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A mutation in the cardiac KV7.1 channel possibly disrupts interaction with Yotiao protein

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ABSTRACT

Here, we characterized the p.Arg583His (R583H) Kv7.1 mutation, identified in two unrelated families suffered from LQT syndrome. This mutation is located in the HC-HD linker of the cytoplasmic portion of the Kv7.1 channel. This linker, together with HD helix are responsible for binding the A-kinase anchoring protein 9 (AKAP9), Yotiao. We studied the electrophysiological characteristics of the mutated channel expressed in CHO–K1 along with KCNE1 subunit and Yotiao protein, using the whole-cell patch-clamp technique. We found that R583H mutation, even at the heterozygous state, impedes I_{Ks} activation. Molecular modeling showed that HC and HD helixes of the C-terminal part of Kv7.1 channel are swapped along the C-terminus length of the channel and that R583 position is exposed to the outer surface of HC-HD tandem coiled-coil. Interestingly, the adenylate cyclase activator, forskolin had a smaller effect on the mutant channel comparing with the WT protein, suggesting that R583H mutation may disrupt the interaction of the channel with the adaptor protein Yotiao and, therefore, may impair phosphorylation of the KCNQ1 channel.

1. Introduction

Hereditary LQTS is a disease caused by mutations in genes encoding ion channels or proteins providing excitation-contraction coupling [1,2], it has an incidence of 1:2000, is associated with severe arrhythmias, demonstrates good genotype-phenotype correlation [3] and is considered the main cause of sudden cardiac death. The most common prolonged QT interval syndrome type 1 (LQTS1) is associated with mutations in the *KCNQ1* gene that cause decreased repolarizing current I_{Ks} [1,3]. The *KCNQ1* gene encodes the alpha-subunit of the Kv7.1 voltage-gated potassium channel. This ion channel is widely expressed in cells of various tissues, but the most crucial and noticeable contribution is its participation in the formation of the action potential of ventricular cardiomyocytes [4,5]. The Kv7.1 alpha subunit possesses a long C-terminus [6], which is complex in both the structure and functional significance. It contains long unstructured flexible loops and rigid regions formed by coiled amphipathic alpha-helices and is responsible for the tetramerization and functional modulation of the channel [7].

The regulation of the activity of ion channels forming the cardiac action potential, including Kv7.1, is vital for the organism [8]. Ion channels are susceptible to modulation by phosphorylation. Yotiao, also known as AKAP9, acts as an anchoring protein for several vital enzymes to form I_{Ks} macromolecular complex, including protein kinase A (PKA), protein phosphatase 1 (PP1), phosphodiesterase PDE4D3, and adenylyl cyclase type 9 (AC9), which modulate phosphorylation and gating of I_{Ks} channel [9]. PKA activation leads to phosphorylation of N-terminal residues S27 [10] and S92 [11] located in the N-terminus of KCNQ1, resulting in the channel opening faster in presence of auxiliary subunit KCNE1 [12]. Previous studies have identified two Kv7.1 binding-regions in Yotiao (N-terminal residues 29–46 and C-terminal residues 1574–1643), as well as S1570L mutation which disrupts association between Yotiao and Kv7.1 [13]. Furthermore, a Leucine zipper motif is required for the interaction between Yotiao and Kv7.1, and this interaction can be disrupted by LQT mutant, G589D [13]. On the other hand, it has been found that number of mutations in helix D of Kv7.1 can impair Yotiao in binding to Kv7.1 [14,15].

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In the present study we discovered and analyzed a novel point mutation in the C-terminus of the Kv7.1 channel (R583H) that leads to LQTS in one patient. We inserted this substitution into an expression plasmid containing Kv7.1 cDNA and studied its influence on the electrophysiological parameters of the Kv7.1 channel in the CHO–K1 cell line using whole-cell patch recordings. Interestingly, the adenylate cyclase activator, forskolin, had a much weaker effect on the mutant channel compared with the WT protein, suggesting that this mutation disrupts the interaction of the channel with the adaptor protein Yotiao and therefore may impair phosphorylation of the channel.

