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KCNJ2 Mutation Causes an Adrenergic-Dependent Rectification Abnormality with Calcium Sensitivity and Ventricular Arrhythmia

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Abstract

Background—*KCNJ2* mutations are associated with a variety of inherited arrhythmia syndromes including CPVT3.

Objective—Detailed cellular and mechanistic characterization of the clinically recognized *KCNJ2* mutation R67Q.

Methods—Kir2.1 current density was measured using the whole-cell voltage clamp technique from COS-1 cells transiently transfected with WT-Kir2.1 and/or R67Q-Kir2.1. Catecholamine activity was simulated with PKA stimulating cocktail exposure. Phosphorylation deficient mutants, S425N-Kir2.1 and S425N-Kir2.1/R67Q-S425N-Kir2.1, were used in a separate set of experiments. HA- or Myc-Tag-WT-Kir2.1 or HA-Tag-R67Q-Kir2.1 were used for confocal imaging.

Results—A 33 year old presented with a CPVT-like clinical phenotype and was found to have *KCNJ2* missense mutation R67Q. Treatment with nadolol and flecainide resulted in complete suppression of arrhythmias and symptom resolution.

Under baseline conditions, R67Q-Kir2.1 expressed alone did not produce I_{K1} while cells co-expressing WT-Kir2.1 and R67Q-Kir2.1 showed rectification index (RI) similar to WT-Kir2.1. After PKA stimulation, R67Q-Kir2.1/WT-Kir2.1 failed to increase peak outward current density; WT-Kir2.1 increased 46% (n=5) while R67Q-Kir2.1/WT-Kir2.1 decreased 6% (n=6), p=0.002. Rectification properties in R67Q-Kir2.1/WT-Kir2.1 demonstrated sensitivity to calcium with decreased RI in high-calcium pipette solution (RI $20.3 \pm 4.1\%$) compared to low-calcium (RI $36.5 \pm 5.7\%$) (p< 0.05). Immunostaining of WT-Kir2.1 and R67Q-Kir2.1 individually and together showed a normal membrane expression pattern and co-localization by Pearson's correlation coefficient.

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All authors report no conflicts of interest.

Conclusion—R67Q-Kir2.1 is associated with an adrenergic-dependent clinical and cellular phenotype with rectification abnormality enhanced by increased calcium. These findings are a significant advancement of our knowledge and understanding of phenotype-genotype relationship of arrhythmia syndromes related to *KCNJ2* mutations.

Keywords

Kir2.1; *KCNJ2*; inherited arrhythmia; CPVT3; ventricular arrhythmia; potassium inward-rectifier channel; genetic arrhythmia

Introduction

In 1995, Leenhardt and colleagues rigorously defined the clinical entity catecholaminergic polymorphic ventricular tachycardia (CPVT) (1) as an inherited cardiac arrhythmia syndrome with characteristic adrenergic-mediated bidirectional (BiVT) or polymorphic ventricular tachycardia (PMVT) resulting in syncope or sudden cardiac death. Importantly, CPVT occurs in the absence of structural heart disease, cardiotoxic medications or prolongation of the QT interval. CPVT was initially linked to mutations in two Ca^{2+} handling protein genes: the ryanodine receptor (*RYR2*), designated CPVT1, and calsequestrin-2 (*CASQ2*), designated CPVT2 (2, 3). The *RYR2* and *CASQ2* genes account for ~30–40% of CPVT cases(4). In 2006, Tester and colleagues performed genomic DNA screening for CPVT-linked mutations in several genes including *KCNJ2*, and discovered mutations in Kir2.1 including R82W and V227F. This was designated as CPVT3 and subsequently additional mutations in *KCNJ2* were reported by other groups including R67W and C101R(5), G144D and T305S(6) and R260P(7), all with a clinical phenotype of CPVT.

