Subunit composition and subunit interactions behind ion channel function

PhD Thesis

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LIST OF PUBLICATIONS

Articles related to the thesis

I. Déri S*, Hartai T*, Virág L, Jost N, Labro AJ, Varró A, Baczkó I, Nattel S, Ördög B. A possible explanation for the low penetrance of pathogenic KCNE1 variants in Long QT syndrome type. *Pharmaceuticals* **2022;**15(12):1550.

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II. Déri S*, Borbás J*, Hartai T, Hategan L, Csányi B, Visnyovszki Á, Madácsy T, Maléth J, Hegedűs Z, Nagy I, Arora R, Labro AJ, Környei L, Varró A, Sepp R, Ördög B. Impaired cytoplasmic domain interactions cause co-assembly defect and loss of function in the p.Glu293Lys KNCJ2 variant isolated from an Andersen-Tawil Syndrome patient. *Cardiovasc Res* **2021;**117(8):1923-1934.

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Articles not related to the thesis

Polyák A, Topal L, Zombori-Tóth N, Tóth N, Prorok J, Kohajda Z, **Déri S**, Demeter-Haludka V, Hegyi P, Venglovecz V, Ágoston G, Husti Z, Gazdag P, Szlovák J, Árpádffy-Lovas T, Naveed M, Sarusi A, Jost N, Virág L, Nagy N, Baczkó I, Farkas AS, Varró A. Cardiac electrophysiological remodeling associated with enhanced arrhythmia susceptibility in a canine model of elite exercise. *Elife* **2023;**12:e80710.

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1 Introduction

Potassium ion channels are widely spread in the living world. They are categorized into three major structural classes: the two transmembrane domains and one P-loop (2TM-1P), the tandem pore domain and four transmembrane domains (4TM-2P), and the six transmembrane domains including the voltage sensor with one P-loop (6TM-1P). In the heart, prominent representatives of the 2TM-1P class are the ion channels driving the I_{K1}, $I_{K,ATP}$ and $I_{K,Ach}$ currents. Interestingly, these ion channels can exist in homo- as well as in heterotetrameric configuration. In the latter case, the ion channel is composed of non-identical subunits which may carry different functional characteristics but are able to co-assemble based on their structural homology. Heteromerization of functionally different, but structurally similar ion channel subunits contribute greatly to the observed functional diversity of all ion channels and particularly potassium channels. Ion channels that are composed of 4TM-2P subunits are represented by potassium channels that belong to the so-called TWIK, TASK, TREK and THIK subfamilies in the heart. While the typical cardiac voltage-gated potassium channels are the ether-a-go-go-related K^+ channels (ERG), Shaker-related channels (Kv1-4), Ca^{2+}-activated K^+ channels and KCNQ channels which belong to the 6TM-1P class.

In a functional K^+ channel, ion channel subunits that belong to either of the above discussed structural classes co-assemble in homo- or heteromeric form and make up the essential core structure of the ion channel, hence the name ‘pore-forming’ or α subunits. With this assembly of pore-forming subunits, however, a various number of additional, so-called auxiliary or β-subunits can associate, giving rise to a multiprotein complex. Most voltage-gated potassium channels are associated with auxiliary subunits, often with different ones depending on the organ, tissue- or cell-type, developmental stage or pathological context. The cardiac I_{Ks} channels are based on the pore-forming subunits encoded by the KCNQ1 gene (6TM-1P class). When KCNQ1 is co-expressed with either of the KCNE subunits (KCNE1–5) in heterologous expression systems, a distinctly different current phenotype can be observed. This indicates that all KCNE regulatory subunits can interact with and modify KCNQ1 function. For example, KCNE1 amplifies current amplitudes and drastically slows down the time course of activation of KCNQ1 channels, largely reproducing characteristics of the cardiac I_{Ks} channels. For this reason, cardiac I_{Ks} channels are thought to be made up by KCNQ1 and KCNE1 subunits. In addition to I_{Ks} channels, KCNE2 and KCNE3 are also able to co-assemble with subunits encoded by the KCNH2 gene that serve as the main pore forming subunit of the rapid component of the delayed rectifier potassium channel (I_{Kr}) and also with α subunits (HCN1-HCN4) of the hyperpolarization-activated cyclic nucleotide gated channel (I_f). These findings exemplify the multiplicity of physiological roles of regulatory ion channel subunits. The stoichiometric ratio of pore-forming and regulatory subunits within the ion

