An amino acid residue whose change by mutation affects drug binding to the HERG channel

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Abstract We did the experiments to search for amino acids that affect quinidine binding to the HERG channel, and have identified an amino acid whose change by mutation affects the binding of various drugs. The residue is located at position 647 in the S6 and is not involved in the recently identified methanesulfonanilide binding pocket. The homology model of the HERG channel indicated that the residue faces toward the outside of the channel pore. We conclude that the residue at position 647 does not interact directly with drug molecules but plays an important role in keeping the binding site's high affinity for drugs. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: HERG; Drug binding site; Quinidine; E-4031; Terfenadine

1. Introduction

The rapidly activating delayed rectifier K^+ current (I_{Kr}) plays an important role in determining cardiac action potential duration (APD). Reduction of I_{Kr} results in prolongation of APD and long QT syndrome (LQTS) that can lead to life-threatening ventricular tachyarrhythmia [1–3]. Prolongation of the QT interval may be congenital or secondary (acquired). Congenital LQTS-2 has been linked to lesions in the human ether-a-go-go-related gene (HERG) that encodes a pore-forming subunit of the I_{Kr} channel [4–6]. Acquired LQTS has been reported to result from a number of medications that inhibit I_{Kr} (e.g. several antiarrhythmic drugs, antipsychotic drugs and antihistaminergic drugs) [7–10]. The class I antiarrhythmic drug quinidine has a broad spectrum of channel-blocking activity and is known to cause QT prolongation which can be both antiarrhythmic and proarrhythmic [11,12]. Quinidine blocks not only the cardiac Na^+ channel but also many cardiac K^+ channels including I_{Kr}. The proarrhythmic activity of quinidine is most likely to be caused by excessive block of I_{Kr}. Molecular analyses for the quinidine binding site have been carried out for the cloned human cardiac K^+ channel hKv1.5 and rat cardiac K^+ channel rKv1.4 [13–16]. For both channels the equivalent residue in the sixth transmembrane segment (S6) has been identified as a molecular determinant of quinidine binding [15,16]. The important role of the S6 in drug binding has been demonstrated not only for the above two K^+ channels, but also Na^+, Ca^{2+} and other K^+ channels [17–22]. Recently, Phe at position 656, which is located on the C-terminal half of the S6, was reported to be a molecular determinant of high affinity dofetilide binding to the HERG channel [23]. Subsequently, an alanine-scanning mutagenesis study identified the MK499 binding site that is composed of five–seven amino acids located on the S6 and the pore helix [24]. In addition to identification of drug binding sites, a number of studies demonstrated that loss of rapid C-type inactivation of the HERG channel resulted in reduced affinity for various drugs [25–27]. The present study was designed to search for amino acid residue(s) that affect quinidine binding to the HERG channel. We have focused on aromatic residues in the S6 domain and have identified an amino acid residue, Ile647, whose substitution with Phe reduces the effect of quinidine probably by an allosteric manner. Although the residue is not in the recently identified methanesulfonanilide binding pocket [24], the I647F mutation affected the binding of not only quinidine but also E-4031 (a methanesulfonanilide) and terfenadine (an antihistaminergic drug).

2. Materials and methods

The plasmid containing HERG cDNA (HERG/pGH19) was generously provided by Dr. G.A. Robertson (University of Wisconsin, Madison, WI, USA) [6]. The origin of the pGH19 vector is the pGHE expression vector constructed by Liman et al. [28].

2.1. In vitro mutagenesis

Point mutations in the S6 of HERG were generated by one-step PCR mutagenesis utilizing two unique restriction sites (*Bgl*II at nucleotide 1913 and *Sph*I at nucleotide 2310). The *Bgl*II site is at the beginning of the S6 and the *Sph*I site is in the C-terminal region. Sense primers containing the desired mutations started two bases upstream of the *Bgl*II site and extended four–seven bases from the mutations. An antisense primer used for construction of the mutants corresponded to nucleotides 2312–2332. In a 100 μl PCR reaction, 50 pmol of each primer, 0.2 μg of HERG cDNA, and 0.125 units of Pyrobest DNA polymerase (Takara, Osaka, Japan) were used. Reaction temperatures were varied using a thermal cycler (Perkin-Elmer, model 2400): 98°C, 25 s; 60°C, 30 s; and 72°C, 90 s for 20 cycles. The amplified fragment was digested with *Bgl*II and *Sph*I and ligated to HERG between the two restriction enzyme sites. In addition to the point mutants, two amino-terminal deletion mutants (HERG WTΔ2-345 and I647FΔ2-345) were constructed by PCR. The sense primer corresponded to nucleotides 1063–1089, and contained an ATG and *Bam*HI site (unique in the multiple cloning site of the vector) at the 5′ end. The antisense primer corresponded to nucleotides 2115–2134. PCR conditions were the same as above. The amplified fragment digested with *Bam*HI and *Bgl*II was ligated to WT or I647F between the two restriction enzyme sites. Sequences of all the fragments generated by PCR in the mutants were verified by the

