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Modulation of Human Ether A Gogo Related Channels by CASQ2 Contributes to Etiology of Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT)

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Key Words
CPVT • Heart • Arrhythmia • Calcium • hERG

Abstract
Rationale - The plateau phase of the ventricular action potential is the result of balanced Ca^{2+} influx and K^+ efflux. The action potential is terminated by repolarizing K^+ currents. Under β-adrenergic stimulation, both the Ca^{2+}-influx and the delayed rectifier K^+ currents I_K are stimulated to adjust the cardiac action potential duration to the enhanced heart rate and to ascertain adequate increase in net Ca^{2+} influx. Intracellularly, a Calsequestrin2 (CASQ2)-ryanodine receptor complex serves as the most effective Ca^{2+} reservoir/release system to aid the control of intracellular Ca^{2+} levels. Currently, it is unclear if disease-associated CASQ2 gene variants alter intracellular free Ca^{2+} concentrations and if cardiac ion channels are affected by it. Objective – The goal of this study is to test if CASQ2 determines intracellular free Ca^{2+} concentrations and to identify cardiac ion channels that are affected by it. Further, we aim to study disease-associated CASQ2 gene variants in this context. Methods and Results - Here, we study the effects of the CASQ2 mutations R33Q, F189L, and D307H, located in highly conserved regions, on the functions of cardiac potassium channels in *Xenopus* oocytes using two electrode voltage clamp. As a result, CASQ2 wild type and CASQ2-mutants modulated hERG functions differently. Free Ca^{2+} measurements and molecular dynamics simulations imply alterations in Ca^{2+} buffer capacity paralled by changes in the dynamic behavior of the CASQ2-mutants compared to CASQ2 wild type. Conclusions - These *in vitro* and *in silico* data suggest a regulatory role of CASQ2 on cytosolic Ca^{2+} and hERG channels which may contribute to the etiology of CPVT.

Introduction

The sympathetic nervous system mediates the fast physiological stress response to physical and psychological stressors. Stimulation of the sympathetic nervous system includes positive chronotropic effects on the heart and positive inotropic effects on the ventricles. The control

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of cardiac function by the sympathetic nervous system is caused by binding of catecholamines to G protein-coupled receptors, including the β1-adrenergic receptor (β1-AR) [1, 2]. Receptor activation results in activation of heterotrimeric G-proteins and subsequent stimulation of adenylate cyclase leading to increased production of cAMP. cAMP activates the cAMP-dependent protein kinase A (PKA) [3] and therewith downstream effectors, including L-type Ca^{2+} channels as well as the delayed rectifier potassium channels KCNQ1/KCNE1 (I_{Ks}) and hERG (I_{Kr}) [4-9]. The plateau phase of the cardiac ventricular action potential is accomplished by a delicate balance of Ca^{2+} influx and K^+ efflux [10], critically depending on L-type Ca^{2+} channels, KCNQ1/KCNE1 and hERG channels [11]. In forms of Long QT syndrome, disruption of e.g. I_{Kr} by mutations in the channel gene or by pharmacological inhibition prolongs the cardiac action potentials, which favors arrhythmogenesis, syncope and cardiac sudden death.

Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT) is a ventricular tachyarrhythmia induced by exercise or emotion without a detectable structural heart disease. In about one third of the CPVT cases a familial history of syncope or sudden death is reported. CPVT usually occurs in otherwise healthy children and young adults and is characterized by stress- or emotion-induced syncope due to the onset of ventricular polymorphic tachyarrhythmias. CPVT affected patients characteristically exert a bi-directional ventricular tachycardia in their ECG during stress but no significant ECG abnormalities at rest. The arrhythmias are reproducibly triggered by emotional stress, exercise or by β-adrenergic stimulation with isoproterenol infusion. Consequently, beta blocker therapy has been suggested as an effective approach in preventing the recurrences of syncope and sudden death.

A decade ago Swan et al. described CPVT as an autosomal dominant arrhythmic syndrome linked to chromosomal locus 1q42-q43 in two families, which is the locus of the human cardiac ryanodine receptor (hRyR2) [12]. Mutations in hRyR2 were found in CPVT families with history of sudden cardiac death [13, 14]. These findings strongly supported the hypothesis that CPVT can result from mutations in hRyR2.

Missense mutations in highly conserved regions of the Calsequestrin2 gene (CASQ2) were linked to an autosomal recessive form of CPVT [15]. The CASQ2 protein serves as the major calcium store in the sarcoplasmic reticulum (SR) of cardiac myocytes. Further, CASQ2 and hRyR2 are part of a protein complex. This complex is functionally involved in the Ca^{2+} release from the sarcoplasmic reticulum [16] in response to the activation of Ca^{2+} influx through L-type Ca^{2+} channels during the plateau phase of the action potential. Since CPVT arrhythmias and β-adrenergically triggered arrhythmias closely resemble the arrhythmias developing during intracellular calcium overload and delayed afterdepolarization [17, 18] the CASQ2/hRyR2 protein complex is a strong candidate gene for CPVT. The three CASQ2 mutations R33Q, F189L, and D307H were linked to the development of CPVT whereas CASQ2 F189L was reported only once in a heterozygous state (summarized in http://www.fsm.it/cardmoc/). The most reported CASQ2 variant D307H was further analyzed by expression in rat cardiac myocytes. The mutant caused impaired Ca^{2+} storing and release functions in the SR and destabilized the Ca^{2+}-induced Ca^{2+} release mechanism. The Ca^{2+}-buffer mechanism involving CASQ2 may be compromised by the mutation. Therefore, the experimental data demonstrated that both genes involved in CPVT pathogenesis, CASQ2 and hRyR2, affect the amount of Ca^{2+} SR-release upon β–adrenergic stimulation. The resulting Ca^{2+} overload may create an instable electrical substrate to favor arrhythmogenesis [19]. Thus, the Ca^{2+} overload seems to play a critical role in arrhythmogenesis, syncope and cardiac sudden death in CPVT and LQTS.

