Overlapping Cardiac Phenotype Associated with a Familial Mutation in the Voltage Sensor of the KCNQ1 Channel

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Key Words
Oocyte • Kv7.1 • KvLQT1 • KCNE • Heart • Arrhythmia

Abstract
Background: Cardiac action potential repolarisation is determined by K+ currents including Ik,1 Ik channels are heteromeric channels composed of KCNQ1 and KCNE β-subunits. Mutations in KCNQ1 are associated with sinus bradycardia, familial atrial fibrillation (fAF) and/or short QT syndrome as a result of gain-of-function, and long QT syndrome (LQTS) due to loss-of-function in the ventricles. Here, we report that the missense mutation R231C located in S4 voltage sensor domain is associated with a combined clinical phenotype of sinus bradycardia, fAF and LQTS. We aim to understand the molecular basis of the complex clinical phenotype. Methods: We expressed and functionally analyzed the respective channels kinetics in Xenopus laevis oocytes. The molecular nature of the residue R231 was studied by homology modeling and molecular dynamics simulation. Results: As a result, the mutation reduced voltage sensitivity of channels, possibly due to neutralization of the positive charge of the arginine side chain substituted by cysteine. Modeling suggested that the charge carrying side chain of R231 is positioned suitably to transfer transmembrane voltages into conformational energy. Further, the mutation altered the functional interactions with KCNE subunits. Conclusion: The mutation acted in a β-subunit dependent manner, suggesting Ik function altered by the presence of different KCNE subunits in sinus node, atria and ventricles as the molecular basis of sinus bradycardia, fAF and LQTS in mutation carriers.

Introduction
Atrial Fibrillation (AF) is the most common cardiac arrhythmia which currently affects over 2 million Americans, a number estimated to increase to over 4 million in the next decade [1]. AF is associated with enhanced prevalence of stroke, heart failure, and death [2]. The causes of AF are polygenic and include a variety of conditions such as cardiac ischemia and aging,
hyperthyroidism, heart failure, valve disease, and hypertension. In contrast, familial forms of AF (fAF) are rare, but well-known and associated with an early onset.

Recently, these forms have been discovered as inherited changes in cardiac ion channel genes (e.g. KCNQ1, SCN5A and KCNJ2) and their subunits (e.g. KCNE2 and KCNA5) as a part of macromolecular complexes [2–7]. These mutations significantly alter cardiac ion channel function and thereby resemble a substrate capable to modify the cardiac action potential [8]. However, atrial disturbance in presence of long QT syndrome (LQTS) has already been shown in a clinical investigation of a small group of patients. During electrophysiology studies prolonged atrial action potential duration as well as effective refractory periods and short episodes of spontaneous polymorphic atrial tachycardia could be identified in patients with LQTS but not in healthy controls [9].

In the heart, the plateau phase of the action potential is enabled by a balanced Ca$^{2+}$ influx and K$^{+}$ efflux, whereas it is terminated (repolarization) by increased K$^{+}$ efflux and reduced Ca$^{2+}$ influx [10, 11]. The two major K$^{+}$ currents involved are $I_{Ks}$ and $I_{Kr}$ [12]. $I_{K}$ channels are formed from heteromeric co-assemblies of $\alpha$-subunits KCNQ1 (also named KvLQT1 or Kv7.1) and $\beta$-subunits KCNE1 (formerly named MinK or IsK) [13, 14]. The KCNQ1 $\alpha$-subunit is structurally similar to classical Shaker-type Kv channels with six transmembrane domains (S1–S6) and a pore-forming region. $\beta$-subunits of the KCNE type have only one $\alpha$-helical transmembrane region with an extracellular N-terminal and intracellular C-terminal end [15, 16]. Subsequently, KCNE2–5 were cloned, which theoretically allows a higher functional diversity of the composition of the KCNQ1/KCNEx channel complexes [17]. KCNE1-5 expression was reported in cardiac tissues [18] and the balance between different KCNE proteins with varying effects on Kv channels may enable variable modulation of cardiac $I_{K}$.