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dideoxy chain termination method using a DNA sequencer (Applied Biosystems, model 310, Tokyo, Japan).

2.2. Expression and current recording

The pGH19 vectors containing the constructs were linearized with *Not*I, and capped cRNAs were prepared from these templates with T7 RNA polymerase (Stratagene, La Jolla, CA, USA). Transcribed RNAs were dissolved in sterile water at a final concentration of 0.2–0.4 μg/μl. Oocyte preparation and electrophysiological measurements were carried out essentially as reported previously [29]. Defolliculated *Xenopus* oocytes (stage V–VI) were injected with 45.6 nl of cRNA using a motor-driven injector (Drummond, PA, USA). The injected oocytes were incubated in Barth's medium supplemented with penicillin G (71.5 units/ml) and streptomycin (35.9 μg/ml) at 18°C for 2–6 days before electrophysiological measurements. The K^+ currents were recorded by a conventional two-microelectrode voltage clamp method with 3 M KCl-filled electrodes. The bath recording solution consisted of ND 96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4). All electrophysiological measurements were carried out at room temperature (21 ± 1°C).

Data are expressed as mean ± S.E.M. The statistical significance was evaluated using Student's unpaired *t*-test. A *P* value smaller than 0.05 was considered to be significant.

3. Results and discussion

3.1. Influence of the S6 mutations on the effects of quinidine

Fig. 1 shows a sequence alignment of homologous regions from the pore-lining helix of selected K^+ channels. It has been demonstrated that the residue at position 505 of hKv1.5 and the equivalent residue of rKv1.4 (at position 529 of rKv1.4Δ3-25) play an important role in determining the binding affinity for quinidine. In hKv1.5, it has been suggested that hydrophobicity of the side chain at the residue 505 is a major determinant of the binding affinity for quinidine: the increased the hydrophobicity the increased the affinity [15]. In rKv1.4Δ3-25, it has been suggested that steric hindrance imposed by the side chain at position 529 is an important factor in determining the affinity: Phe (a bulky hydrophobic residue) at position 529 markedly decreased the affinity for quinidine [16]. Therefore, we first investigated whether the equivalent residue of HERG (M651) is responsible for quinidine binding. Since I_{Ks} is much less sensitive than I_{Kr} to many drugs including quinidine, we have substituted the M651 with the corresponding residue of KvLQT1 that encodes a pore-forming subunit of I_{Ks} [30–32]. Fig. 2A and C show representative current traces before and after application of quinidine and *I–V* relationships for HERG WT and the M651F, respectively. When effects of 10 μM quinidine were compared, WT currents were inhibited by 59.7 ± 2.7%, whereas M651F

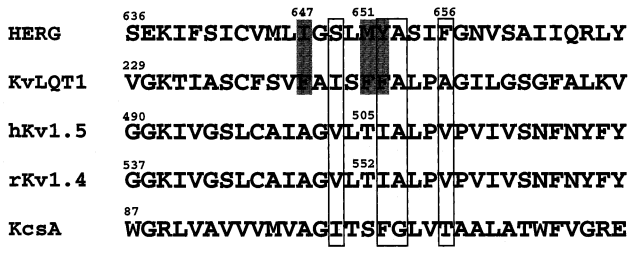

Fig. 1. Alignment of K^+ channel pore-lining helices. Mutated amino acids of HERG and the corresponding residues of KvLQT1 are shaded. T505 of hKv1.5 and T552 of rKv1.4 (T529 of rKv1.4Δ3-25) play an important role in quinidine binding. F656 of HERG was reported to be a molecular determinant of dofetilide binding. Residues predicted to line the channel pore, based on the KcsA structure, are boxed.