channel complex may vary greatly. For example, for complexes of HCN and KCNE subunits, a cryo-electron microscopy study showed variable stoichiometry between 4:1 and 4:4, depending on expression levels of subunits and mutations in genes encoding subunits. Importantly, KCNQ1 and KCNE1 and complexes can also exist in different stoichiometric ratios, displaying different functional behaviors. These findings highlight the importance and complexity of interaction between pore-forming and regulatory ion channel subunits. However, despite all the above evidence, the stoichiometric ratio of distinct subunits within the ion channel macromolecular complex remain incompletely understood and actively studied. Quite interestingly, deletion of KCNE2 caused QT prolongation only when particular environment factors were present, such as western diet or aging. Furthermore, the absence of KCNE2 could also lead to sudden cardiac death (SCD) in presence of certain environmental factors. These evidence suggest that the interaction between the pore-forming and auxiliary subunits might be a rather dynamic process under the influence of cellular and/or environmental factors. Because of the vast number of possible combinations between potassium channel subunits and particularly, due to the lack of appropriate research methods, the exact role of auxiliary subunits in the physiology of potassium channels, as well as their involvement in pathological processes remains incompletely known.

There exist substantial differences in the electrophysiology of cardiomyocytes in mammalian species, which hampers interpretation of findings from animal models in the human context. Therefore, in addition to their clinical relevance, cardiac channelopathies have long served as unique models for ion channel research providing invaluable clues on ion channel physiology and pathophysiology. The experiments and conclusions drawn in this study are especially relevant in the context of LQT5 and ATS, hence the genetics and pathophysiology of these diseases will be briefly discussed in the following.

The LQT5 is caused by loss off function mutations in the KCNE1 gene which encodes a single transmembrane domain protein that is able to associate with KCNQ1 and is thought to be an essential regulatory subunit of cardiac I_{Ks} channels. While approximately 75% of patients with LQT1–LQT3 genotypes are symptomatic the overall penetrance of LQT5 mutations was only 20%. Low penetrance in LQT5 implies that QT prolongation is not always observed, making it difficult to establish an LQTS diagnosis without genetic screening. Therefore, LQT5 can be particularly dangerous, because symptoms can suddenly develop and/or exaggerate in patients unaware of their condition upon exposure to additional factors affecting repolarization, such as QT-prolonging drugs. The mechanisms responsible for the low penetrance of LQT5 mutations, however, are currently unknown. The first genetic LQT5 animal model has been recently developed with the pivotal contribution of our department and has provided new insights into the pathomechanisms of LQT5. The LQT5 rabbit model is based on the heart-specific overexpression

of the well-known LQT5 KCNE1 variant Gly52Arg-KCNE1. In the transgenic animals, QT prolongation was modest but significant, however, arrhythmia susceptibility increased remarkably in the presence of QT prolonging drugs as compared to wild type (WT) animals, consistent with clinical observations in LQT5 cases. Gly52Arg-KCNE1 has a strong dominant negative effect on KCNQ1 current amplitudes, when co-expressed heterologously with KCNQ1 and WT-KCNE1. Based on this, decreased I_{Ks} amplitudes could be expected in cardiomyocytes isolated from LQT5 rabbits, expressing Gly52Arg-KCNE1. Surprisingly, the amplitude of I_{Ks} was not different in LQT5 as compared to WT cells, only an accelerated rate of current deactivation was identified by detailed characterization. These seemingly contradictory findings from the LQT5 rabbit model and in vitro experiments suggest that factors that are present in cardiomyocytes, but not in heterologous expression systems ameliorate the effects of the dominant negative Gly52Arg-KCNE1 variant, translating eventually to very mild symptoms at baseline conditions and low penetrance of the Gly52Arg mutation. The nature of such factors rescuing the LQTS phenotype, however, remain elusive.