Loss-of-function mutations in $I_{Ks}$ and $I_{Kr}$ encoding ion channel subunit genes were predominantly found in patients with inborn cardiac repolarization disorders such as congenital long QT syndrome ($I_{Ks}$): due to mutations in KCNQ1/LQT-1, or KCN1/LQT-5; $I_{Kr}$: due to mutations in KCNH2/LQT-2). From a few observations [8, 19, 20], a higher than expected portion (estimated: 2%) of patients with congenital LQTS might also have atrial fibrillation with early onset and, in some, polymorphic wave forms have been recorded in the atria that raise the question of similar arrhythmogenic presentations of cardiac repolarization disorders at the ventricular and atrial level [19].

Vice versa, gain-of-function mutations in the $I_{Ks}$ or $I_{Kr}$ channel genes have been associated with shortened ventricular repolarization (short QT syndrome, SQTS) [21]. In patients with SQTS, there also is higher incidence of atrial fibrillation. Recently, Chen et al. first reported the KCNQ1 (Kv7.1, KvLQT1) mutation S140G in a familial form of atrial fibrillation [18]. Patients were not reported to have any ventricular repolarization abnormality. Later, additional KCNQ1 mutations including KCNQ1 (V141M), KCNQ1 (S209P), KCNQ1 (V307L) and KCNQ1 (Q147R) were associated with AF [20, 22, 23]. Recently, also mutations in the KCNE subunits have been proposed as a cause for atrial fibrillation e.g., KCNE2 (R27C), and KCNE5 (L65F) [24, 25]. Common to some of these mutations in $I_{Ks}$ subunits is a gain-of-function phenotype based mainly on alteration of channel gating and altered functional $\beta$-subunit modulation upon heterologous expression experiments. Although, care has to be taken to interpret effects on KCNQ1/KCNE channel features in heterologous expression [26], the gain-of-function phenotypes may allow for the development of AF as a result of increased $I_{Ks}$ and resultant action potential shortening [27, 28]. Very recently, a combined phenotype of SQTS and atrial fibrillation was reported for carriers of the mutation R231C [29].

In the present study, we identify a heterozygous KCNQ1 mutation (R231C) in two independent German families with a mixed phenotype of fAF, LQTS and additional sinus bradycardia. In order to understand the molecular basis of the KCNQ1 (R231C)-associated clinical phenotypes, we introduced the disease-associated mutation into human KCNQ1 and studied the effects on homomeric and heteromeric mutant KCNQ1 channels possibly closely resembling the situation in the heart.

**Materials and Methods**

**ECG analysis**

Standard 12-lead ECG recording was done (paper speed 25 or 50 mm/s). For analysis, recordings were digitalized by scanning in a high-resolution graphic format and were imported into a graphic measure program (DatInfin® Measure, Germany). ECG parameters were measured in three consecutive beats and provided as mean values. ECG and arrhythmia analyses were independently performed by two cardiologists.

**Genetic analysis**

DNA was isolated from EDTA blood samples of all available family members. Mutation screening of all coding
sequences from several ion channel genes (KCNQ1 (LQT-1), KCNH2 (LQT-2), SCN5A (LQT-3), KCNE1 (LQT-5) and KCNE2 (LQT-6), KCNJ2 (LQT-7) and KCNA5 (AF)) was performed by sequencing (ABI3500) after directed and selective PCR amplification using standard procedures. Family members gave a written informed consent before genetic analysis that is in concordance with recommendations of the local ethics committee and current legal regulations. Obtained nucleotide sequences were compared with published wild type sequences (KCNQ1: NM_000218, isoform 1) and cross-checked with cardiac ion channel web databases and PUBMED entries (http://www.fsm.it/cardmoc). We used DNA from 380 healthy, unrelated individuals as control samples; mutations were, when absent in the control population, checked for evolutionary conservation.