2. Methods

2.1. Clinical and genetic evaluation

Genetic testing was performed in accordance with Helsiki declaration and by direct request of proband. Informed consent was obtained from the patient for the research publication of any anonymous data included in this manuscript. The medical assessment involved a physical examination, gathering personal and family medical history, performing biochemical blood tests, monitoring of resting and standing ECG 24 h Holter ECG, and echocardiography (EchoCG). DNA samples for the proband genetic analysis were isolated from the venous blood leukocytes, according to standard protocol. Full-exome sequencing of archival DNA samples from patients was performed by sequencing a new generation of protein-coding genes on the DNBSEQ-G400 (MGISEQ-2000) platform (BGI Group, China) with SureSelect Human All Exon V7 library enrichment kit (Agilent, USA). Sequencing data were analyzed using a bioinformatic pipeline based on a customized combination of shared access programs for Linux family of operating system distributions. The pathogenicity of variants identified in the KCNQ1 gene was assessed according to the ROMG (2018) and ACMG (2015) guidelines.

2.2. Introduction of a point mutation into a channel sequence

A point mutation (R583H) was introduced into the Kv7.1 channel

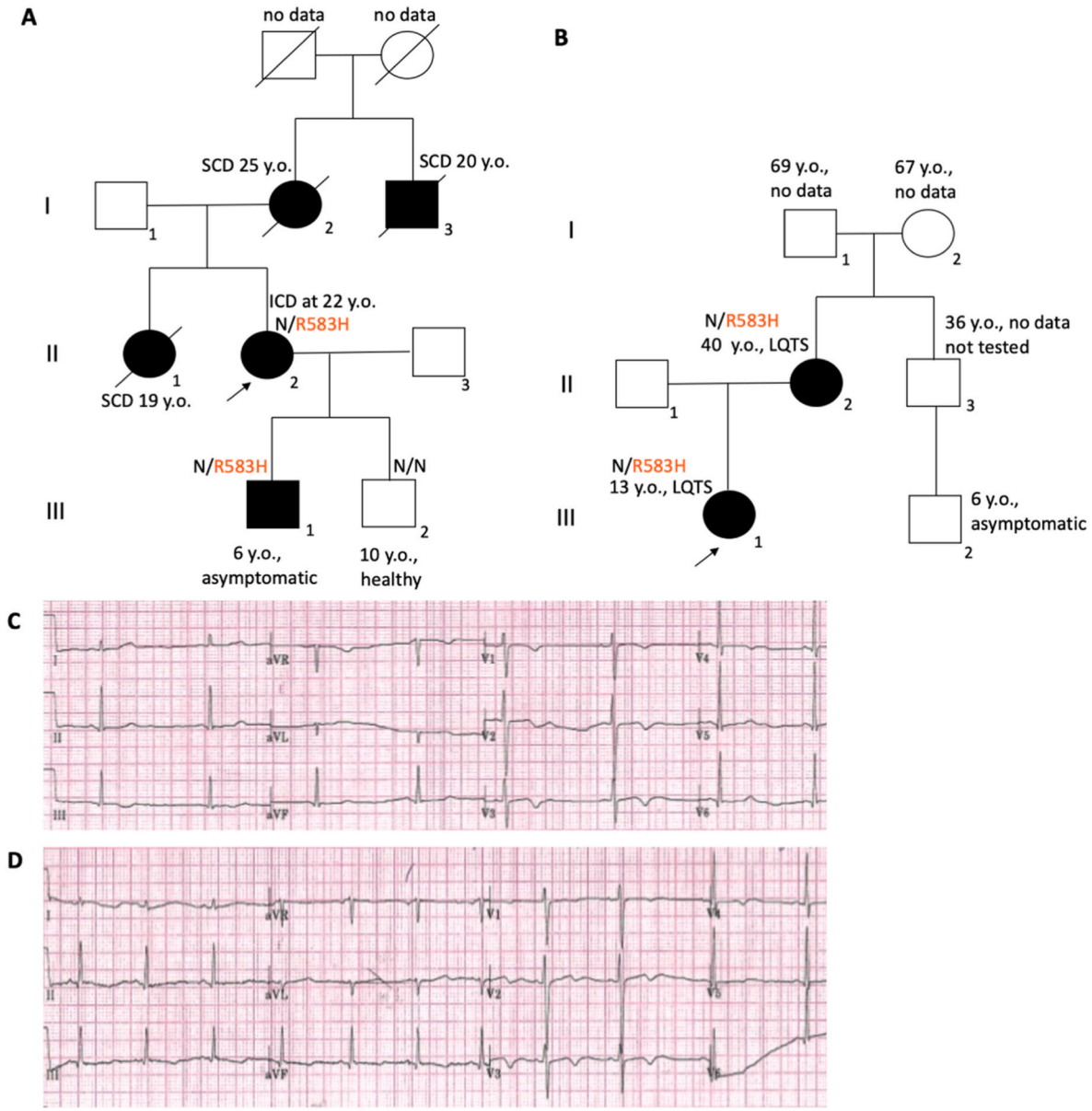

Fig. 1. Pedigrees of families with R583H mutations. Closed symbols represent affected family members, opened symbols represent healthy and non-tested family members, probands are marked by arrows. **A**. Family LQTS201. **B**. Pedigree LQTS6. **Fragment of ECG of the patient III.1 (family LQTS6). C**. Resting ECG: HR 48–51 bpm, QTc 431–485 ms.**D**. ECG after physical exercise: HR 67–72 bpm, QTc 493–539 ms. Pronounced repolarization abnormalities.

sequence by PCR, as described earlier [16]. Plasmids from the obtained clones were checked by Sanger sequencing (Sangon Biotech, China) for the presence of the necessary mutation and absence of additional single-nucleotide substitutions. An endotoxin-free DNA purification kit (Omega Bio-Tek, USA) was used for preparative purification. The purified plasmid was stored under sterile conditions and used to transfect eukaryotic cells for electrophysiological experiments.