ATS is a rare genetic disorder that is characterized by a triad of symptoms, including periodic paralysis, dysmorphic features and cardiac manifestations. Cardiac symptoms may include QT prolongation, a prominent U-wave, premature ventricular beats and bidirectional ventricular tachycardia. Over 70 mutations causing ATS1 have been identified. Almost all ATS-causing KCNJ2 mutations cause loss of function via various mechanisms, including structural changes, gating alterations and defective protein trafficking.

Our colleagues at the 2nd Department of Internal Medicine, University of Szeged have carried out the genetic analysis of a patient with typical ATS1 phenotype. Sequencing of a panel of candidate genes revealed a de novo mutation of the KCNJ2 gene that causes a glutamic acid – lysine substitution at position 293. The functional effects of Glu293Lys or its potential pathogenic role in ATS were unknown. Glu293, as it is discussed later is located in the cytoplasmic domain of KCNJ2 subunits, within to the so-called cytoplasmic domain interface (CD-I) region, the protein surface which lies in between two adjacent KCNJ2 subunits. Interestingly, the function of the CD-I region of inward rectifier potassium channels has been a matter of detailed investigation. In the laboratory of Professor Colin G. Nichols it has been shown, that the conformation of the cytoplasmic domain of the functional channels is stabilized by a network of salt bridges. Some of these salt bridges are formed between two amino acids that are located in adjacent subunit, thereby linking the two subunits together. Impairment of the CD-I salt-bridge network reduces greatly the channel activity and its sensitivity to phosphatidylinositol 4,5-bisphosphate (PIP2), an allosteric activator of inward rectifier potassium channels. They concluded that the cytoplasmic domain can exist in a 'tight' conformation when the CD-I salt bridge network is unaffected. The 'tight'

conformation is associated with higher channel activity in the case of KCNJ2. Genetic disruption of the CD-I salt bridge network, however, results in the dislocation of CD-I amino acids consistent with a more loose conformation and reduced channel activity. The possible role of Glu293 in the structure-function relationships of KCNJ2 channels, however, has not been investigated.

2 Aim of the study

The recent findings obtained from the transgenic LQT5 rabbit model, as well as the discovery of the potentially pathogenic Glu293Lys KCNJ2 mutation in an ATS patient have provided us with unique research possibilities to obtain novel insights into the structure-function relationships of two major cardiac ion channels responsible for I_{Ks} and I_{K1}, respectively. In order to seize these opportunities, we conducted two parallel studies to answer the following questions.

In the context of LQT5, we considered the surprising finding from the transgenic rabbit LQT5 model on the lack of inhibitory effect of the Gly52Arg-KCNE1 allele on I_{Ks} amplitudes, despite the strong dominant negative effect of Gly52Arg-KCNE1 on KCNQ1 current that has been confirmed by several studies in the literature and in our laboratory as well. Similarly strong evidence from the literature indicates that all KCNE subunits are able to modify KCNQ1 function and all KCNE genes, although at varying levels, are expressed in the heart. On these grounds we asked, is it possible that KCNE subunits take part in the regulation of I_{Ks} channels? Do they modify the development of the LQT5 phenotype? To elaborate on these possibilities, we have designed and conducted experiments that explore the potential regulatory roles of the most likely candidates from the KCNE gene family on KCNQ1-based ion channels.

In the context of ATS, we first asked, could Glu293Lys be the pathogenic mutation causing the ATS phenotype? In order to establish the causative role of Glu293Lys in ATS, we aimed at characterizing the functional effects of Glu293Lys on KCNJ2 currents. Furthermore, considering its intriguing localization with the CD-I region of KCNJ2 channels and that no functional role has been attributed to Glu293 so far, we designed experiments to explore the molecular mechanisms underlying the functional consequences of the clinically relevant Glu293Lys mutation.