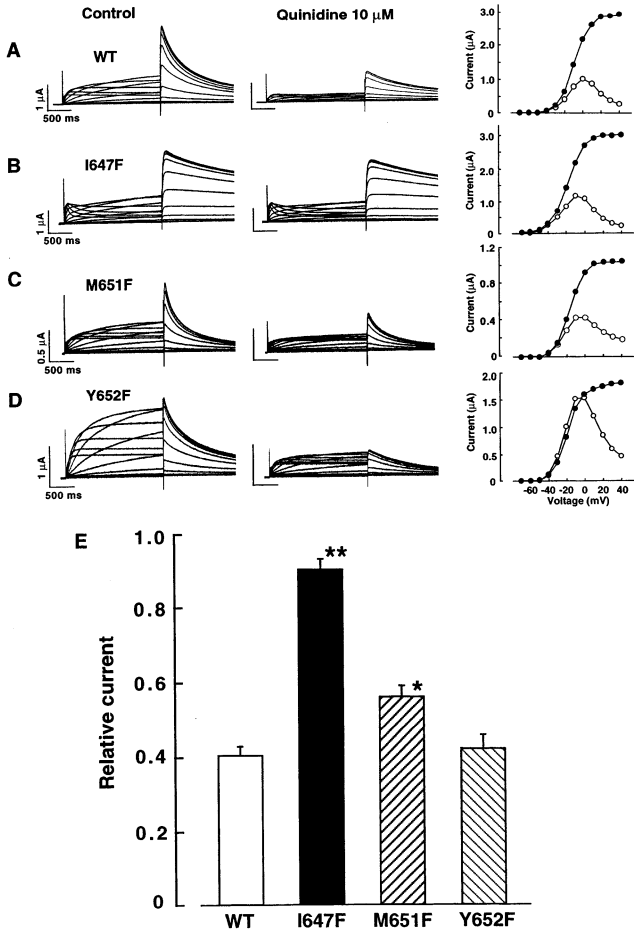

Fig. 2. I647F mutation reduces effects of quinidine. Current traces before and after application of 10 μM quinidine are shown in the left and middle panel: HERG WT (A), I647F (B), M651F (C), and Y652F (D). Respective control *I–V* relationships for currents at the end of depolarizing pulse (open circle) and peak tail currents (closed circle) are shown in the right panel. Currents were elicited by 2 s depolarizing pulses ranging from −70 mV to +40 mV in 10 mV increments. The holding potential was −80 mV and return potential was to −60 mV. E: Effects of 10 μM quinidine were evaluated by tail currents at −60 mV following the depolarizing pulse to 0 mV after 20 min exposure to the drug. Currents are expressed as relative values to control currents before quinidine application. WT ($n = 10$), I647F ($n = 5$), M651F ($n = 7$), and Y652F ($n = 9$). $^*P < 0.05$, $^{**}P < 0.01$.

currents were inhibited by 44.0 ± 3.2% (Fig. 2E). The difference between these values was significant but small, which suggests that the equivalent residue (M651) does not play a crucial role in quinidine binding to the HERG channel. Recently, Sanguinetti's group have identified the methanesulfonanilide binding pocket of the HERG channel using alanine-scanning mutagenesis and have demonstrated the importance of benzene rings in drug binding (not only for methanesulfonanilides but also for other drugs) [24]. In addition, quinidine binding to rKv1.4 is reduced by Phe substitution [16]. Therefore, we have focused on aromatic residues in the S6 of both HERG and KvLQT1, and substituted the residues of the HERG with the corresponding residues of the KvLQT1. We have found that substitution of Ile647 of the HERG with Phe reduced the effects of quinidine. Representative current traces and *I–V* relationships of the I647F are shown in Fig. 2B.