2.3. Cell culture and transfection

Heterologous expression of wild type (WT) and mutant Kv7.1 alpha-subunits in CHO–K1 cultured cells were performed according to the protocol, described earlier [16]. Briefly, we studied three experimental groups: WT, transfected with plasmid containing WT Kv7.1 cDNA, the homozygote (R583H/R583H), transfected with plasmid containing R583H mutantion and the heterozygote (WT/R583H), co-transfected with 1:1 WT and R583H plasmids. All groups were simultaneously co-transfected with plasmids containig axilliary subunit (KCNE1) and Yotiao protein (AKAP9) (1:2:4 ratio, respectively). The total amount of the DNA was the same for all experiments. After 24 h cells were seeded for electrophysiological recordings, which were performed 48–54 h after transfection.

2.4. Electrophysiological experiments

I_{Ks} currents in transfected CHO–K1 cells were recorded using the standard whole-cell patchclamp technique [16] using an Axopatch 200A amplifier (Molecular Devices). The current was elicited using a standard protocol of two-step square-pulse depolarization from the holding potential of −80 mV to −60 - +120 mV with 20 mV increments (Fig. 3, inset). The second step was repolarization to −40 mV, necessary to measure I_{Ks} tail current. The duration of the first step was 5 s, the second step was 2.5 s. During the data analysis, current-voltage curves were plotted by peak absolute values of tail current. The normalized peak amplitudes of tail currents were used to build the steady-state activation curves fitted with a Boltzmann equation, y = Imax/(1 + exp((V50 – V)/k)). The membrane potential for half-maximal activation (V50) and the slope factor (k) were found and compared between different groups of cells. Time courses of tail current deactivation and step current activation were fitted with a single exponential function to obtain the time constants of deactivation (τ_D) and activation (τ_A), respectively.

2.5. Molecular modeling

Molecular modeling of HC-HD region (residues 535–625) was performed with Alphafold2-multimer [17,18] via ColabFold web service (https://github.com/sokrypton/ColabFold) [19] with manual correction for improving fitness with an electron density map [6].