KCNJ2 encodes the α -subunits that co-assemble to form the potassium inward rectifier channel Kir2.1, which conducts the inward rectifier current, I_{K1} . Four distinct arrhythmia syndromes have been associated with Kir2.1 mutations; Andersen-Tawil Syndrome (ATS1, also denoted as Long QT Syndrome type 7 (LQT7)(8), Short QT syndrome (SQT3)(9), familial atrial fibrillation (FAF)(10) and CPVT3(4). Mutations associated with ATS1 have been functionally characterized by us and others as “loss of function” mutations (decreased I_{K1}) (11). ATS1 is characterized by a phenotypic triad consisting of cardiac abnormalities (premature ventricular complexes, ventricular bigeminy, supra-ventricular and ventricular tachycardia, Torsades de Pointes, prolonged QT intervals and prominent electrocardiographic U waves), in addition to dysmorphic features and periodic paralysis(12). First designated Type 7 Long QT syndrome (LQT7), it was noted that the QT intervals were minimally prolonged yet with the presence of other unique features such as prominent U waves, the designation ATS1 might be preferred to LQT7 (12). In contrast to this, other Kir2.1 mutations associated with CPVT3 that were functionally characterized demonstrated that decreased I_{K1} occurred with beta-adrenergic stimulation, and in 2009 our group published biophysical data showing PKA-dependent decrease in I_{K1} for the *KCNJ2* V227F mutation in a patient with CPVT3 (13).

In the current study we present unique clinical data of a family with a CPVT-like phenotype found to have a *KCNJ2* R67Q mutation. We characterized this *KCNJ2* mutation's

association with a beta-adrenergic dependent cellular loss of Kir2.1 function, as well as characterized the molecular mechanism underlying channel phosphorylation.

Methods

Clinical Presentation

The proband is a 33-year-old female with a 20-year history of stress-related syncope and ventricular arrhythmias. She was referred for evaluation to the University of Wisconsin Inherited Arrhythmias Clinic from an outside hospital (OSH). Her initial evaluations at an OSH included ECG, Holter monitor, exercise treadmill test. Imaging studies included an echocardiogram, coronary angiogram and a cardiac MRI, all of which were normal. Her resting 12-lead ECG demonstrated a normal QT interval with prominent U waves (Figure 1A). A Holter monitor revealed exercise related polymorphic ventricular ectopy, non-sustained polymorphic ventricular tachycardia and bidirectional ventricular ectopy (Figure 1B). She had no other arrhythmias, no history of periodic paralysis and on physical exam she had no dysmorphic features. The patient had undergone two unsuccessful ablations for PVC's at two separate OSHs. Gene-targeted sequencing was performed in a commercial laboratory (GeneDx) and included LQTS1–12, *RyR2*, *CASQ2*, and *KCNJ2*, and revealed a heterozygous missense mutation in *KCNJ2*, R67Q-Kir2.1. The patient was diagnosed with CPVT3. She was treated with nadolol and flecainide, and has not had further syncope. Subsequent ETT on nadolol and flecainide demonstrated complete suppression of ventricular ectopy. She does not participate in competitive sports and is pharmacologically restricted to heart rates less than 130 bpm. Because of her excellent response to medical therapy, an implantable cardioverter defibrillator (ICD) was not recommended. There was no family history of sudden cardiac death, ventricular arrhythmias, ICD or pacemakers, syncope, SIDS, congenital hearing loss, or seizure disorders. Available genotyping of her family members revealed that her 7-year-old son harbors the R67Q mutation. He has had no dysmorphic features, a normal ECG, exercise test without ventricular arrhythmias, and no dysmorphic features. He had 9 PVCs on Holter that suppressed at peak HR (190–200 bpm). Due to lack of symptoms or identifiable arrhythmia currently he has no exercise restrictions.

