:Y652A] (ΔG = 3.0 kJ/mol) and from td[wt:F656A] to td[wt:Y652AF656A] (ΔG = 2.7 kJ/mol) and the $\Delta\Delta G_{int}$ value was 0.3 kJ/mol. For the double-mutant cycle in the bottom panel, the $\Delta\Delta G_{int}$ value was −3.1 kJ/mol. As seen from the double mutants for both cycles, the coupling energy represents interactions between Tyr_{652} and Phe_{656} in the same subunit and in the adjacent subunits. Significant interaction suggests that Tyr_{652} and Phe_{656} from different subunits located in proximity. B, chemical structure of cisapride and a hypothetical binding topology. Red lines indicate positive interaction, whereas the blue dotted line indicates negative contributions. In this scheme, bindings are restricted to the adjacent two subunits.

function of the cisapride concentrations (Fig. 7A). If the blocking kinetics is described by a simple two-state model, the rate constant of block should be linear. Sublinearity of the blocking rate constant can be accounted for by the presence of an intermediate state of block. Therefore at least three states are necessary to describe the kinetic data, which is compatible with the linear 3-state model proposed for the voltage-dependent block. Sublinearity of the kinetic data demands that the model has a fast low-affinity binding process followed by a slow transition.

$$O \underset{k_2}{\overset{[B]k_1}{\rightleftharpoons}} B_1 \underset{k_4}{\overset{k_3}{\leftrightarrow}} B_2$$

where k_1 through k_4 represent the rate constants, and the white arrow indicates fast transitions.

The eq. 9 (see *Materials and Methods* and Supplemental data) for the apparent blocking rate was used to fit the data and values of K_f (the dissociation constant for the initial binding site), k_3 and k_{off} (= k_4) were obtained (Fig. 7B, also see Supplemental Data). For the high-affinity channels (WT, td[wt:wt], td[wt:F656A], td[wt:Y652A], and td[wt:Y652AF656A]), the k_{off} values were very slow (< 0.32/s; Fig. 7A legend). This slow unblocking accounts for the apparent absence of the unblocking at the time of peak for tail current measurements: the high affinity blocked state (B_2) did not return to the open state through B_1 during the early course of the tail currents. K_i values evaluated from the tail currents thus reflect blocking at depolarized potentials. k_3 was so slow that the high-affinity binding site scarcely appeared during short depolarizing pulses. Slow appearance of the high-affinity site and slow release from it led to slow accumulation of blocking during repeated depolarizations.

The relationships between the inactivated state and the high-affinity binding state have been discussed. The inactivation state is a state for a nonconducting selectivity filter, whereas the high-affinity binding state represents arrangements of aromatic residues in the central cavity. From a spatial perspective, they are closely located. However, the kinetic analyses suggest that the high-affinity site appears later (Fig. 7A, legends) than the fast inactivation. Then it is likely that the inactivation may induce the transition to the high-affinity state. This is in agreement with the previous papers that inactivation controlled the high-affinity binding (Numaguchi et al., 2000).

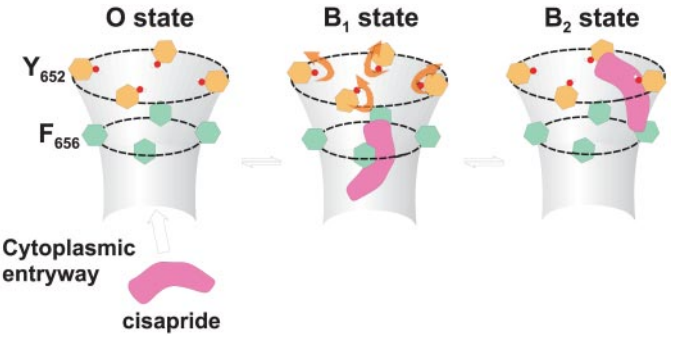

Fig. 9. A hypothetical binding model for cisapride. A scheme for the longitudinal section of the HERG pore from the cytoplasmic entryway to the central cavity is shown. B_1 and B_2 represent the low- and high-affinity binding states. Orange arrows indicate voltage-dependent rearrangements of Tyr_{652} residues.

For F656A, the K_f and k_{off} (Fig. 7, B and C) values were significantly greater, which led to the low affinity of this mutant. Voltage dependence of this channel indicates that the transition between B_1 and B_2 was retained. Then, the absence of current decay in the envelope experiment is related to the fast transition from the B_1 to the B_2 state. This feature suggests that Phe_{656} residues may retard the rearrangements of Tyr_{652} residues.

An overview of the kinetic features of channel species revealed that only in F656A did the initial binding site show a reduced affinity and the other channels did not. All of the latter possess at least two diagonal Phe_{656} residues in common. Therefore, it is suggested that the diagonal Phe_{656} residues may form the initial binding site for cisapride.

Conclusion

Combining the steady-state block data, its voltage dependence and the kinetic data suggest the hypothetical binding process shown in Fig. 9. Cisapride binds to the low-affinity site constituted by diagonal Phe_{656} residues. Meanwhile, voltage-dependent conformational changes reorient the Tyr_{652} residues with Phe_{656} and form the high-affinity site produced by two adjacent Tyr_{652} residues and Phe_{656} but hindered by adjacent Phe_{656}.

In this study, a topological picture of the interaction between HERG and cisapride was obtained. Systematic approaches using a set of mutants and thermodynamic and kinetic analyses mapped the binding site and the blocking process. This approach will be applied to understand better the mechanism of action between HERG channels and the broad spectrum of blocking substances.

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