3. Results

3.1. Clinical cases

We had under observation two unrelated families, *LQTS6* and *LQTS201*, both R583H mutation carriers. Proband *LQTS6* (female, 22 y. o.) first visited our clinic in 2013 with entering diagnosis “Unexpected syncope, cryptogenic epilepsy”. She underwent complete instrumental investigation including EEG, brain CT, EchoCG, ECG, Holter monitoring and endocardial electrophysiological (EPI) study without significant findings. Resting and 24-h ECG was practically normal with maximal QTc 460 ms, no ventricular tachycardia was induced on endocardial EPI. Genetic counseling revealed family history burdened with 3 SCD cases at the age of 19–25 years (Fig. 1A). Heterozygous genetic variant R583H in the *KCNQ1* gene was found but was classified as a variant of uncertain significance at the time of detection (2013). Taking together family history, upper level of QTc duration, unexpected syncope, and VUS in *KCNQ1* gene, a cardioverter defibrillator (ICD) Maximo II DR D284DRG (Medtronic) has implanted. She had 2 stress-induced appropriate shocks due to polymorphic ventricular tachycardia in 16 months of follow-up. Beta-blockers was prescribed and no syncope or additional shocks was

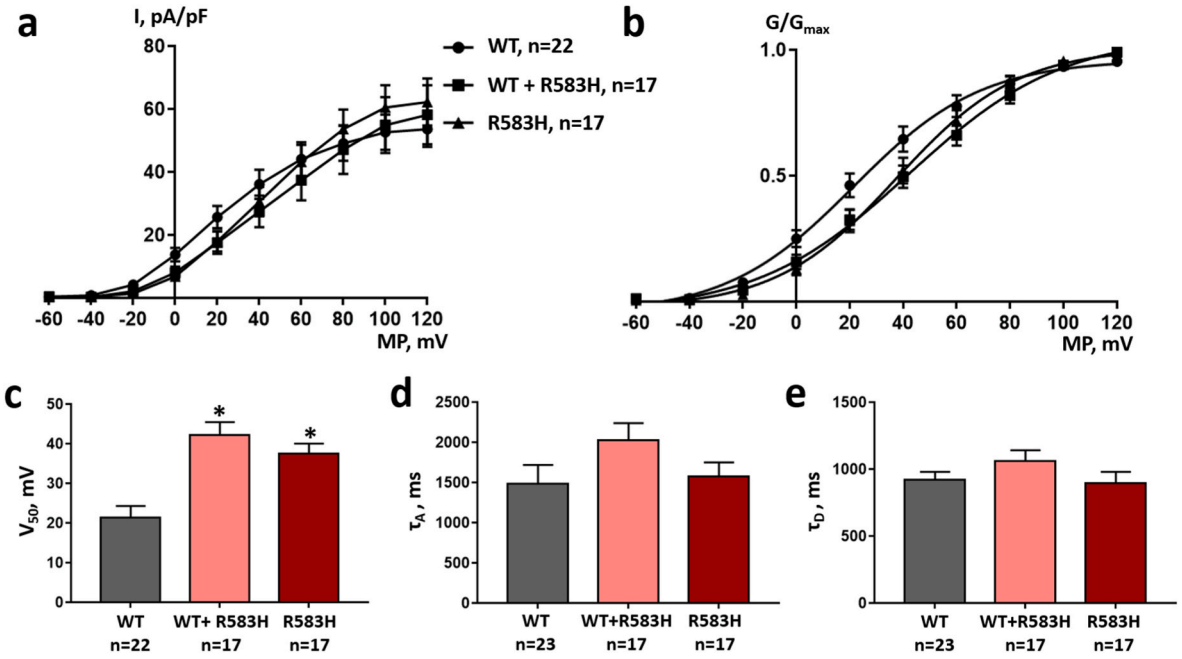

Fig. 2. Electrophysiological characteristics of the current transferred by $K_V7.1$ channels with R583H mutation (homo- and heterozygotes). (**a**) Comparison of mean ± s.m.e. I–V curves of tail I_{Ks} current obtained from 3 groups of cells: the cells co-transfected with KCNE1 and WT KCNQ1 gene only (WT), by KCNE1 and mutant KCNQ1 gene only (R583H) or co-transfected with KCNE1 and both KCNQ1 genes in 1:1 ratio (WT + R583H). Inset: activation voltage protocol used (one sweep every 15 s). (**b**) Activation curves, obtained from tail currents using the protocol shown in **a**, in 3 groups of cells. (**c, d, e**) Comparison of mean ± s.m.e. half-activation voltage V_{50} (**c**), activation time constant τ_A (**d**) and tail current deactivation time constant τ_D (**e**) in 3 experimental groups. * - significant difference from WT group, one-way ANOVA, $p < 0.05$.