3 Materials and methods

3.1 Heterologous expression systems

Transient transfections were carried out as follows. A total amount of 4 µg plasmid DNA were mixed with 16 µg of polyethyleneimine (PEI) in 1.5 ml serum-free medium and incubated at room temperature for 30 minutes. Then, the transfection mixture was added to the cells (CHO-K1 or HEK-293) and the culture dishes were moved back to the CO_2 incubator for 2 hours. At the end of the incubation period with the transfection mixture, the cells were washed twice with serum-

free medium and growth medium was then added. All experiments (e.g. patch clamp or NanoBiT assays) were carried out 48 hours following transient transfections.

3.2 Electrophysiology techniques

CHO-K1 cells were superfused with normal Tyrode solution. Micropipettes were fabricated from borosilicate glass capillaries using a horizontal puller. The pipette solution was contained (mmol/L): KOH 110, KCl 40, K2ATP 10, HEPES 5, EGTA 5, and $MgCl_2$ 0.1; pH was adjusted to 7.2 with aspartic acid.

Currents were recorded at 37°C from GFP positive cells. Current densities were calculated as the current amplitude – cell capacitance quotient. All recordings were analysed off-line using Clampfit software.

3.2.1 Characterization of KCNQ1-based currents

KCNQ1-based currents were elicited by 5s long test pulses between -20 and 50 mV in 10 mV increments from the holding potential of -80 mV, followed by a repolarizing step to -40 mV to record deactivating tail currents.

3.2.2 Measurement of KCNJ2-based potassium current

KCNJ2-based currents were elicited by step pulses between -120 mV and +40 mV in 10 mV increments from the holding potential (-80 mV). Current amplitudes were recorded as a Ba^{2+}- sensitive (cc: 30 μM) current at the end of the 300 ms step pulse.

3.3 Immunocytochemistry

CHO-K1 were fixed with 4% formaldehyde solution. After fixation, the cell membrane was immunolabelled with Wheat- Germ Agglutinin Texas Red-X Conjugate (1:400 dilution) at room temperature for 10 minutes. Immunolabelling was accomplished by incubating the cells with anti-KCNJ2 primary antibody (1:1000 dilution) overnight at 4°C. The next day, cells were incubated with FITC-Conjugated Anti-Rabbit IgG secondary antibody (1:450 dilution). Two types of negative control were used to check the specificity of immunostaining ('no primary AB' control and no transfection control).

Fluorescent images were captured by LSM880 laser scanning confocal microscope. Images were quantitatively analysed by the ImageJ software (1.52p).

3.4 Prediction of salt bridges in the cytoplasmic domain of KCNJ2

The salt bridges within the cytoplasmic domain (CD) of homotetrameric KCNJ2 channels were predicted using ESBRI software in the 1U4F and the 2GIX crystal structure. The models were accessed from the RCSB Protein Data Bank (https://www.rcsb.org/) and were graphically visualised using RasWin Molecular Graphics Software (2.7.5.2).

3.5 Protein:protein interaction assay procedures

Protein:protein interaction assays were carried out by following instructions given in the NanoBiT kit. Briefly, transfection was carried out as it was described previously. 48 hours after the transfection procedure. 1.6×10^5 cells were loaded in each well in a 96-well plate. The cell-permeable NanoGlo Live Cell Reagent was prepared and was added to each well in a reduced light environment. The plate was promptly moved into a FLUOstar Optima Microplate Reader preheated to 37°C and the measurement was started in luminescence mode.

3.6 Statistical analysis

Statistical tests were computed by using GraphPad Prism Software (version 8; GraphPad Software Inc., San Diego, CA, USA). In case of the LQT5 study, data are presented as mean ± 95% confidence interval (95% CI) and the group means were statistically compared by one-way ANOVA followed by Holm-Sidak's post hoc tests in each experimental arrangement. In case of ATS study, data are presented as mean ± 95% CI. Luminescence values were log-transformed to attain homogenous distribution of variance. Group means were statistically compared by Student's T-test or one-way ANOVA followed by Bonferroni post hoc tests for multiple comparisons.