In the present study, we tested whether CASQ2 modulates cardiac repolarizing potassium channels. We studied the effect of coexpression of cardiac potassium channels with wild-type (wt) and mutant forms of CASQ2. Whereas the channels Kv4.3, KCNQ1/KCNE1 and Kir2.1-3 were not affected by coexpression with CASQ2, hERG channels were functionally modulated by CASQ2-variants. We show that CASQ2-effects are dependent on intracellular calcium levels. In order to gain insights into the structural changes that lead to altered hERG-modulation depending on the respective CASQ2-mutation we performed Molecular Dynamics (MD) simulations. Our results concerning the interaction of CASQ2, hERG and Ca^{2+} can contribute to a better understanding of CPVT.

Materials and Methods

Molecular Biology

The molecular biological procedures were the same as previously described [20]. Human *Kv1.5, Kv4.3, Kir2.1, Kir2.2, Kir2.3 and KCNQ1* were subcloned into the oocyte expression vector pSGEM. Human *ERG* and *KCNE1* were subcloned into

pSP64, and CASQ2 (NM_009814) was subcloned into pCR4 TOPO. The CASQ2-clone was mutated at the positions mentioned in the text by QuickChange site directed mutagenesis kit (Stratagene). All constructs were confirmed by automated DNA-sequencing. The vectors were linearized using *NheI, EcoRI* or *EcoRV*. *In-vitro* synthesis of cRNA was performed with T7 mMessage mMachine kit (Ambion).

Electrophysiology

Xenopus laevis oocytes were harvested in accordance with German laws as described previously [21]. Animals were ethyl 3-aminobenzoate methanesulfonic acid- (tricaine) anesthetized, ovarian lobes were removed from *Xenopus laevis* and digested with collagenase (Type II, Worthington, 1 mg/ml in calcium-free Barth's solution) for about 120 min. Stage V and VI oocytes were collected and injected with ~60 nl of cRNA solution. Oocytes were injected with 6 ng of wild type hERG cRNA alone or with 6 ng CASQ2 wt cRNA or 6 ng mutant CASQ2 cRNA. The oocytes were stored for 3 days at ~18 °C in Barth's solution containing (in mmol/L): 88 NaCl, 1.1 KCl, 2.4 $NaHCO_3$, 0.3 $Ca(NO_3)_2$, 0.3 $CaCl_2$, 0.8 $MgSO_4$, 15 HEPES-NaOH, penicillin-G (31 mg/L), gentamycin (50 mg/L), streptomycin sulfate (20 mg/L), pH 7.6. TEVC recordings were performed at ~22 °C using a Turbo Tec-10CX (NPI, Germany) amplifier equipped with a Digidata 1322A AD/DA-interface and pCLAMP 9.0 software (Axon Instruments Inc. / Molecular Devices, USA). The data were analyzed with Clampfit 9.0 (Axon Instruments Inc. / Molecular Devices, USA) and Origin 6.0 (Additive, Germany). Recording pipettes were filled with 3 M KCl and had resistances of 0.4-1 MΩ. Channel currents were recorded in ND96 recording solution containing (in mmol/L): 96 NaCl, 4 KCl, 1.8 $MgCl_2$, 1.0 $CaCl_2$, 5 HEPES; pH 7.6. Reagents where purchased from Sigma unless otherwise stated.

Data analysis

The activation curves were obtained by plotting the normalized peak tail current amplitudes (y) versus the respective test potential. These curves were fitted to a Boltzmann equation of the form:

$$y = \frac{A_1 - A_2}{1 + e^{(x-x_0)/dx}} + A_2$$

x_0 is the voltage of half-maximal activation (or $V_{1/2}$); dx is the slope factor; x is the voltage of the test pulse; A_1 and A_2 are the maximal and minimal current amplitudes, respectively. Statistical analysis was performed with student's t-test (unpaired) or Anova.

Determination of intracellular free Ca^{2+} concentrations

Xenopus laevis oocytes were injected with 6 ng CASQ2 wt cRNA or 6 ng mutant CASQ2 cRNA. The thus injected and uninjected oocytes were stored for 3 days at ~18 °C in Barth's solution. The oocytes were washed in cold distilled H_2O. 15 oocytes were transferred into 1.5 ml Eppendorf tubes. The extracellular solution was increased up to 11 µl/oocyte by adding distilled H_2O. The oocytes were homogenized using a pistil. The suspension was centrifuged and the aqueous phase was taken for the concentration measurements.