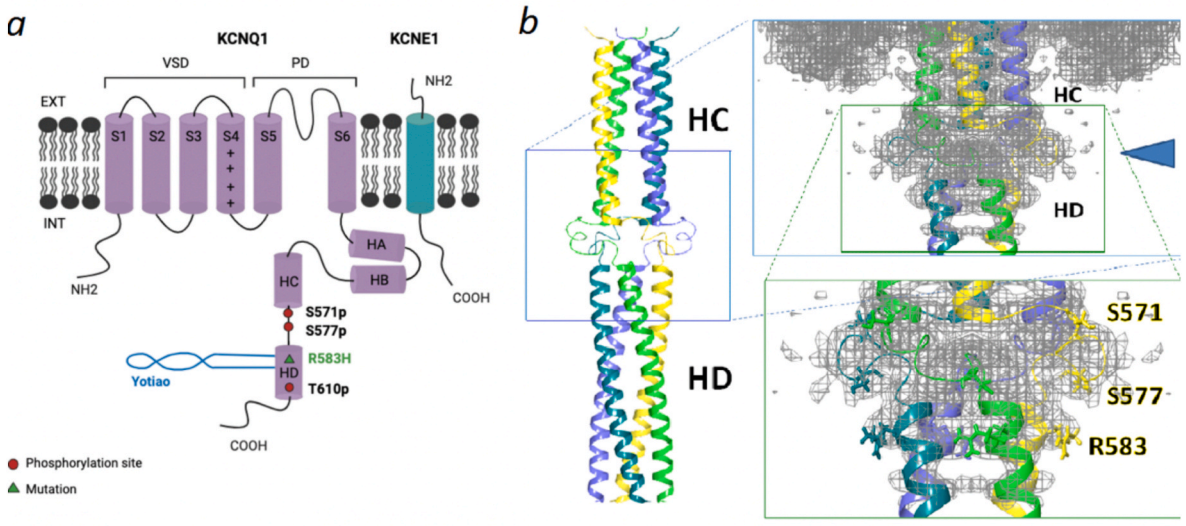

Fig. 3. (a) Diagram of Kv7.1 ion channel organization. One alpha (KCNQ1) and one beta (KCNE1) subunit are shown. VSD—voltage-sensing domain; PD—pore domain; (b) A ColabFold model for HC-HD region. Electron density map (gray mesh), of the human KCNQ1 cryo-EM structure (pdb ID 6v00). Chain swap is shown (top right). Location of the S571, S577, and R583 can be fitted to the original electron density map for this channel (bottom right). HC and HD – cytoplasmic helixes.

registered later on the therapy. During 10 years of follow-up, she got two sons without any ECG abnormality but one of them also carries R583H variant.

Proband *LQTS201* (female, 13 y.o.) underwent genetic counseling and testing due to transient QTc prolongation detected during routine prophylactic ECG screening. Family history was unremarkable (Fig. 1B). At the time of investigation (2021) variant R583H was re-classified as Likely Pathogenic (IV) according to ACMG(2015) criteria and refining criteria [20] but without strong experimental data. Close instrumental investigation revealed strong worsening of repolarization processes in myocardium after physical exercises (Fig. 3A, B). We also detected this variant in proband's mother who also has slight resting QTc prolongation. Both carriers takes beta blockers and has no cardiac events. Mutation carriers of LQTS201 family had neither experienced any syncope or cardiac arrest/sudden death.