4 Results and discussion

4.1 Subunit interactions within the I_{Ks} ion channel complex

KCNQ1 forms a slowly-activating ion channel when co-assembled with KCNE1 and based on this, cardiac I_{Ks} channels are thought to be primarily made up by heteromeric complexes of KCNQ1 and KCNE1. LQT5 KCNE1 variants, such as Gly52Arg-KCNE1 used in the transgenic LQT5 rabbit model to induce the LQT5 phenotype and the Asp76Asn-KCNE1 variant with well-established pathogenic role in LQT5 have strong dominant negative effects and inhibit current amplitudes of KCNQ1+WT-KCNE1 channels. KCNE3 renders KCNQ1 channel constitutively active and has been reported to activate currents driven by KCNQ1/KCNE1 complexes. This latter effect makes KCNE3 a likely candidate of an auxiliary subunit that could rescue the current phenotype from the inhibitory effects of the LQT5 KCNE1 alleles (e.g. Gly52Arg-KCNE1) seen in the LQT5 transgenic rabbit model. In order to explore a possible role for KCNE3 in the modulation of KCNQ1-base ion channels, KCNQ1 was co-expressed with KCNE1, with or without additional regulatory subunits, including the LQT5 KCNE1 variants.

4.1.1 KCNE3 rescues current densities in the LQT5 context

Average current density in group 2 expressing KCNE3 together with KCNQ1 and WT-KCNE1 was similar to that of group 1 expressing KCNQ1 and WT-KCNE1 only. In group 3 and 5, two LQT5 KCNE1 variants, the Gly52Arg-KCNE1 that was used in the LQT5 transgenic rabbit model and Asp76Asn-KCNE1, a KCNE1 variant with well-established pathogenic role in LQT5 have

been co-expressed together with KCNQ1 and WT-KCNE1. These groups would represent the heterozygous LQT5 genotype if one assumed that KCNE1 is the only auxiliary subunit regulating cardiac I_{Ks} channels. In case of both LQT5 KCNE variants, current densities decreased significantly (group 3 and 5) compared to group 1. Average current density in the group 3 (KCNQ1 + WT-KCNE1 + Asp76Asn-KCNE1) was 25.5 pA/pF and in cells that were co-transfected with KCNQ1, WT-KCNE1 and Gly52Arg-KCNE1 (group 5) was 31.7 pA/pF, both significantly lower compared to current densities seen in group 1 (74.9 pA/pF). These data indicate strong dominant negative effects of Asp76Asn- and Gly52Arg-KCNE1 variants over the channels based on KCNQ1 and WT-KCNE1.

Next, KCNE3 was co-expressed with KCNQ1 in the presence of WT- KCNE1 and either of the LQT5 mutant KCNE1 variants. Interestingly, KCNQ1-based currents seemed to be activated by KCNE3 even in the presence of the LQT5 KCNE1 variants. Current densities were significantly increased when KCNE3 was added to Asp76Asn-KCNE1 genetic background in group 4 and Gly52Arg-KCNE1 genetic background in group 6, compared to the group 3 and 5, representing the corresponding LQT5 genetic backgrounds without KCNE3. Moreover, average current densities were not statistically different in groups which included KCNE3 in the presence (group 4 and 6) or absence (group 2) of Asp76Asn- or Gly52Arg-KCNE1 variants. These data indicate that inhibitory effect of LQT5 KCNE1 variants on KCNQ1-based channels was rescued by KCNE3 on both distinct LQT5 genetic backgrounds.

4.1.2 Deactivation kinetics is accelerated in the presence of LQT5 KCNE1 variants

The average half decay times do not show significant differences between group 1 (KCNQ1+WT-KCNE1) and group 2 (KCNQ1+WT-KCNE1+KCNE3). However, groups 3 to 8 which contained additional cDNAs showed significantly decreased half decay times compared to the group 2. Accelerated deactivation kinetics were observable in the presence of LQT5 KCNE1 variants regardless of the presence of KCNE3.