The free Ca^{2+} concentrations of the samples were determined using a Kwik-Tip Ca^{2+}-selective electrode (World Precision Instruments) and a high resistance amplifier. The Ca^{2+}-selective electrode was filled with a solution containing 100 mM KCl and 30 nmol/L $CaCl_2$. An Ag/AgCl-half cell was used as the reference electrode. A reference curve using a NaCl-based buffer (100 mM NaCl) with the free Ca^{2+} concentrations 100 pmol/L, 1 nM, 10 nmol/L, 100 nmol/L, 1 µmol/L, 10 µmol/L and 100 µmol/L was constructed. The free Ca^{2+} concentrations of the respective samples were determined using this reference curve.

Molecular dynamic simulations of CASQ2 mutant proteins

CASQ2 (pdb-ID: 1SJI) was received from the protein data base, incorporated in a simulation box filled with NaCl-H_2O. A 10 ns all-atoms-mobile simulation was performed with CASQ2 wt to reach a stable conformation using YASARA Structure version 10.1: An unrestrained high-resolution refinement with explicit solvent molecules was run, using AMBER03 force field and the result was validated to ensure that the refinement did not move the model in the wrong direction. The resulting CASQ2 model was taken as it was or mutated at the respective residue and standard MD simulations were run for 25 ns with 1.2 fs time steps. The following settings were used for the MD simulations: force field AMBER03, temperature was 298 K, pressure at 1 bar, pH 7.0, Coulomb electrostatics at a cutoff of 7.86, 0.9 % NaCl, solvent density 0.997. The structures at the beginning and after 25 ns simulations were used to calculate the RMSD for the models.

Results

hERG channels were expressed in *Xenopus laevis* oocytes and generated characteristic voltage dependent, strongly inactivating and potassium selective channel currents. Coexpression with CASQ2 reduced channel currents by about 25-40 % (Fig.1, 2). To evaluate whether these changes in current amplitude were associated with changes in channel kinetics we analyzed different parameters in hERG gating in the absence and presence of CASQ2. We found that the voltages of half maximal activation were -12.5 ± 0.2 mV and -13.5 ± 0.1 mV (n=56/58) for hERG expressed alone and coexpressed with CASQ2 wt, respectively.

CASQ2 contains three thioredoxin (TRX) fold domains. These structural motifs allow for the high capacitance calcium binding by CASQ2. CASQ2 may exert its effects on hERG channels by chelation and thereby effective reduction of the free intracellular calcium. Indeed, Tang et al. showed recently that hERG

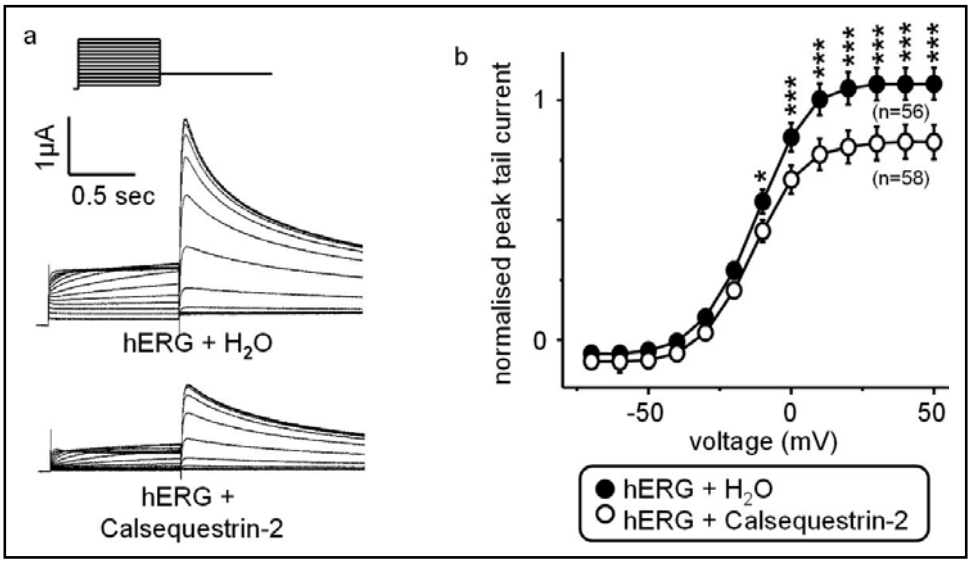

Fig.1. hERG channel currents in oocytes are reduced upon coexpression with CASQ2. hERG channels were expressed alone or in combination with CASQ2, and currents were recorded 3 days after injection. A. Representative current traces. B. hERG currents were analyzed and mean data ± SEM indicate inhibition of hERG peak tail currents upon coexpression with CASQ2. Asterisks indicate significances (* $p \leq 0.05$, *** $p \leq 0.001$).

channels are inhibited by the Ca^{2+} chelators BAPTA and, to a lesser extend, EGTA [21]. To explore whether the CASQ2 inhibition of hERG channels depends on the free intracellular Ca^{2+} concentration we tested inhibition of hERG in the presence of the Ca^{2+} chelator BAPTA. Consistent with the findings of Tang *et al.* [21] we found that BAPTA inhibited hERG (Fig. 2). The inhibition by BAPTA was not increased upon coexpression with CASQ2. These data indicate that CASQ2 and BAPTA act in a similar way by reducing the free intracellular Ca^{2+} concentration.