3.2. Electrophysiological properties of WT and mutant channels

The R583H mutation did not cause a decrease in the amplitude of the I_{Ks} current compared with WT either alone or when co-transfected with WT (Fig. 2a). There were also no significant changes in the kinetics of I_{Ks} activation (Fig. 3d) and tail current deactivation (Fig. 2e). However, this mutation led to a 17–21 mV rightward shift in the steady-state activation curve of the recorded current compared with that carried by WT channels (Fig. 2b and c). A significant difference in the half-maximal activation potential from that in the WT group was observed in both experimental groups. Thus, R583H, even when co-transfected with the wild-type gene, impedes I_{Ks} activation; at the same degree of depolarization, the current carried by mutant channels will be less than that carried by normal I_{Ks}.

3.3. Molecular simulations of HC-HD superhelix

Mutation R583H is located in the HC-HD flexible linker (Fig. 3a) which contains binding sites for modifiers [21]. All published structures of Kv7.1 channels [6,22–25] did not resolve HD helix due to flexibility of a HC-HD connection. A structure of the isolated HD helix was solved using X-ray crystallography [26]. Here we used ColabFold to predict a structure of HC-HD superhelix (Fig. 3b).

Docking of the Initial AlphaFoldmodel into the density map of the cytoplasmic region of the human KCNQ1 (pdb ID 6v00) revealed that the predicted HC-HD linker (residues 567–587) may not fit into the available density (Fig. 3b, blue arrowhead). Therefore, we suggested that the HD helices should be two turns longer (residues 581–620) than it is presented in a known structure (pdb id 3bj4 [26]), which in turn leads to shortening the linker (to residues 567–580) and better fit into available electron density. The investigation of the electron density map in the vicinity of HC-HD linker revealed that there should be a swap between four subunits of the channel. That is, the HC of the yellow subunit is followed by HD of the green subunit (Fig. 3b, left), the HC of the blue subunit is followed by HD of the yellow subunit etc. Proposed model is supported by experimental data and good consistency of residues 581–588 of HD helix with the available electron density map (EMD 20966) (Fig. 3). According to databases dbPTM [27] and Phosposite [28] residues S571 and S577 are phosphorylated or could be phosphorylated [29]. For that, they must be exposed, and our model supports this (Fig. 3b, bottom). Residue T610 is also exposed in the experimental structure (pdb id 3bj4) and in our model (not shown), and is available for phosphorylation [28]. Finally, our model suggests that R583 residue is exposed on the surface and thus ready to participate in interactions with modifier proteins.

3.4. Changes in an adenylate cyclase activity

We proposed that the mutation R583H in the C-terminal coiled-coil of the Kv7.1 channel may impair interactions with the adaptor protein Yotiao, whose known binding site is in close proximity. In order to test this hypothesis, we co-expressed the channel in CHO–K1 cells together with the adaptor protein Yotiao and used the adenylate cyclase activator, *forskolin* [30], to study the effect of the mutation in the channel on its response to elevation of intracellular cAMP level (Fig. 4). The effect of *forskolin* is mediated via activation of cAMP-dependent intracellular target proteins, such as PKA. In the cells transfected with WT *KCNQ1* we observed marked augmentation of I_{Ks} produced by 1 μM forskolin (Fig. 4 a,b). However, *forskolin*-induced increase of I_{Ks} was insignificant in cells transfected with mutant channel (Fig. 4 c), suggesting that R583H mutation impairs the interaction between PKA and Kv7.1 channel.