**Molecular Biology**

Molecular biological procedures were described before [30]. Briefly; human KCNQ1 (variant 1), KCNE1 (NM_000219.3), KCNE2 (NM_172201), KCNE3 (NM_005472), KCNE4 (NM_080671) and KCNE5 (NM_012282) were subcloned into the oocyte expression vectors pSGEM or pSP64 or pXOOM [31]. The KCNQ1 clone was mutated at R231 to cysteine using the QuickChange site directed mutagenesis kit (Stratagene) and confirmed by automated DNA sequencing of the complete clone. The vectors were digested using Nhe1 and in vitro synthesis of cRNA was performed with T7 mMessage mMachine kit (Ambion).

**Electrophysiology**

Stage V *Xenopus laevis* oocytes were harvested according to German law as described previously [32]. Oocytes were injected with 4 ng of wt or mutant KCNQ1 cRNA alone or with 2 ng wt KCNQ1 cRNA plus 2 ng R231C KCNQ1 cRNA plus 4 ng of KCNEx cRNA. The stoichiometry of the mix was 180:10:47:180:10:47:1:10:1:10:1:10:1:1 for E1:E2:E3:E4:E5. The oocytes were stored for 3-4 days at 17°C in Barth’s solution containing 88 mM NaCl, 1.1 mM KCl, 2.4 mM NaHCO3, 300 μM Ca(NO3)2, 300 μM CaCl2, 800 μM MgSO4, 15 mM HEPES-NaOH, streptomycin sulfate (20 mg/l), penicillin-G (31 mg/l) and gentamycin (50 mg/l) at a pH of 7.6. Standard TEVC recordings were performed at 22-23°C using a Turbo Tec-10CX amplifier (NPI, Germany) combined with an Digidata 1322A interface and pCLAMP 8.0 software (Axon Instruments Inc./ Molecular Devices, USA). Recordings were analyzed using ClampFit 8.0 (Axon Instruments Inc./ Molecular Devices, USA) and Origin 7.0 (Additive, Germany) combined with an Digidata 1322A interface and pCLAMP 8.0 software (Axon Instruments Inc./ Molecular Devices, USA). Recording pipettes were filled with 3 M KCl and had resistances of 0.5-1.5 MΩ. Potassium channel recordings were collected in ND96 recording solution containing 96 mM NaCl, 4 mM KCl, 1.8 mM MgCl2, 1.0 mM CaCl2, 5 mM HEPES; pH 7.6. Reagents where purchased from Sigma unless otherwise noted.

**Data analysis**

The activation curves were obtained by plotting initial (determined ~5 ms after the end of the test pulse) normalized tail current amplitudes (Itail) versus the respective test potential. These curves were fitted to a Boltzmann equation of the form:

\[ I_{\text{tail}} = \frac{A_1 - A_2}{1 + \exp \left( \frac{V - V_{1/2}}{k} \right)} + A_2 \]  (formula 1)

\[ V_{1/2} \] is the voltage of half-maximal activation; k is the slope factor; V is the voltage of the test pulse; A1 and A2 are the maximal and minimal current amplitudes, respectively.

Activating and deactivating current traces were fitted using the simplex algorithm to one (formula 2) or two (formula 3) exponential functions of the form:

\[ y = A_0 + A_1 * \exp (-t/\tau_1) \]  (formula 2)

\[ y = A_0 + A_1 * \exp (-t/\tau_1) + A_2 * \exp (-t/\tau_2) \]  (formula 3)

Where the time constant(s) \( \tau_{1,2} \) and the corresponding amplitude(s) \( A_{1,2} \) and a steady-state current \( A_0 \) are taken into account.