Mutation in the CASQ2 gene can result in CPVT. The effects were classically assigned to altered interaction of CASQ2 mutants with ryanodine receptors. However, we showed that CASQ2 is a functional modulator of hERG channels and therefore tested whether disease associated CASQ2 mutants show changes in modulation of hERG channels. We coexpressed hERG channels with the CASQ2 mutants R33Q, F189L and D307H. We found that the CASQ2 mutants R33Q and F189L lead to altered hERG modulation compared to CASQ2 wt. Whereas CASQ2 D307H did not modulate hERG channel currents differently than CASQ2 wt, R33Q reduced hERG currents less and F189L reduced hERG function stronger than CASQ2 wt (Fig. 3).

As mutations in proteins can potentially change protein function, mutations in CASQ2 could change the capacity of Ca^{2+} binding effectively influencing intracellular Ca^{2+} storage. This could affect hERG function as hERG seems to be sensitive to intracellular Ca^{2+} buffering. To test this hypothesis we injected EGTA after coexpression of hERG with wt and mutant CASQ2 cRNAs. The apparent sensitivity of hERG to the Ca^{2+} buffer EGTA is supposed to be variable if the CASQ2 variants would buffer Ca^{2+} to different degrees. The underlying mechanism would be a competition for free intracellular Ca^{2+} between EGTA and the respective CASQ2 variant. Indeed, we found that 4.6 mM EGTA

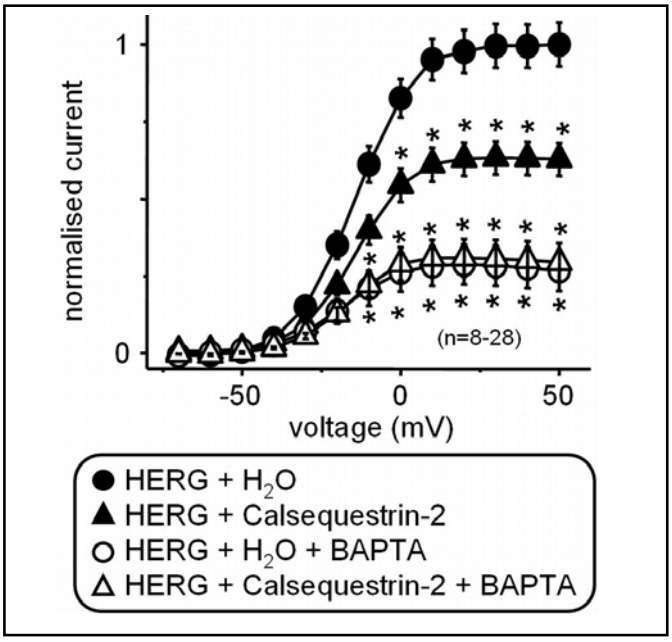

Fig. 2. Calcium chelation nullifies hERG channel inhibition by CASQ2. hERG channels were expressed alone or in combination with CASQ2, and currents were recorded 3 days after injection. BAPTA (final intracellular concentration about 1 µM) was injected and currents were recorded again. The hERG currents were analyzed and mean data ± SEM indicate inhibition of hERG peak tail currents upon coexpression with CASQ2 and BAPTA. The channel currents in the presence of BAPTA were not dependent on CASQ2. Asterisks indicate significances (* $p \leq 0.05$).

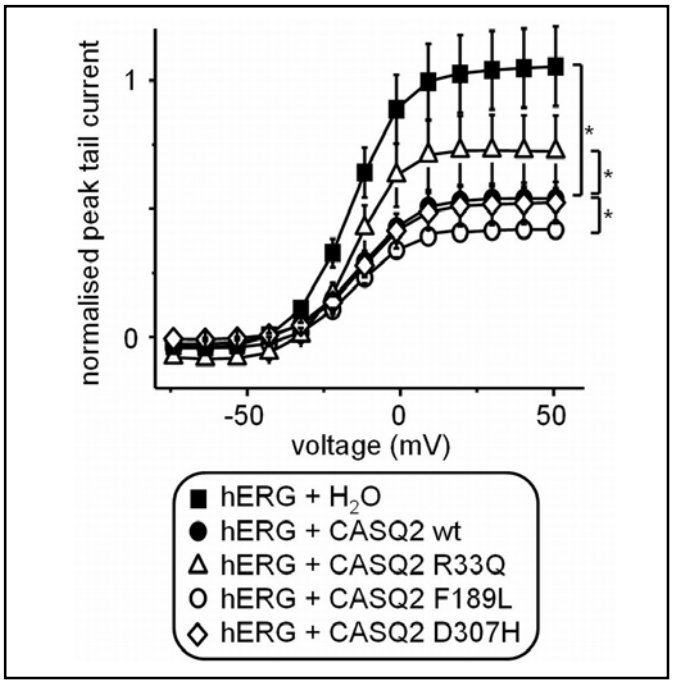

Fig. 3. hERG channels are modulated by CASQ2 mutants. hERG channels were expressed alone or in combination with CASQ2 wt and mutants. Currents were recorded 3 days after injection, peak tail currents were analyzed and mean data ± SEM indicate variable effects on hERG peak tail currents. Asterisks indicate significances (* $p \leq 0.05$).