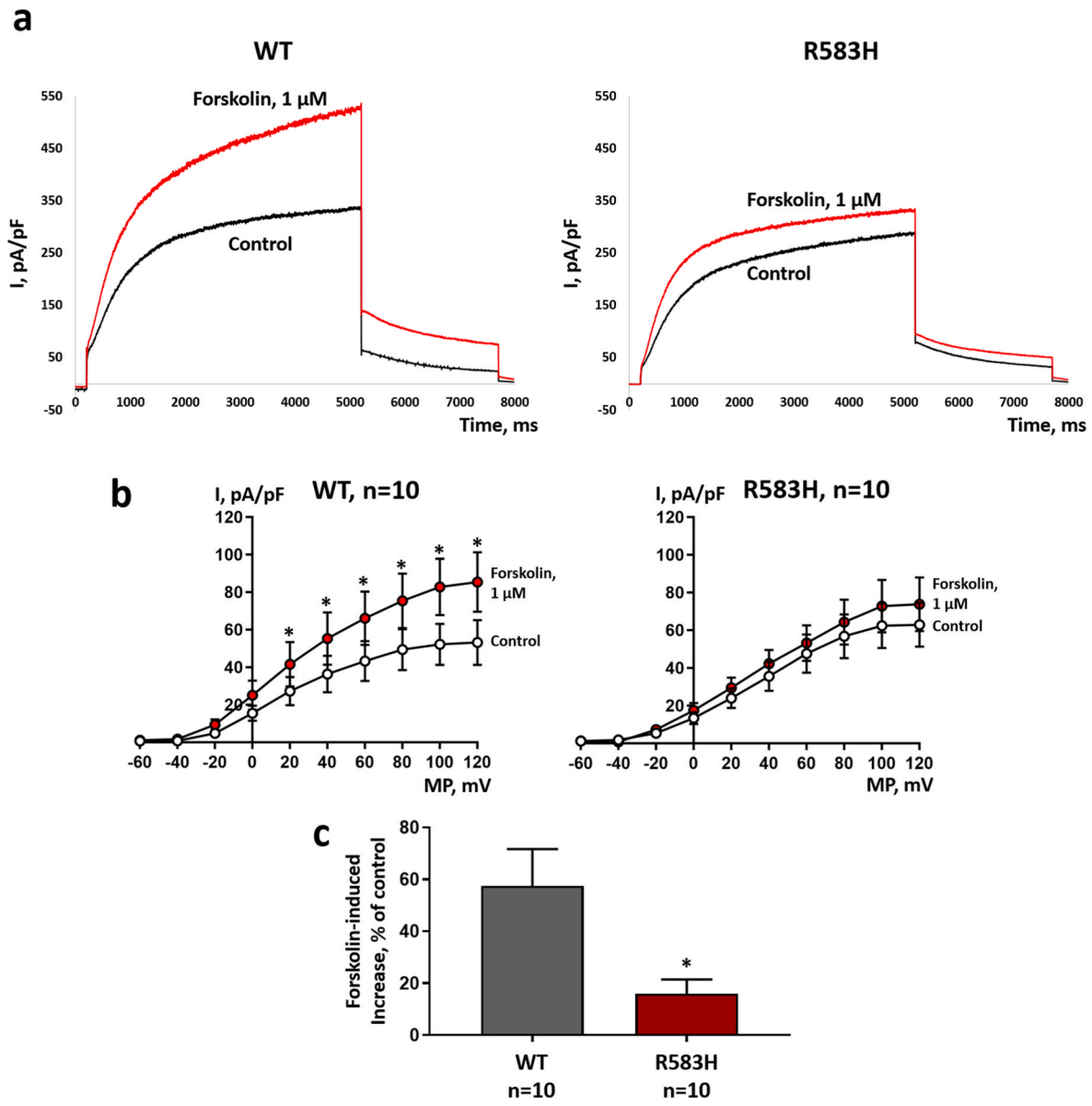

Fig. 4. cAMP-dependent augmentation of I_{Ks} current transferred by WT and mutant channels. Representative, superimposed recordings (**a**) and mean ± s.m.e. I–V curves (**b**) of the I_{Ks} current in the cells cotransfected with KCNE1, WT KCNQ1 and *yotiao* (WT, left panels) and cells co-transfected with KCNE1, mutant KCNQ1 and *yotiao* (R583H, right panels), obtained in control conditions and in the presence of 1 μM forskolin. The current was elicited by a single step (+100 mV) of protocol shown in the inset Fig.1. * - significance of forskolin effect, paired *t*-test, $p < 0.01$. (**c**) The comparison of relative forskolin-induced increase in the tail current measured after depolarization to +100 mV in two groups of cells. * - comparison between WT and R583H, *t*-test, $p < 0.05$.