4.1.3 Activation kinetics remained unchanged in the presence of LQT5 KCNE1 variants or KCNE3

Average time constant was similar among all experimental groups, as neither any of the LQT5 KCNE1 variants, nor KCNE3 had detectable effect on activation kinetics.

4.1.4 Voltage dependence of activation

Voltage dependence of activation was characterized by extracting voltage of half maximal activation (V1/2) from non-linear curve fit of steady-state activation curves. The half maximal activation shows no significant differences in between group 1 (KCNQ1+WT-KCNE1) and group 2 (KCNQ1+WT-KCNE1+KCNE3) which contained KCNE3. Observable changes do not occur

in the presence of the Gly52Arg-KCNE1 variant. However, in the presence of the Asp76Asn-KCNE1 variant, the V1/2 values were significantly shifted to the positive direction compared to group 1, independent of KCNE3. The right shift of steady-state activation curves with the Asp76Asn-KCNE1 variant is a known phenomenon and it contributes to the dominant negative loss of function nature of this mutation.

In summary, KCNE3 has no effect on KCNQ1 and WT-KCNE1-based currents, but it prevents the inhibitory effect of both LQT5 KCNE1 variants in the LQT5 context. The rescue effect, however, is incomplete, as the deactivation kinetics remained accelerated in case of both LQT5 KCNE1 variants in the presence of KCNE3, and the Asp76Asn-KCNE1 variant cause a shift of steady state activation to the positive direction regardless of the presence of KCNE3.

4.1.5 KCNE3 shifts subunit stoichiometry in the KCNQ1-based channel complex

By the whole-cell configuration of the patch clamp technique used in this study, the sum of all transmembrane currents driven by all ion channels present in the cell membrane are recorded. Thus, this method does not provide information on whether KCNE3 and KCNE1 are present in a distinct ion channel population or co-assemble in the same ion channel complex. The NanoBiT protein:protein interaction assay was used to gain insight into this question. The LgBiT and SmBiT fragments of luciferase enzyme were fused to the C-termini of KCNQ1 and WT-KCNE1 resulting in the KCNQ1-LgBiT and KCNE1-SmBiT reporter constructs, respectively. The KCNQ1-LgBiT and KCNE1-SmBiT reporters were co-expressed in HEK-293 cells with varying amount of KCNE3. In these experiments, the KCNQ1, KCNE1 and KCNE3 cDNA copy number ratio was 1:2:0, 1:2:1 and 1:2:2.

The average relative luminescence (RLU) was 158.4 in group 1 (1:2:0 cDNA ratio) which was significantly different from group 2 (1:2:1 cDNA ratio) (129.3 RLU). However, in the group 3 (1:2:2 cDNA ratio), where the HEK-293 cells were contained a same amount of KCNE1 and KCNE3, the reduction of average RLU was more pronounced (96.7 RLU) compared to the group 1. The lower RLU values in the presence of KCNE3 indicate that KCNE3 reduces the amount KCNE1 within the KCNQ1-base ion channel complex. Because there only a limited number of binding sites within the KCNQ1 tetramer that can be occupied by KCNE-like auxiliary subunits, this might mean that KCNE3 is able to occupy the same binding sites used by KCNE1. The relative affinity of these subunits towards the binding sites, however, should be addressed in future studies.

In summary, KCNE3 prevents the inhibitory effect of LQT5 KCNE1 variants when co-expressed heterologously. Furthermore, subunit compositions representing the heterogenous LQT5 genetic background completed with KCNE3 generate currents with accelerated deactivation kinetics, thereby recapitulating the properties of I_{Ks} observed in the transgenic LQT5 rabbit model. KCNE3 achieves these effects by replacing KCNE1 within the macromolecular complex of I_{Ks} channel.