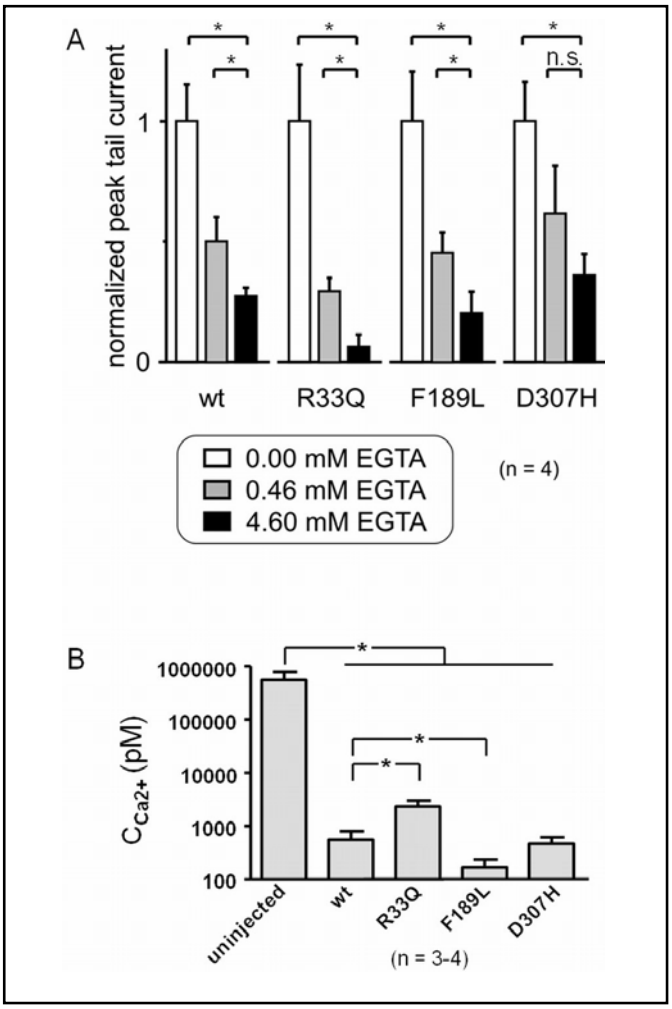

Fig.4. hERG + CASQ2 wt/mutants determine intracellular Ca^{2+} concentrations which modulates hERG channel function. A. hERG channels were expressed in combination with CASQ2 wt and CASQ2 mutants. Peak tail currents were recorded 3 days after injection. EGTA (final intracellular concentration about 0.46/4.6 mM) was injected before recording the currents. The hERG currents were analyzed and mean data ± SEM indicate differences in inhibition of hERG peak tail currents upon coexpression with CASQ2 and injection of EGTA. B. The intracellular Ca^{2+} concentration of oocytes expressing CASQ2 wt or CASQ2 mutants were determined as described in materials and methods. All CASQ2 variants reduce the free Ca^{2+} concentration in oocytes whereas differences in putative buffer capacity are measured. Asterisks indicate significances (* $p \leq 0.05$).

reduced hERG currents stronger in the background of R33Q and F189L compared to CASQ2 wt or D307H (Fig. 4A). To clarify whether the CASQ2 variants buffer Ca^{2+} to differing degrees direct intracellular Ca^{2+} concentration of oocytes expressing CASQ2 wt or CASQ2 mutants were determined. All CASQ2 variants reduce the free Ca^{2+} concentration in oocytes compared to uninjected oocytes. However, differences in free Ca^{2+} concentrations were observed in oocytes expressing the different CASQ2 variants suggesting altered buffer capacities as a result of the respective gene variant (Fig. 4B).

All three mutations are located in one of the three TRX fold domains, the structural motifs allowing for efficient Ca^{2+} binding. In order to gain insight into the molecular nature of the altered CASQ2 function by the mutations we performed molecular dynamics simulations. The CASQ2 structure was incorporated into a simulation box filled with aqueous NaCl solution. After 25 ns simulations the structures of CASQ2 and its mutated forms were compared. Overlay of the structures showed that those of the CASQ2 variants diverged. The root mean square deviation (RMSD) of atom positions is a good measure of precision and provides insight into the movement/flexibility of a structure in a dynamic simulation. The mean RMSD of CASQ2 wt and D307H were in the range of about 2.2-2.4 Å whereas the mean RMSD of F189L was significantly reduced to about 1.8 Å, and the mean RMSD of R33Q was dramatically increased to