**Homology modeling and molecular dynamics simulations**

A consensus homology model of human KCNQ1 was generated for the KCNQ1 residues 91-358. Several individual modeling steps (using YASARA Structure version 10.1.) were required. The consensus homology model is based on the respective amino acid sequence and refinement of a high-resolution model using a CASP approved protocol [33]. We chose to model KCNQ1 in homology to Kv1.2 (pdb: 2A79). Five independent models were built based partly on SSALN scoring matrices [34]. An indexed version of the PDB was used to determine the optimal loop anchor points and collect possible loop conformations [35] and loops were optimized. An unrestrained high-resolution refinement for the KCNQ1 models with explicit solvent molecules was run using AMBER03 force field and the result was validated to ensure that the refinement did not move the models in the wrong directions. A final hybrid model was built; bad regions in the top scoring model were iteratively replaced with corresponding fragments from the other models. The resulting consensus homology model represents the transmembrane region of KCNQ1 in an open/inactivated state. The KCNQ1 model was inserted into a membrane within a simulation box and the box was then filled with H2O and NaCl. A molecular dynamics (MD) simulation was run for 40 ns using YASARA 10.1. The following settings were used: force field AMBER03, time step 1 fs, temperature was 298 K, pressure at 1 bar, pH 7.0, Coulomb electrostatics at a cutoff 7.86, 0.9% NaCl, solvent density 0.997. The average KCNQ1 structure was determined and was used to calculate the RMSFs for the model.

KCNQ1(R231C) Causes Combined Cardiac Phenotype
Results

In two unrelated families, we identified a heterozygous KCNQ1 missense mutation with a nucleotide substitution of cytosine to thymine at position 691 (c.691C>T) that leads to an amino acid exchange from arginine to cysteine (p.R231C). This mutation could not be found in 380 European control samples and was shown to be orthologously highly conserved. Beside eight patients with proven mutation, three additional family members were obligate mutation carriers (Fig. 1). Brief clinical information is presented in Figure 1 and Table 1.

In family 772, the R231C mutation was present in 6 family members and three additional patients were obligate mutation carriers. Genetic screening was gradually performed after presentation of fetal bradycardia in patient 772_5 and knowledge of LQTS phenotype in the mother (772_2) with a QTc interval of 475 ms (Fig. 1). However, beside sinus bradycardia the boy kept asymptomatic and showed no signs of atrial rhythm abnormality or QT prolongation in rest ECG. This phenotype was exactly the same in the one year younger brother (772_6). Two years after diagnosed LQTS and with the age of 40 years the mother (772_2) developed several times persistent atrial fibrillation that had to be terminated by electrical cardioversion (Fig. 1). Her brother (722_9) also suffered from atrial fibrillation that was diagnosed by chance during medical examination for military service at the age of 19 years. However, although he was a mutation carrier he only showed phenotypical signs for atrial fibrillation and not for QT prolongation. Other mutations that may be responsible for atrial
fibrillation (e.g. a mutation in the KCNA5 gene) could be excluded. Two further mutation carriers (patient 772_8 and 772_10) were complete asymptomatic regarding atrial fibrillation, QT prolongation, sinus bradycardia, syncope or SCD.

However, one uncle (772_18) of the patient 772_8 suddenly died during swimming at the age of 23. As already known, swimming and stress are typical triggers for ventricular arrhythmia in patients with mutations in KCNQ1 leading to LQTS, therefore it can be reasoned that he also was a mutation carrier. Furthermore the mother (patient 772_17) of the patient 772_8 phenotypically showed sinus bradycardia and suddenly died at the age of 70 years due to ventricular fibrillation and his brother (patient 772_15) was diagnosed for LQTS, however, further details are unclear, including the genetic status.

In a second family (family ID 32) the same KCNQ1 mutation as mentioned above could be found in two family members. A 38 year old female was first diagnosed with LQTS after surviving cardiac arrest. The ECG showed a QTc interval of 502ms in rest. Further screening of first degree family members identified the asymptomatic daughter at age of nine with a QTc interval of 484 ms.