KCNJ2 construction and mutagenesis

Wild-type (WT) human Kir2.1 was isolated from human cardiac cDNA, and *KCNJ2* mutations were constructed using the Stratagene ExSite site directed mutagenesis, as previously described(11, 13). WT and mutant DNA was sub-cloned into mammalian expression vector pcDNA3.1 (Invitrogen) for electrophysiological experiments. HA tagged construct of Kir2.1 was a gift from Dr. A. George (Vanderbilt University). Myc tag was sub-cloned into Kir2.1 using the gBlock Gene Fragments technology (Integrated DNA technologies). The myc tag (ATG GCA TCA ATG CAG AAG CTG ATC TCA GAG GAG GAC CTG) sequence was inserted in between amino acids 115 and 116, the same location where the HA tag is in the Kir2.1 sequence (Ballester et al, 2006). All constructs were verified by sequence analysis.

Transfection and cell culture

COS-1 cells were cultured in DMEM (Invitrogen, Carlsbad, CA) with 10% FBS and then were transiently transfected with a total of 1.5 µg of DNA using FuGENE 6 reagent (Promega, Madison, WI) according to manufacturer specifications. The transfections contained WT-Kir2.1 cDNA alone, mutant-Kir2.1 cDNA alone or co-transfections of two different Kir2.1 cDNAs with total DNA content maintained as the same for each experiment. For all electrophysiological experiments, a green fluorescent protein (GFP) cDNA in a pMax vector (GFP-pMax, Lonza, Basel, Switzerland) was transfected along with Kir2.1 cDNA. Twenty-four hours after transfection, cells were transferred to cover slips.

Electrophysiological experiments

I_{K1} was recorded 48 hours after transfection using the ruptured patch whole cell technique (Hamill, 1981) at room temperature with an Axopatch-200B amplifier. Borosilicate glass pipettes were pulled to resistances of 2–4 MΩ. Successfully transfected cells were identified by GFP fluorescence under fluorescent microscope (Nikon). Bath solution contained (mM): NaCl 148, KCl 5.4, CaCl₂ 1.0, MgCl₂ 1.0, HEPES 15, NaH₂PO₄ 0.4, and D-glucose 5.5 (pH 7.4, NaOH). Pipette solution contained (mM): KCl 148, MgCl₂ 1, EGTA 5, Na₂ATP 5, HEPES 5, creatine 2, and phosphocreatine 5 (pH 7.2, KOH). From a holding potential of –80 mV, voltage clamp steps were applied from –120 to 30 mV in 10 mV increments for 500 ms. Data were filtered at 10 kHz and digitized using a Digidata 1440A (Axon Instruments). Current amplitude was normalized to cell capacitance. After recording in the control bath solution, the bath was immediately perfused with a PKA cocktail (PKA-CT) (100 µmol/L forskolin + 10 µmol/L 3-isobutyl-1-methylxanthine + 28 mmol/L DMSO). I_{K1} was then recorded again after perfusing with PKA-CT for at least 8 minutes. In a separate arm of experiments, cells were incubated in PKA-CT for 2 hours prior to recording currents and compared with cells incubated in control solution. All currents in the incubation experiments were recorded while perfusing the control solution. To assess the rectification properties of the channels, a rectification index was calculated. This index was defined as the ratio of the outward current at –60 mV divided by the absolute value of the inward current at –100 mV multiplied by 100(14). For a subset of experiments, 4.2 mM CaCl₂ was added to the above pipette solution. Based on the WEBMAXC standard calculator (<http://www.stanford.edu/~cpatton/CaMgATPEGTA-NIST-Plot.htm>), this provides a free calcium concentration of 1 µM in a cell free system. This solution is referred to as the high-calcium pipette solution in the results section. Analysis of data was done using pClamp 10 (Axon Instruments) and Origin 8.6 (OriginLab Corp.).