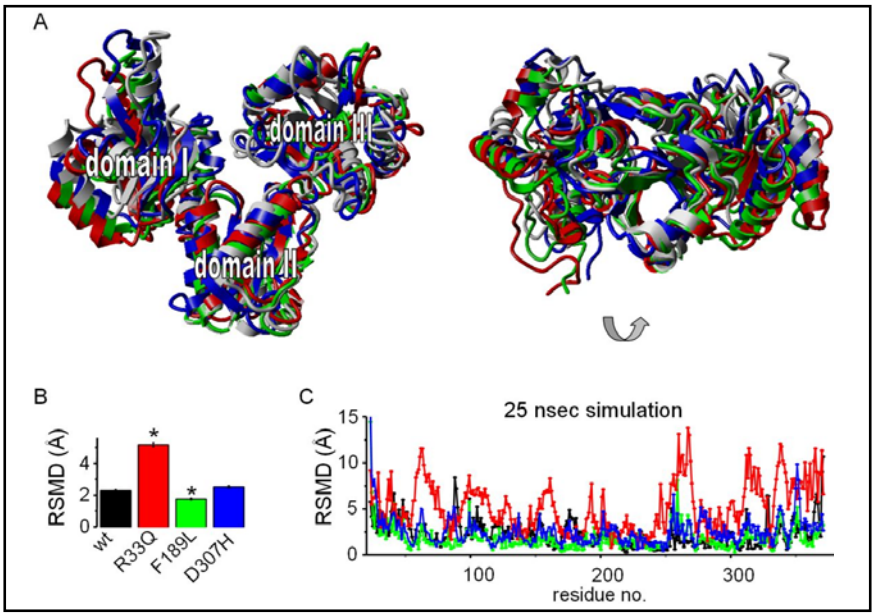

Fig. 5. Molecular dynamics simulations of CASQ2 wt and mutants. CASQ2 (pdb-ID: 1SJI) was incorporated in a simulation box filled with NaCl-H_2O. A 10 ns simulation was performed with CASQ2 wt to reach a stable conformation. This structure or the respective mutation (R33Q, F189L or D307H) was incorporated in a simulation box filled with aqueous NaCl solution and subsequent 25 ns simulations were performed. Overlays of the final structures are shown in A. The mean root mean square deviations (RMSD) ± SEM of the complete models compared to the initial structures are shown in B. The RMSD of the respective residues compared to the initial structural parameters are shown in C. Asterisks indicate significances (* $p \leq 0.05$).

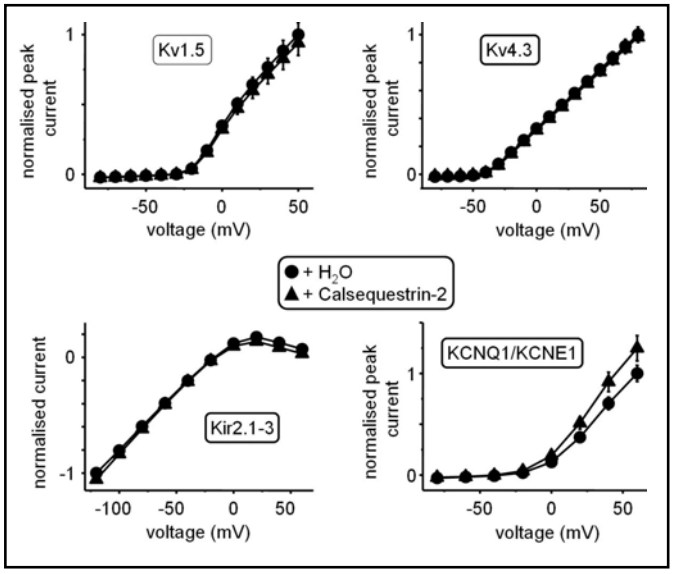

Fig. 6. CASQ2 shows no or only small effects on Kv, Kir and KCNQ1/KCNE1 cardiac potassium channel currents in oocytes. Kv1.5, Kv4.3, Kir2.1/Kir2.2/Kir2.3 or KCNQ1/KCNE1 channels were expressed alone or in combination with CASQ2 wt. Channel currents were recorded 3 days after injection using the pulse protocols indicated. Currents were analyzed at the end of pulses (KCNQ1/KCNE1 5 s pulses) and mean data ± SEM are shown (n = 7 - 28).

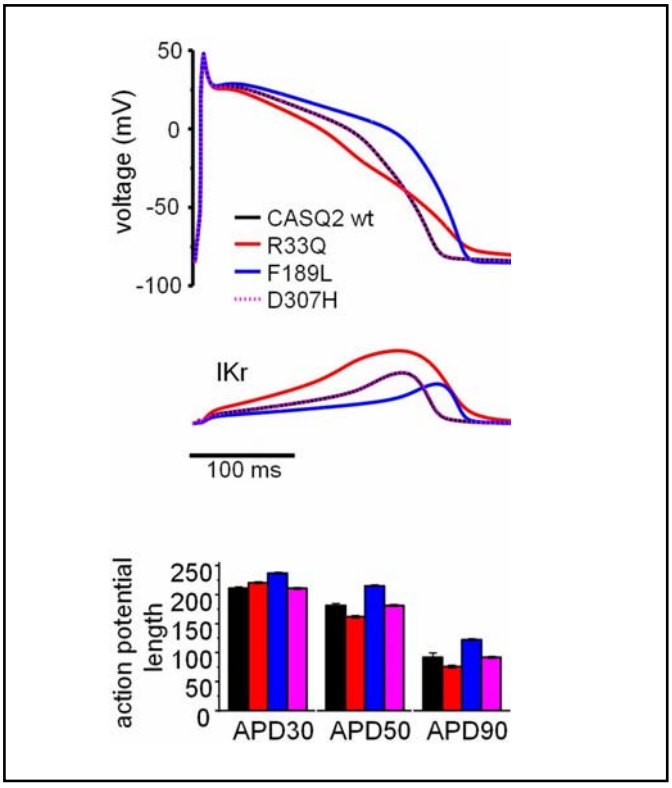

Fig. 7. Luo-Rudy II simulations suggest CASQ2 induced alterations in ventricular action potential shape. Luo-Rudy II simulations were performed using LabHEART 4.9.5 [21]. Simulated action potentials and hERG currents (IKr) are shown. The putatively reduced calcium binding capacity of CASQ2 mutants were accounted for by changing respective K_m values. Calculated action potential durations (APD30, APD50 and APD90) were calculated and are shown below. Specific parameters used were: CASQ2 wt – I_{Kr} 50% ± 7%, Kμ 0.9 μmol/L; CASQ2 R33Q – I_{Kr} 75% ± 3%, K_m 3,0 μmol/L; CASQ2 F189L – I_{Kr} 40% ± 1%, K_m 0.3 μmol/L; CASQ2 D307H – I_{Kr} 50% ± 2%, K_m 0.9 μmol/L.

about 5.2 Å (Fig. 5B). These data indicate that the mutations R33Q and F189L may change the flexibility of CASQ2, i.e. the flexibility is increased by R33Q and decreased by F189L. To understand in which region the changes by R33Q and F189L become effective we calculated the RMSD for each residue in the structure. The analysis indicates that R33Q induced the strongest

increase in RMSD in domains I and III (TRX fold domains I and III) whereas F189L seemed to change the pattern of RMSD (Fig. 5). Interestingly, with the exception of a few residues the pattern of CASQ2 wt and D307H are similar.