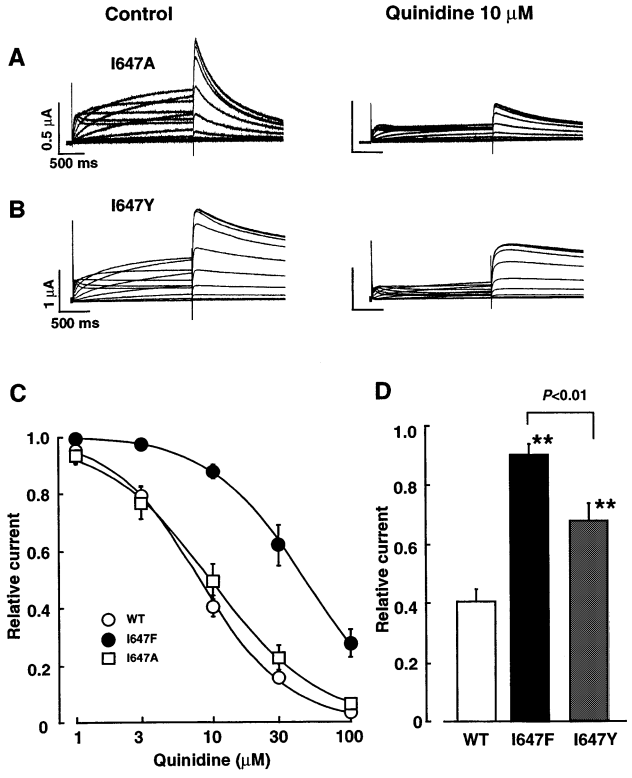

Fig. 3. I647Y mutation reduces effects of quinidine but I647A mutation has no influence. A (I647A), B (I647Y): Current traces before and after application of 10 μM quinidine are shown in the left and right panel, respectively. Pulse protocols are the same as in Fig. 2. C: Concentration–inhibition curves for HERG WT (open circle; $n=8$), I647F (closed circle; $n=5$) and I647A (open square; $n=7$). Quinidine was applied in a cumulative manner. D: Effects of 10 μM quinidine on I647Y were compared with those on WT and I647F. Data on WT and I647F are the same as in Fig. 2E.
←

Quinidine (10 μM) inhibited the I647F currents only by 9.6 ± 3.1% (Fig. 2B, E). Another mutation which substituted Tyr652 with Phe (Y652F) markedly affected the gating kinetics but had little influence on block by quinidine (Fig. 2D, E). This result indicates that loss of the hydroxyl side group has no influence on quinidine binding, and is in line with the important role of the aromatic residue at position 652 in drug binding, which has been reported in the alanine-scanning study [24].

3.2. Influence of other substitutions of I647

We constructed other point mutants of I647 to investigate whether Ile at position 647 is essential for high affinity quinidine binding. Fig. 3C shows concentration–inhibition curves for HERG WT, the I647F and I647A. The I647F mutation increased the IC_{50} for quinidine 6-fold: 8.6 ± 0.68 μM for WT and 49.1 ± 8.64 μM for the I647F. In contrast, the I647A had little influence on the effects of quinidine. This result indicates that the I647 is not essential for high affinity quinidine binding. Another mutation, I647Y, that is less hydrophobic than I647F but introduces an aromatic ring at 647 reduced effects of quinidine. Inhibition of I647Y currents by 10 μM quinidine