4.2 Subunit interactions in the KCNJ2 ion channel complex

The de novo, previously uncharacterized mutation Glu293Lys has been discovered in an ATS patient that causes amino acid substitution in the CD-I region of KCNJ2 ion channels. In order to establish the potentially pathogenic role of Glu293Lys mutation in ATS and gain insight into the role of Glu293 in ion channel function, as well as into the molecular mechanisms leading to ion channel dysfunction in case of the Glu293Lys variant, a series of experiments were carried out as follows. The Arg218Gln KCNJ2 variant with well documented loss-of-function dominant negative effects and a pathogenic role in ATS has been included as a positive control.

4.2.1 Electrophysiological characterization of the Glu293Lys KCNJ2 variant

For the characterization of the functional effects, WT and mutant KCNJ2 variants expressed heterologously in CHO cells and the currents were characterized by the whole-cell patch clamp technique. No current was detected in cells expressing the Glu293Lys variant alone, indicating loss-of-function effect of the mutation. Under the same conditions, cells expressing the WT KCNJ2 variant alone or the combination of WT and Glu293Lys variants exhibited robust inward current with strong inward rectification, with a prominent outward component at the physiologically relevant membrane potential range between -80 and 0 mV. Average current densities measured at -60 mV test pulses were the highest in cells expressing WT KCNJ2 alone. Co-expression of WT and a mutant KCNJ2 variant resulted in significantly decreased average current densities with both the Arg218Glu and the Glu293Lys variant compared to homomeric WT channels.

In conclusion, these data indicate a prominent dominant-negative effect of the Glu293Lys variant over WT channels, resulting in loss of function on the heterozygous genetic background.

4.2.2 Intersubunit salt bridges predicted in the vicinity of Glu293

Observing the Glu293 within the ion channel complex using the crystallographic models of the cytoplasmic domain of KCNJ2 channels (1U4F; 2GIX) revealed that Glu293 is located at the CD-I. Using the ESBRI software revealed that Glu293 may participate in the formation of a salt bridge network, with the involvement of four amino acids all together. Each of these four salt bridges are formed between amino acids that reside in different subunits and therefore connect two subunits to each other and were similarly predicted in two independent crystallographic models of KCNJ2. The substitution of the positively charged glutamic acid to a negatively charged lysine at position 293 may be expected to impair this intersubunit salt bridge network, which may in turn translate into disturbed subunit-subunit interactions.

4.2.3 Assessment of subcellular localization of KCNJ2 subunits

An impaired salt bridge network at CD-I in the Glu293Lys variant may affect co-assembly of KCNJ2 subunits, which could result in the cytoplasmatic accumulation of misfolded protein complexes. To assess this possibility, immunocytochemical experiments were carried. The average immunofluorescence intensity of KCNJ2 was significantly higher in the membrane region compared to the cytoplasmic region in all three experimental groups, while the ratios of average KCNJ2 signals detected in the membrane and cytoplasmic regions were not different among the experimental groups. Interestingly, colocalization of the KCNJ2 and WGA-TxRed fluorescence showed no difference for the WT and the Arg218Gln KCNJ2 variants. The Glu293Lys, however, were significantly lower compared both to WT and Arg218Gln groups. These data suggest that membrane accumulation of Glu293Lys variant is less efficient compared to the WT and Arg218Gln variants. However, clear evidence of cytoplasmic accumulation was absent. Therefore, we suggest that oligomerization of Glu293Lys variant into a tetrameric complex may be insufficient and the misfolded Glu293Lys subunits present preferentially in the submembrane area.

4.2.4 Assessing physical interaction between KCNJ2 subunits

To gain further mechanistic insights into the deficient oligomerization of the Glu293Lys variant, two variations of the NanoBiT assay were developed. In the NanoBiT system, Nanoluc luciferase enzyme is split into two fragments, the so-called large (LgBiT) and small (SmBiT) fragments. LgBiT and SmBiT associate and complement each other to create a functional enzyme when the two fragments can move freely or are in proximity to each other. Interestingly, by genetic engineering, the two Nanoluc fragments can be fused to proteins of interest and used as 'proximity sensors'. In this configuration, the chemiluminescent signal increases, when the proteins of interests tagged with Lg- and SmBiT fragments are in physical proximity. In this study, we applied this strategy to investigate subunit interactions within the ion channel complex.