As hERG is only one of the major players in the ion channel ensemble determining the action potential shape and duration we tested further cardiac potassium channels. We found that the channels Kv1.5, Kv4.3 and Kir2.1-3 underlying the native cardiac currents I_{Kur}, I_{to} and I_{K1} are not modulated by CASQ2. On the other hand, KCNQ1/KCNE1 channel currents were mildly stimulated upon coexpression with CASQ2 (Fig. 6). These data indicate that the inhibiting effect of CASQ2 is selective for hERG channels.

To estimate the impact of altered hERG function under the influence of the CASQ2 variants on the cardiac action potential we introduced our experimental data into the Luo-Rudy ventricular action potential model [22]. We assumed that effects on hERG are the result of altered Ca^{2+} affinity of the CASQ2 mutants. To account for this fact we modified the respective K_m values of Ca^{2+} in the model calculations which resulted in increased cytosolic Ca^{2+}. The respective K_m values are roughly estimated from the effects on hERG channels and intracellular free Ca^{2+} concentrations (Fig. 4AB). The effects on the hERG channels were directly taken from the experiments in Fig. 3. Clearly, according to these semi-quantitative model calculation these manipulations alter both action potential shape (note differences of R33Q in APD30/50/90 compared to CASQ2 wt) and duration depending on the CASQ2 variant (Fig. 7).

Discussion

The present study reveals the modulation of the activity of the cardiac hERG K^+ channels by the calcium binding protein CASQ2 in a heterologous expression system. The current amplitude of hERG channels expressed in *Xenopus laevis* oocytes depends on the presence of CASQ2 wt (Figs. 1, 2). CASQ2 reduced hERG activity to about 25-40 %. Injection of BAPTA was reported to result in inhibition of hERG channels [21]. This inhibition was modulated by the presence of two mutations either in the inner helix S6 (Y652A) or in the pore helix (S631A). This fact caused the authors to suggest a pore blocking mechanism as the molecular basis of hERG inhibition. As both mutations result in strong alterations in hERG gating these data are very difficult to interpret. However, buffering of intracellular calcium could be an alternative explanation for hERG inhibition. Here, we show that application of BAPTA abrogates CASQ2 effects (Fig. 2). As CASQ2 is far too large to enter the central cavity of the hERG channel BAPTA and CASQ2 cannot compete for a binding site within the central cavity of hERG channels. A plausible explanation for these findings is that both BAPTA and CASQ2 buffer intracellular Ca^{2+} which leads to a reduction of hERG function. The CASQ2 protein exerts a high Ca^{2+} binding capacity and may serve as the major calcium store in the sarcoplasmic reticulum of cardiac myocytes. This increases the likelihood of the proposed competition mechanism for free Ca^{2+} by BAPTA and CASQ2.

Missense mutations in the highly conserved TRX fold regions of CASQ2 were linked to an autosomal recessive form of CPVT [15]. Mutations in CASQ2 are associated with alterations in intracellular Ca^{2+} levels possibly by altered Ca^{2+} storage [13]. This raises the question of how the intracellular Ca^{2+} level influences hERG function. The first possibility is a direct activation of hERG channels by Ca^{2+} as reported for cobalt and lanthanum [23]. However, cobalt and lanthanum alter gating of hERG potassium channels which was not seen for effects by CASQ2. Alternatively, CASQ2 may influence hERG trafficking: Misfolded and incompletely assembled proteins, including hERG ion channel proteins, are recognized by the cellular quality control and results in the retention of these proteins in the ER. Some ER-associated chaperones and adaptors have been shown to assist hERG protein folding and forward trafficking. These include HSP70/90 [24], 14-3-3 [25], Sar1, GM130, ARF1 [26], GTPases [27] and calnexin [28]. Calnexin is a membrane bound ER enriched protein which binds to specific sugars in a calcium dependent fashion [28, 29]. Calnexin recognizes glycan sugars of incompletely folded proteins to aid complete protein folding. It is possible that Calnexin function is regulated by CASQ2 wt / mutants via modulation of intracellular Ca^{2+} levels to control hERG channel trafficking and function. However, more research is required to test this.

If calcium chelators like BAPTA and EGTA compete for Ca^{2+} with CASQ2 wt/mutants to reduce hERG function, altered CASQ2 binding affinity can be expected to modulate calcium chelator sensitivity. We tested this hypothesis and found indeed that CASQ2 wt and mutants lead to differences in Ca^{2+} sensitivity (Fig. 4A). Especially hERG channels coexpressed with CASQ2 R33Q were sensitive to Ca^{2+} buffering, which indicates that under these conditions there is a higher concentration of free

Ca^{2+} available which in turn allows increased EGTA sensitivity. These results are consistent with differences in Ca^{2+} buffer capacity of CASQ2 variants determined by direct free Ca^{2+} measurements (Fig. 4B).