In order to understand the molecular mechanisms of the clinical phenotype of R231C in vitro experiments were performed: The mutation was introduced into the human cardiac KCNQ1 and the channels were expressed in *Xenopus laevis* oocytes. We found that the R231C mutation reduced K+ channel function. The homomeric KCNQ1 (R231C) channels activated and deactivated faster and higher potentials were required to activate the channels (Fig. 2, 3). These effects may be the result of reduced capability to sense voltages by the missing positive charge normally provided by the positive side chain of R231 (note: cysteine does not contain a positive side chain).

| Patient- ID | Age (y) | Gender | Mutation | HR (1/min) | QT (ms) | QTc (ms) | Clinical presentation |
|-------------|---------|--------|----------|------------|---------|----------|----------------------|
| 32_1        | 38      | f      | KCNQ1    | 61         | 496     | 502      | survived cardiac arrest |
| 32_7        | 9       | f      | KCNQ1    | 62         | 477     | 484      | asymptomatic |
| 772_2       | 38      | f      | KCNQ1    | 71         | 436     | 475      | atrial fibrillation |
| 772_5       | 0       | m      | KCNQ1    | 58         | 427     | 421      | neonatal bradycardia |
| 772_6       | 0       | m      | KCNQ1    | 56         | 451     | 437      | neonatal bradycardia |
| 772_8       | 67      | f      | KCNQ1    | 51         | 471     | 434      | asymptomatic |
| 772_9       | 19      | m      | KCNQ1    | 63         | 413     | 424      | asymptomatic, atrial fibrillation |
| 772_10      | 35      | f      | KCNQ1    | 46         | 487     | 426      | asymptomatic, sinus bradycardia |
| 772_15      | unknown | m      | unknown  | -          | -       | -        | LQTS |
| 772_17      | -70     | f      | unknown  | -          | -       | -        | sinus bradycardia, SCD |
| 772_18      | 23      | m      | unknown  | -          | -       | -        | SCD (swimming), suspected LQTS |

**Table 1.** Clinical and genetic data of the two families with the heterozygous KCNQ1 (R231C) mutation. *propositus in bold; †age at the time of diagnosis / symptoms ; m, male; f, female; SCD, Sudden Cardiac Death

Fig. 2. KCNQ1 (R231C) causes reduced K+ currents. Wt and mutant channels were expressed in *Xenopus laevis* oocytes and channel functions were analyzed with the two-electrode voltage clamp technique. a. Representative current traces of wt and mutant KCNQ1 channel currents. Currents were activated by a pulse protocol as indicated (2s pulses; -120 mV – 60 mV in 20 mV increments). b. I/V curves were obtained by normalization of the currents to the mean peak currents of the KCNQ1 wt at 40 mV (n = 8-38, error as ± SEM). Significant differences (p<0.05) are marked with *. 

KCNQ1(R231C) Causes Combined Cardiac Phenotype

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As atrial and ventricular I_{Ks} channels may differ in their specific β-subunit composition, we tested the effects of the respective KCNE β-subunit on KCNQ1 wt and KCNQ1(R231C). As a result we found that KCNE1 increases mutant channel currents compared to wt channels, whereas KCNE2 and KCNE4 reduced heteromeric channel functions (Fig. 4). Bendahhou et al. (2005) suggested a specific stoichiometry of KCNEs for human ventricular tissue [17]. We mixed cRNAs in a similar stoichiometry (180:10:47:110:1 for E1:E2:E3:E4:E5) to mimic the ventricular situation and found that mutant heteromeric channels exert smaller channel currents in the range of -100mV to -40mV compared to wt heteromeric channels (Fig. 4). These data suggest that KCNQ1(R231C) may act to reduce I_{Ks} and thus lengthen the ventricular action potential length.

The localisation of the sinus bradicardia/fAF/LQTS-associated mutation R231C is predicted in the KCNQ1 S4 transmembrane α-helix as suggested by sequence comparison with other Kv channels. This region comprises the central part of the voltage sensing machinery. We performed homology modelling to generate a 3D model. Subsequently the model was incorporated in a membrane, the resultant system was filled with water and a short molecular dynamic simulation was performed to relax the molecular system (Fig. 5a,b). Analyses of this system shows that the residue R231 is positioned in a stable crevice and is in contact water from the extracellular space (Fig. 5b,d). This position is perfectly suited to sense transmembrane voltages.