4. Discussion

Cytoplasmic domains play a significant regulatory role in the functioning of ion channels [31,32]. In the current study we found a missense R583H mutation between HC and HD helixes of the C-terminus of Kv7.1 channel. Many pathological mutations, which decrease electric charge, were previously described in this area [26,33–37], suggesting electrostatic contribution to the interaction with modulators. Currently, a detailed reconstruction of the distant C-terminal region of Kv7.1 channel is absent due to an increased flexibility of this region. To visualize the possible interaction sites, we modeled the full-length HC-HD tetramer with a linker and docked it partially into the available cryo-EM density [6]. For that we added two more alpha-helical turns (residues 581–587), which lead to shortening of the flexible linker. Interestingly, while no domain swap was detected for membrane and cytoplasmic HA domains for Kv7.1 channel in general [6], we suggested a swap of helices in a HC-HD tetramer coiled coils (Fig. 3b). Given the shorter linker, the mutation of interest is located in the upper part of HD helix (Fig. 3a) and the corresponding amino acids are exposed to the outside of the superhelix.

We suggested that this mutation may disrupt the interaction of the Kv7.1 channel with the regulatory protein, Yotiao, or AKAP9. In the heart, AKAP9 helps to bring together the ion channel and PKA, PP1, PDE4D3, AC9 to achieve the regulation of I_{Ks} current during β-adrenergic stimulation [38]. The anchoring of PKA at $K_V7.1$ pore-forming subunit facilitates the phosphorylation of channels by PKA and increases the density of I_{Ks} [39]. To mimic the effects of β-adrenergic stimulation in CHO–K1 cells, which lack functional β-adrenoreceptors, we used *forskolin*, a potent activator of adenylate cyclase which launches the cAMP signaling cascade in various animal cells. In cells transfected with a plasmid containing WT KCNQ1 gene 1 μM *forskolin* produced marked augmentation of I_{Ks} current in a wide range of positive voltages

(Fig. 4a and b, left panels). On the contrary, the same effect of forskolin was very modest and below the level of significance ($p > 0.01$) in the cells transfected with R583H KCNQ1 gene (Fig. 4a and b, right panels). The relative *forskolin*-induced increase in I_{Ks} (at +100 mV) tail current amplitude was significantly larger in WT than in the R583H channel (Fig. 4c).

Thereby, mutation R583H strongly hampers the cAMP-dependent stimulation of I_{Ks} current, which is a crucial mechanism delimiting the AP prolongation under sympathetic stimulation of the heart. It is well-known that β-adrenergic stimulation leads to phosphorylation of L-type Ca^{2+} channels by PKA and augmentation of I_{CaL}, which in turn elevates the AP plateau level and provokes prolongation of AP phase 2. Since L-type Ca^{2+} channels are characterized by prominent window-current at potentials close to AP plateau level, this β-adrenergic effect is potentially dangerous, supporting the development of early after-depolarizations (EADs) in human cardiomyocytes [40]. β-adrenergic stimulation of I_{Ks} serves as a negative feedback loop, limiting AP phase 2 prolongation and protecting the heart from EADs. R583H modestly hampers the activation of I_{Ks} current, which is not enough for marked QTc prolongation in a patient in normal conditions (Fig. 1C). However, that mutation breaks the protective mechanism of β-adrenergic augmentation of I_{Ks} and thereby leads to abnormal response to sympathetic activation – increased QTc during physical activity (Fig. 1D) and provokes life-threatening ventricular arrhythmias.

Institutional review board statement

Clinical and genetic evaluation was performed in accordance with the principles of the Declaration of Helsinki.

Informed consent statement

Informed consent was obtained from all adult subjects involved in the study.

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CRediT authorship contribution statement

Bowen Li: Writing – original draft, Investigation. **Maria Karlova:** Supervision, Investigation. **Han Zhang:** Investigation. **Oksana B. Pustovit:** Methodology, Investigation. **Lisha Mai:** Investigation. **Valery Novoseletsky:** Supervision, Investigation, Formal analysis. **Dmitry Podolyak:** Validation, Methodology. **Elena V. Zaklyazminskaya:** Writing – review & editing, Project administration, Methodology, Conceptualization. **Denis V. Abramochkin:** Writing – review & editing, Supervision, Investigation. **Olga S. Sokolova:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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