The extraordinarily high Ca^{2+} binding capacity of CASQ2 is the result of three TRX (thioredoxin) fold domains which structure has been solved [30]. The three mutations tested here are positioned in this structure: R33Q close to TRX domain I, F189L in TRX domain II and D307H in TRX domain III. Here, we used the published structural coordinates for MD simulations to gain insights into the protein stability as results of the specific mutations. In these simulations the mutations did not disrupt the global structure of the protein but resulted in mild structural changes of CASQ2 (Fig. 5A). The mutations R33Q and F189L resulted in an increase or decrease in the global RMSD, respectively (Fig. 5B). In the case of R33Q the RMSD in domains I and III were particularly increased indicating that these regions may gain structural flexibility (Fig. 5C). On the contrary, the pattern of residue-resolved RMSD changed in F189L (Fig. 5C). These changes could not be seen in D307H (Fig. 5B,C) which corresponds to the *in vitro* results (Fig. 3, 4). These data indicate that the mutations R33Q and F189L resulted in local changes of molecule dynamics which may be crucial for Ca^{2+} binding affinity. Possibly, these changes in protein flexibility change the ability to polymerize, a process regulating Ca^{2+} binding by CASQ2. This may change the Ca^{2+} buffer function of CASQ2 and alter the free Ca^{2+} concentration to regulate hERG channels (Fig. 3,4).

A major point not accounted by these measurements is a severe deficiency of CASQ2 protein demonstrated in murine models of recessive CPVT, caused by either D307H or R33Q CASQ2 mutations [31, 32]. Thus, hERG channel activity related to these mutations might be better represented by the CASQ2-deficient state than oocytes coexpressing hERG and mutant/wt CASQ2. This model would also explain why mutations with different effects on the buffering capacity give rise to a similar phenotype.

An obvious point of discussion is the fact that *Xenopus laevis* oocytes supposedly do not posses a sarcoplasmic reticulum, where CASQ2 is localized *in vitro*. However, the endoplasmic reticulum of oocytes shares significant functional similarities to the sacrcoplasmic reticulum and in the context of Ca^{2+} buffer function of sarcoplasmic proteins the *Xenopus* expression system was used before [33-35]. Provided that both hERG channels and CASQ2 variants behave similarly in cardiac myocytes and the oocyte expression system predictions for cardiac action potentials can be made. hERG channels represent the molecular correlate of the cardiac I_{Kr} suggesting that hERG modulation by CASQ2 could be relevant for the cardiac action potential. In order to estimate the effects of CASQ2 on the cardiac action potentials we tested for CASQ2 sensitivity of further cardiac potassium channels. We expressed Kv1.5 (I_{Kur}), Kv4.3 (I_{to}), Kir2.1/Kir2.2/Kir2.3 (I_{K1}) and KCNQ1/KCNE1 (I_{Ks}) alone or together with CASQ2. We found that only KCNQ1/KCNE1 channels were mildly stimulated whereas the other channels were not affected by coexpression with CASQ2 (Fig. 6). These data suggest that the CASQ2 inhibition is specific to hERG channels. However, since there is no precise quantification of the relative expression levels of CASQ2 it remains unclear if the results described here are caused from different sensitivities of the channels. These observations allowed us to try mathematical modeling of the ventricular action potential. Using the Luo-Rudy II model we incorporated the effects on hERG currents from the *in vitro* experiments described here. Further, our experiments suggested alterations in Ca^{2+} binding affinity of CASQ2 as a result of the respective mutation. We addressed this by altering the respective K_m. Clearly, these estimations are only semi-quantitative and thus the modeled data may be regarded as an approximation. Under the described conditions we find that the mutations in CASQ2 may change basic features of the ventricular action potentials like action potential duration and size (Fig. 7). However, as mentioned before this model does not take the CASQ2 protein deficiency demonstrated in murine models [31, 32] or altered CASQ2-ryanodine receptor interaction [19] into account and is only intended to incorporate effects suggested by the oocyte experiments presented in this study. The model predictions suggest changes in shape and duration of the ventricular action potential that are possibly pro-arrhythmic.

Summarizing, we report that hERG channels are modulated by CASQ2 in a heterologous expression system. Molecular dynamics simulations suggest differences in the protein dynamics in CPVT-associated CASQ2 mutants which could contribute to different hERG-modulation of the respective CASQ2 variant. The differences in hERG modulation by CASQ2 wt and CPVT-associated CASQ2 mutants may result in altered ventricular action potentials as suggested by Luo-Rudy II action potential modeling.

Together with the reported altered ryanodine receptor function, a CASQ2 protein deficiency, and intracellular Ca^{2+} overload by CASQ2 mutants, the modulation of

hERG channels increases the likelihood of ventricular arrhythmias. Thus, modulation of hERG channels by CASQ2 mutants may contribute to the etiology of catecholaminergic polymorphic ventricular tachycardia in patients.

Abbreviations

CPVT (Catecholaminergic Polymorphic Ventricular Tachycardia), wt (wild type), β-AR (β-adrenergic receptor), BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid), EGTA (glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid), TEVC (two-electrode voltage clamp).

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