Immunostaining

Cells on coverslips were fixed 48 hours after transfection using 4% paraformaldehyde in 1X-PBS. Cells were permeabilized with 0.1% Triton-X-100 and then blocked with normal goat serum for one hour at room temperature (RT). Cells were then incubated with primary antibody overnight at 4°C. Anti-HA monoclonal (Covance) antibody and Anti-Myc polyclonal (Cell Signaling technology) antibody were used at 1:100 dilutions. The next day the cells were washed with PBS containing 0.05% Tween-20 (PBS-T) and incubated with fluorophore conjugated secondary antibodies for one hour at RT. We used Alexa 488 goat

anti-mouse and Alexa 567 goat anti-rabbit at 1:500 dilution from Life Technologies. DAPI (Life Technologies) was used to stain the nucleus. The cells were washed again in PBS-T and then mounted using ProLong Gold antifade reagent (Life technologies). Images of immunostained cells were captured on a Leica confocal microscope. Pearson's co-localization coefficient was calculated using ImageJ software.

Statistical Analysis

Data are expressed as mean $\pm$ S.E. unless otherwise specified. Data were analyzed using an unpaired Student's *t*-test calculated with Microsoft Excel. Values of $p < 0.05$ were considered significant.

All research has been reviewed and approved by the Institutional Review Board, University of Wisconsin, Madison.

Results

R67Q-Kir2.1 does not alter WT-Kir2.1

We previously reported that homomeric R67Q-Kir2.1 channels are non-functional and produce no measurable current, and that co-expression of R67Q-Kir2.1 with WT-Kir2.1 does not produce a dominant negative effect on I_{K1} (11). Figure 2 shows the voltage clamp protocol and example current traces for WT-Kir2.1, R67Q-Kir2.1, and co-transfected R67Q/WT-Kir2.1 along with current-voltage plots for these. WT-Kir2.1 transiently transfected into COS-1 cells demonstrated a typical inward-rectifier N-shaped current-voltage relationship with a maximal outward current at -60 mV, whereas R67Q-Kir2.1 did not generate current. For cells co-expressing R67Q/WT-Kir2.1 the current amplitude was reduced. At -60 mV current density was 7.7 ± 1.3 pA/pF compared to 14.0 ± 2.2 pA/pF for cells expressing WT alone ($p < 0.05$), a 45% reduction. The rectification properties of the R67Q/WT-Kir2.1 channels are similar to WT channels. The rectification indices for the R67Q/WT-Kir2.1 and WT-Kir2.1 were $36.5 \pm 5.7\%$ and $41.1 \pm 5.1\%$, respectively ($p > 0.05$).

R67Q-Kir2.1 alters WT-Kir2.1 Response to PKA Stimulation

In additional experiments, we used a PKA-activating cocktail (PKA-CT, 100 μ mol/L forskolin + 10 μ mol/L 3-isobutyl-1-methylxanthine) for beta-adrenergic stimulation in COS-1 cells transfected with either WT-Kir2.1 or R67Q/WT-Kir2.1. As shown in Figure 3A, perfusion with PKA-CT for at least 8 minutes resulted in an increase in outward current density for WT-Kir2.1. The peak outward current density (-60 mV) increased from 13.3 ± 2.1 pA/pF to 19.4 ± 3.7 pA/pF ($p = 0.14$). In R67Q/WT-Kir2.1, peak outward current density (-60 mV) decreased from 8.5 ± 1.3 pA/pF to 7.9 ± 1.2 pA/pF with PKA-CT (Figure 3B, $p = 0.49$). The relative changes, a 46% increase for WT-Kir2.1 compared to a 6% decrease for R67Q/WT-Kir2.1 were statistically different ($p = 0.002$).

Because the acute exposure to PKA-CT seemed to affect WT-Kir2.1 or R67Q/WT-Kir2.1 channel currents differently, we simulated the chronic effect of beta-adrenergic stimulation by incubating transfected cells for 2 hours in media containing PKA-CT and compared this

to cells in control media. As shown in Figure 4A, incubation with PKA-CT for 2 hours increased outward current for WT-Kir2.1 channels. At -60 mV, WT-Kir2.1 current density increased from 14.0 ± 2.2 pA/pF to 26.9 ± 5.0 pA/pF ($p < 0.05$). In contrast, for R67Q/WT-Kir2.1 incubation for 2 hours in PKA-CT did not affect the current. At -60 mV, current density was 7.7 ± 1.3 pA/pF for control conditions and 8.0 ± 1.2 pA/pF following incubation in PKA-CT (Figure 4B, $p = 0.91$).