First, the LgBiT and SmBiT tags were fused to the cytoplasmic C-termini of KCNJ2 variants, giving rise to the 'intracellular' configuration. In this case, our goal was to assess association of the cytoplasmic domains of adjacent subunits within the KCNJ2 ion channel complex. In the second variation, the so-called 'extracellular' configuration, we have engineered an extra transmembrane domain, the so-called Snorkel tag (-Sn-) between the C-termini of KCNJ2 variants and the LgBiT or SmBiT fragments. In this configuration, the LgBiT and SmBiT reporter tags are displayed extracellularly, providing us with the opportunity to investigate whether heteromeric complexes are transported to and present in the membrane.

With the intracellular NanoBiT reporters expressed in homomeric configuration, the average relative $\log_e$ luminescence (lnRLU) values were similar in the WT and in the Arg218Gln group. However, in case of Glu293Lys group lnRLU values were significantly lower compared to the

WT or Arg218Gln groups. Furthermore, the luminescent values seen in the Glu293Lys group were not different from the negative control.

Regarding the heteromeric configurations intended to model the heterozygous genotype, mean lnRLU values were not different in cells expressing WT-LgBiT and Arg218Gln-SmBiT compared to the positive control. However, the luminescence values of the WT-LgBiT with Glu293Lys-SmBiT group were significantly lower compared to the positive control, yet significantly higher than the negative control. Collectively these data indicate that Glu293Lys subunits are able to form heteromers with WT subunits, but with lower efficacy compared to the WT or the Arg218Gln variant. The possible explanation for this observation may be the impairment of oligomerization in the presence of Glu293Lys.

With the 'extracellular' NanoBiT reporters, average lnRLU were similar in positive control in the Arg218Gln-Sn-LgBiT+Arg218Gln-Sn-SmBiT group and in cells expressing Glu293Lys-Sn-LgBiT+Glu293Lys-Sn-SmBiT too. However, mean values in these groups were significantly higher than that of the negative control group. Data with the extracellular NanoBiT reporters indicate that both the Arg218Gln and Glu293Lys variants co-assemble with WT subunits and these heteromers exist in the cell membrane.

5 Conclusion and new findings

5.1 Subunit interactions within the I_{Ks} ion channel complex

1. Co-expression of the KCNQ1, WT-KCNE1 and Gly52Arg-KCNE1 variants together with KCNE3 recapitulates an ion current phenotype in vitro that is consistent with the I_{Ks} phenotype observed in the LQT5 transgenic rabbit model in vivo.

2. KCNE3 shifts the subunit stoichiometric ratio of KCNQ1- and KCNE1-based ion channels by reducing the average amount of KCNE1 within the same ion channel complex.

3. Taken together, our findings strongly support the hypothesis that in addition to KCNE1, other structurally related auxiliary subunits, such as KCNE3, are involved in the formation and regulation of I_{Ks} channels in cardiomyocyte.

5.2 Subunit interactions in the KCNJ2 ion channel complex

1. The Glu293Lys KCNJ2 mutation causes loss of function and exerts dominant negative effect on KCNJ2 currents. These results provide evidence for the causative role of the Glu293Lys KCNJ2 variant in ATS1.

2. Glu293 plays a role in mediating co-assembly of KCNJ2 subunits and in maintaining channel conductivity, likely by participating in the formation of an intersubunit salt bridge network at the CD-I region.

5.3 Development of molecular assays for studies on ion channel subunit interactions

Two different configurations of the NanoBiT assay have been developed and applied for the investigation of K^+ channel subunit interactions, thereby expanding the range of relevant research methods in the field.

1. The intracellular configuration of the NanoBiT assay was applied to study interactions between pore-forming and regulatory subunits in the context of the LQT5 and the inter-subunit interactions in the context of ATS1.

2. The extracellular configuration of NanoBiT assay was applied to detect membrane presentation of KCNJ2 subunits.

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