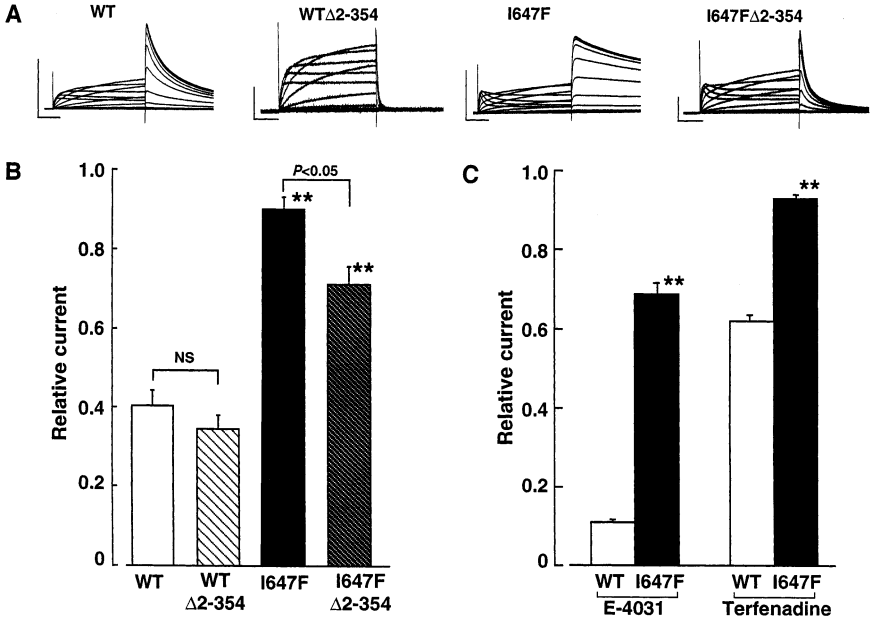

Fig. 4. Restoration of deactivation in I647F does not restore effects of quinidine. A: Current traces of WT, WTΔ2-354, I647F, and I647FΔ2-354 are shown. Pulse protocols are the same as in Fig. 2. The N-terminal deletion accelerated the deactivation both for WT and I647F. When the decaying phase of the tail current at −80 mV was fit with double exponential function, the rapid component of time constant was 171.2 ± 17.25 ms for WT ($n=8$), 20.5 ± 1.54 ms for WTΔ2-354 ($n=5$), and 56.9 ± 45.3 ms for I647FΔ2-354 ($n=4$). The tail current of the I647F could not be fit well with double exponential function and was fit with single exponential function; the time constant was 928.9 ± 45.5 ms ($n=5$). B: Effects of 10 μM quinidine on WTΔ2-354 and I647FΔ2-354 were compared with those on WT and I647F. Data on WT and I647F are the same as in Fig. 2E. C: Effects of E-4031 (10 μM) and terfenadine (1 μM) on HERG WT and I647F currents were similarly evaluated. $^{**}P<0.01$.

was 32.0 ± 5.40%, which is significantly smaller than that of WT (59.7 ± 2.67%) (Fig. 3D). Since inhibition of I647Y by quinidine was larger than that of the I647F currents, the hydroxyl side group might have favorable influence on quinidine binding. However, these results suggest that an aromatic ring at 647 reduces quinidine binding.

3.3. Effects of quinidine on amino-terminal deletion mutants

As shown in Figs. 2B and 3A and B, the I647F and I647Y mutations resulted in slowing of deactivation, whereas the I647A mutation had no marked effects on the deactivation. Therefore, we investigated whether the change in deactivation kinetics itself is responsible for the decreased affinity of the mutants for quinidine. Since it has been reported that the amino-terminus regulates deactivation of HERG current and the deletion of the domain results in acceleration of deactivation [33], we constructed the amino-terminal deletion mutant of I647F (I647FΔ2-354). As expected, deactivation of the currents of I647FΔ2-354 was accelerated (Fig. 4A). Fig. 4B shows the effects of 10 μM quinidine on the currents of WT, WTΔ2-354, I647F and I647FΔ2-354. The effects of quinidine tended to be smaller in the deletion mutant than in its parent channel; the inhibition was 59.7 ± 2.67% (WT), 65.5 ± 3.84% (WTΔ2-354; NS vs. WT), 9.7 ± 3.42% (I647F) and 28.8 ± 4.95% (I647FΔ2-354; $P < 0.05$ vs. I647F). However, when HERG WT and I647FΔ2-354 were compared, the effect of quinidine was much smaller in I647FΔ2-354, while the currents of I647FΔ2-354 deactivated faster than WT. Thus, although the slower deactivation kinetics might be partly involved in the reduced affinity for quinidine, the I647F mutation affects quinidine binding besides changing the deactivation kinetics.

3.4. Effects of E-4031 and terfenadine on the I647F

The alanine-scanning study has demonstrated that Y652 and F656 of the HERG channel are crucial for high affinity binding of both methanesulfonanilides and other unrelated compounds. When mutated to Ala, only the two residues markedly reduced the affinity simultaneously for a methanesulfonanilide (MK499) and other drugs (terfenadine and cisapride). In contrast, mutation of two other residues (Val625 and Gly648) to Ala dramatically reduced the affinity for MK499 without affecting the affinity for terfenadine and cisapride. The mutations of V625 and G648 were thought to disrupt the MK499 binding site by altering the size or shape of the methanesulfonanilide binding pocket. Since the I647F mutation reduced the effects of quinidine, we examined the effects of E-4031 and terfenadine on the I647F currents. E-4031 (10 μM) inhibited WT currents by 89.1 ± 0.74%, whereas it inhibited the I647F currents by 31.3 ± 1.84% (Fig. 4C). In our experimental conditions, 1 μM terfenadine inhibited WT currents by 38.0 ± 1.84% and I647F currents only by 6.9 ± 1.20% (Fig. 4C). Thus, the I647F mutation affected binding of a variety of drugs including methanesulfonanilides.