**Discussion**

The mutation R231C was reported to be associated with severe QTc prolongation [36]. The mutation is positioned in the center of the S4 voltage sensor transmembrane domain. It neutralizes a positive charge crucial for channel activation (Fig. 5). The modeling results suggest that R231C is presented stably at the surface/in a crevice that makes it an attractive candidate for a transmembrane sensing residue in the voltage sensor of open KCNQ1 channels. During the opening/closing of the channels the conformation of the S4 segment is
expected to undergo substantial changes: In the closed/resting state the side chains of the residues are expected to partially interact with the surrounding α-helices, the transmembrane α-helix of KCNEs and the lipid phase. Mutations in KCNQ1 can disturb the equilibrium between these states leading to complex functional consequences [37]. As a result mutations leading to a closed state shifted equilibrium lead to reduced channel function and long QT syndrome [38]. On the other hand, a mutation which causes increased channel conduction may result in shortening of cardiac action potential length. Depending on the location of the channels the increased channel functions may cause a short QT syndrome (channels in ventricles) or fAF [18, 20, 22–25, 27]. However, there is also evidence that fAF is evoked by prolongation of atrial action potential duration and effective refractory periods that could be identified by clinical studies as well as cell and animal experiments [9, 39, 40]. Here, we present clinical data indicating that KCNQ1 (R231C) leads to atrial fibrillations and LQTS similar to a very recent report by Bartos [29]. However, in addition to to atrial fibrillations and LQTS we identity sinus bradycardia in the R231C patients. The above presented thoughts are based on loss-of-functional I$_{Ks}$ in ventricles leading to LQTS and on gain-of-functional I$_{Ks}$ in the atria to fAF. The obvious question arising is, how the mutation R231C can possibly cause a gain-of-function and at the same time a loss-of-function. A possible explanation is that the compositions of KCNQ1 subunits with specific KCNE subunits in atria and ventricles are different and that the specific KCNE determines if the mutation causes a gain- or a loss-of-function. This hypothesis is supported by the finding that coexpression with KCNE1 increases heteromeric channel function whereas KCNE2 and KCNE4 coexpression suppresses potassium conduction of heteromeric channels (Fig. 5). However, the data on specific composition of atrial and ventricular I$_{Ks}$ channels is not sufficiently studied to make a clear statement. Bendahhou et al. (2005) reported a stoichiometry of KCNEs based on qRT-PCR on cardiac tissue [17]. Mixing KCNE cRNAs in a similar composition and coexpressing it with KCNQ1 cRNA to mimic the ventricular situation suggested that I$_{Ks}$ function may indeed be reduced by the R231C mutation, explaining the LQTS by loss-of-function. Bendahhou et al. (2005) also reported up to two-fold differences in KCNE expression in atrium compared to ventricle [17]. If KCNE1 or/and KCNE3 are more abundant in atrial I$_{Ks}$ channel
complexes, a resulting gain-of-function of the channels would lead to shortened atrial APs and increased likelihood of fAF as seen in the patients of our two independent families (Fig. 1, 2). Little is known about the function of KCNQ1/KCNE channels in sinus nodes. A gain of function of KCNQ1/KCNE channels in the sinus node could result in sinus bradycardia seen in patients as well. Clearly, more clinical and molecular data on the exact expression patterns of KCNEs are required to prove this hypothesis.

**Conclusion**

The neutralization of the positive charge carried by R231 leads to largely reduced voltage dependence of KCNQ1 channels and altered functional KCNE sensitivity, which cause diverse functional effects depending on the specific KCNE subunit. These effects may be causative for the clinical manifestation of sinus bradycardia, fAF and LQTS reported here in two independent German families.
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