The Mechanism of Adrenergic Response is related to S425 Phosphorylation

Kir2.1 contains a known PKA consensus motif for channel phosphorylation involving the serine at position 425, which has been shown to regulate PKA effects on I_{K1} (15). To assess the molecular mechanism of the PKA-CT response in these experiments, we generated this phosphorylation deficient mutants (13). Using constructs S425N-Kir2.1 and R67Q-S425N-Kir2.1/S425N-Kir2.1 transfected into COS-1 cells, currents were recorded using the same voltage clamp protocol as described above. The current densities measured in the S425N mutant under control conditions were similar to the WT-Kir2.1: at -60 mV current density was 15.4 ± 2.2 pA/pF in the S425N-Kir2.1 mutant compared to 14.0 ± 2.2 pA/pF for WT-Kir2.1 (Figure 4C, $p = 0.68$); and 6.9 ± 1.2 pA/pF in R67Q-S425N-Kir2.1/S425N-Kir2.1 mutant compared to 7.7 ± 1.3 pA/pF in R67Q-Kir2.1/WT-Kir2.1 (Figure 4D, $p = 0.68$). Following incubation with PKA-CT for 2 hours, the current density at -60 mV in S425N-Kir2.1 was similar to baseline, 13.5 ± 2.4 pA/pF (Figure 4C, $p = 0.59$), as was that in R67Q-S425N-Kir2.1/S425N-Kir2.1, 6.6 ± 2.9 pA/pF (Figure 4D, $p = 0.92$). These results suggest that the response to PKA-CT is dependent on S425 phosphorylation.

Calcium differentially affects R67Q-Kir2.1

Divalent cations alter the rectification properties of Kir2.1 channel current(16). Additionally, a mutation that alters Kir2.1 sensitivity to PIP_2 has been shown to exaggerate the inhibition of Kir2.1 by Mg^{2+} (14). Finally, CPVT is considered mediated and dependent on Ca_2 overload for arrhythmia induction(3, 17). Therefore, we hypothesized that the R67Q-Kir2.1 mutation found in our patient may alter the rectification properties of Kir2.1 when exposed to calcium.

To test this hypothesis, we measured I_{K1} in cells incubated in control solution or PKA-CT for 2 hours using pipette solution with either high or low-calcium (as specified in the Methods section). The results of these experiments are shown in Figure 5. The rectification index (RI) of WT-Kir2.1 under control conditions was $41.1 \pm 5.1\%$ and did not change significantly with high-calcium in the pipette solution ($40.8 \pm 7.6\%$, $p = 0.97$). After incubation in PKA-CT, there was a statistically significant increase in RI for WT-Kir2.1, to $61.9 \pm 8.1\%$. With the high-calcium pipette solution and PKA-CT incubation, RI also increased for WT-Kir2.1, to $54.8 \pm 7.5\%$, but it did not reach significance ($p = 0.15$). R67Q/WT-Kir2.1 expressing cells had a RI of $36.5 \pm 5.7\%$ under control conditions. As stated above, this was not statistically different than WT-Kir2.1 ($p = 0.57$). In contrast, R67Q/WT-Kir2.1 when studied with the high-calcium pipette solution resulted in a reduction in RI, from $36.5 \pm 5.7\%$ to $20.3 \pm 4.1\%$ ($p < 0.05$). Likewise the R67Q/WT-Kir2.1 cells incubated in PKA-CT for 2 hours and studied with the high-calcium pipette solution, had a reduced RI of $21.3 \pm 2.5\%$ ($p = 0.05$). Thus, the presence of high calcium results in a

reduction of the RI, a marker of repolarization capability, only in the presence of the R67Q-Kir2.1 mutation and not WT-Kir2.1. In CPVT, which is considered dependent on Ca^{2+} overload for arrhythmia induction, this may confer unique arrhythmic capabilities of the R67Q mutation.