3.5. A homology model

Sanguinetti's group created a homology model of the HERG channel by using the KcsA structure as the template. Their model has demonstrated that three residues in the S6 (G648, Y652, and F656) whose mutation to Ala substantially reduced the sensitivity to MK499 face toward the inside of the channel pore. With the model and electrophysiological data, they have suggested that the two aromatic residues directly interact with drug molecules and G648 forms the one end of the methanesulfonanilide binding pocket. We generated a similar homology model of the HERG I647F channel by using

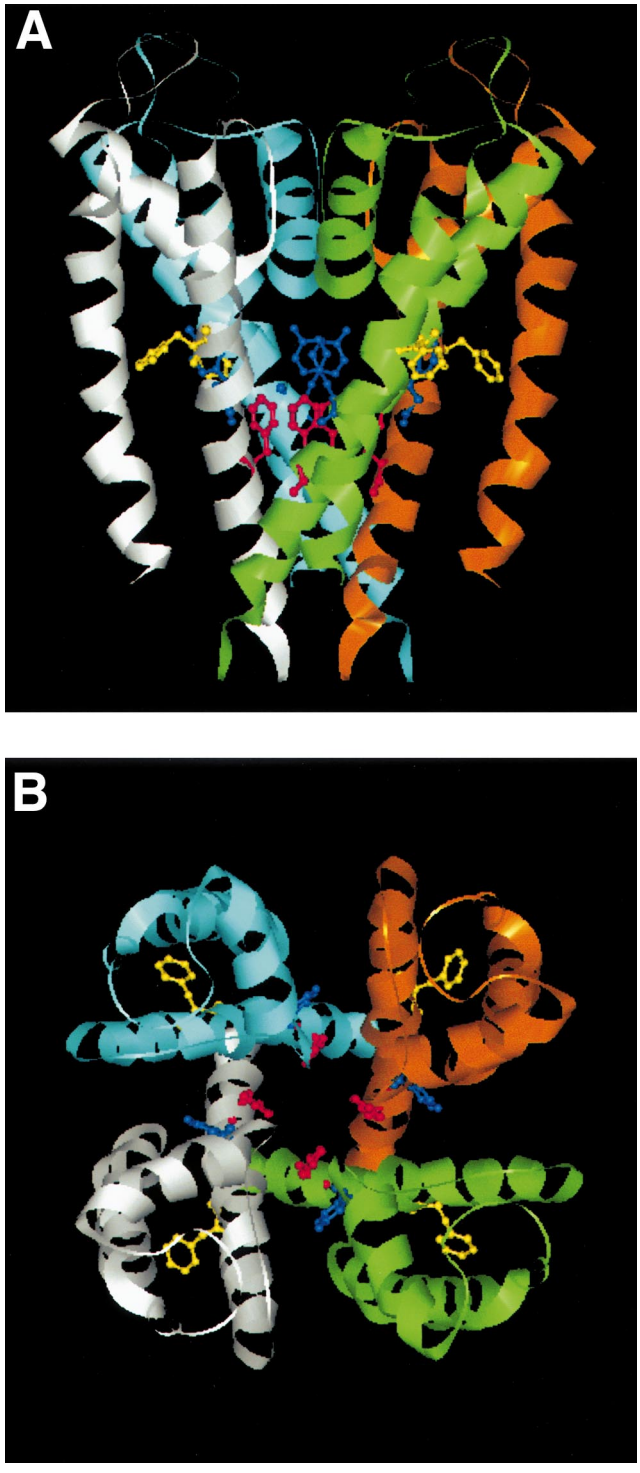

Fig. 5. A homology model of the HERG I647F mutant channel. Side view (A) and top view (B) of the I647F channel. F647, Y652 and F656 are shown as a ball and stick model: F647 is yellow, Y652 is blue, and F656 is red. F647 faces toward outside of the channel pore. The KcsA structure retrieved from the Protein Data Bank was used as the template structure for a homology model. The homology model was created by MOE (2000.02; Chemical Computing Group Inc., Montreal, QC, Canada).

MOE (2000.02; Chemical Computing Group Inc., Montreal, QC, Canada) to visualize location of F647 (Fig. 5). The model shows that F647 faces toward the outside of the channel pore to the outer helix of the S5. Therefore the F647 could not directly interact with drug molecules. It has been reported that F656, one of the important aromatic residues in the methanesulfonanilide binding site, reduced the affinity not only for dofetilide but also for quinidine when mutated to Val [23]. Influence of the F656V mutation on the effects of quinidine is greater than that of the I647F mutation we studied, which is consistent with the indirect influence of the I647F mutation on the binding. Our results indicate that an amino acid residue at position 647 plays an important role in terms of keeping the binding site of high affinity for drugs. Phe, a bulky aromatic residue, at position 647 might cause shift of the S6 to change the shape of the drug binding site, which results in the reduced effects of various drugs.

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