R67Q-Kir2.1 and WT-Kir2.1 Exhibit Normal trafficking and Colocalization

In order to differentiate WT-Kir2.1 from two known mutations in the R67 position, R67Q-R67W-Kir2.1, we tagged WT-Kir2.1 with Myc-Tag and R67Q and R67W-Kir2.1 with HA-Tag. Single transfections were done to determine the pattern of expression and to test the antibodies (Figure 6). The co-localization of WT-Kir2.1 with R67Q- or R67W-Kir2.1 in combination (Figure 7) was quantified using Pearson's correlation coefficient and ImageJ software, where values range between -1.0 and 1.0 and values >0.5 indicate significant correlation. The calculated Pearson values for HA-WT-Kir2.1 with Myc-WT-Kir2.1 was 0.88 ($n=7$), HA-R67Q-Kir2.1 with Myc-WT-Kir2.1 was >0.92 ($n=6$) and HA-R67W-Kir2.1 with Myc-WT-Kir2.1 was >0.5 ($n=5$). This pattern indicates that R67Q- and R67W-Kir2.1 mutants do not affect the pattern of localization of WT-Kir2.1 and that the colocalization of R67Q or R67W is present with WT-Kir2.1 suggesting heterotetrameric channels.

Discussion

In this study we present a patient with exertion-induced PMVT, bidirectional VT and syncope harboring a Kir2.1 mutation R67Q. This mutation significantly differs from WT Kir2.1 with an adrenergic dependent loss of function despite normal surface expression of the protein. Furthermore, we have demonstrated that the mechanism of loss of function is dependent on phosphorylation of the Kir2.1 C-terminal phosphorylation site, S425 and that there is a significant affect on the RI in the presence of higher Ca^{2+} only in the presence of the R67Q mutation. We propose that the phosphorylation of S425 in the presence of the R67Q mutation induces functional loss of repolarization reserve leading to arrhythmogenesis. This phenotype-genotype relationship advances our understanding of the diverse arrhythmia syndromes associated with *KCNJ2* mutations.

Biophysical Phenotype

Unlike WT-Kir2.1 that showed increased I_{K1} in response to PKA stimulation, the combination of WT-Kir2.1 with R67Q-Kir2.1 showed no difference in baseline I_{K1} rectification properties, but failed to show the increase in I_{K1} with PKA stimulation. Mutating the known PKA-phosphorylation site at S425 (15) in Kir2.1 showed that the mechanism of this effect is due failure of the phosphorylated channel to increase outward current as in the WT state.

The mechanism of CPVT-related arrhythmias is considered to be dependent on a calcium overload state (3, 17). In this study we evaluated the affect of high and low calcium on both WT-Kir2.1 and R67Q/WT-Kir2.1 by changing the concentration of calcium in the pipette solution. The presence of the R67Q mutation affects the rectification profile of the channel and this is enhanced with high pipette calcium. The arrhythmic correlation to this finding

could thus be that in a high cell calcium state, the channel has even further hindrance to repolarization, leading to DAD's and subsequent ventricular arrhythmia, as seen by our patient.

Additionally, the confocal imaging shows that the WT-Kir2.1 and R67Q-Kir2.1 are present in the same membrane location, suggesting that the proteins associate as heterotetramers. It is interesting to note that an ATS-related *KCNJ2* mutation R67W, demonstrated a dominant negative phenotype at baseline(18). We also performed confocal imaging with R67W-Kir2.1 with WT-Kir2.1 and found a similar result with normal membrane trafficking and colocalization, suggesting heterotetrameric association. What creates this apparent biophysical change between these two amino acid switches is not clear. It is important to note that Andelfinger, et al. studied R67W-Kir2.1 in *Xenopus oocytes*, which may have different biophysical properties than studying this in a mammalian cell. Alternatively, the change in our patient's amino acid sequence varied in the 67 position from a positively charged arginine (R) to uncharged side chain glutamine (Q). This may have incurred a unique protein folding or cellular interaction characteristics in comparison with the arginine to tryptophan (W), which is hydrophobic amino acid with large aromatic ring. The potential protein conformational change with these two different amino acid substitutions will be studied more thoroughly in future modeling experiments in our laboratory.

Molecular, Cellular, and Phenotype Controversy

The physiologic result of this mutation is that only under adrenergic stimulation, myocytes expressing the R67Q mutations will have reduced control over repolarization and inward rectification. The loss of repolarization capability is increased with higher cell calcium, with the overall effect of increasing arrhythmia potential.

This is an important finding for phenotypic disease characterization. The signature arrhythmia of CPVT, bi-directional VT is induced with exercise or high adrenergic states such as fright. In contrast the bi-directional VT reported in ATS1 is suppressed at peak exercise (19). Kir2.1 mutations that have been "associated" with CPVT include R82W and V227F (4), R67W and C101R(5), G144D and T305S(6) and R260P(7). Some of these cases have been referred to with the diagnosis of CPVT(4) or ATS with polymorphic VT(5) or as "phenocopies" of CPVT(20). It is debatable if *KCNJ2* related arrhythmia syndromes are a spectrum of ATS1 mimicking the CPVT phenotype or if they are true CPVT with a worse clinical outcome and with different underlying molecular and cellular mechanisms. Another interpretation is that arrhythmia syndromes associated with *KCNJ2* mutations have been unintentionally misclassified for lack of a better definition for these syndromes. Disease classification and phenotypic characterization is imperative since this affects the treatment plan, risk stratification and even device therapy(21).

From a functional characterization standpoint, most ATS1 mutations exhibit a dominant-negative Kir2.1 current suppression when co-expressed with WT Kir2.1. Both V227F and R67Q had an atypical molecular phenotype in that they showed no dominant-negative effect but following adrenergic stimulation markedly reduced outward I_{K1} . Moreover, eliminating PKA phosphorylation by inserting the S425N mutation abrogated the change in I_{K1} . The two calcium handling proteins associated with CPVT, ryanodine receptor and calsequestrin, also

require adrenergic stimulation for arrhythmic induction and CPVT has been considered to be critical to cellular calcium [Ca^{2+}] overload(2, 3, 17). While there is a similar dependence on adrenergic stress to elicit these pathognomonic arrhythmias, it is unclear if in the Kir2.1 related syndromes there is a dependence on Ca^{2+} overload. The mechanisms at play have not been fully elucidated in RYR2 related syndromes since in the RYR2^{R4496C/+} transgenic mouse blockade of Ca^{2+} transients and sparks was not determined not to be the antiarrhythmic mechanism, but rather flecainide sodium channel blockade which was the primary target for effective arrhythmia suppression(22). An alternative mechanism is related to the observation that flecainide, via a cysteine residue at position 311, has been shown to decrease Kir2.1 channel affinity for intracellular polyamine blockade, which functionally results in an increase in outward current(23). This, in essence, corrects the effect that the R67Q mutation induces and, thus, flecainide's clinical action may also be directed on Kir2.1 channels. We propose that this unique molecular phenotype with direct adrenergic-dependence on loss of I_{K1} might underlie a unique clinical phenotype but is best managed and treated as clinical CPVT.

Cellular Characterization to Understanding Clinical Phenotype?

In this particular case, the mechanism for managing the patient's clinical phenotype was in large part based on her clinical phenotype of exercise induced syncope (24). However, characterization of the cellular phenotype abnormality as dependent on adrenergic stimulation allowed us better ability to guide treatment and lifestyle changes, such as exercise restriction, drug compliance, avoidance of sympathomimetic agents (caffeine) and long-term surveillance with exercise testing (21, 25, 26). Gene positive family members have also been restricted from vigorously competitive sports and beta-blocker treatment will be initiated if symptoms develop.

Conclusions

These findings are a significant advancement of our knowledge and understanding of phenotype-genotype relationship of arrhythmia syndromes related to *KCNJ2* mutations. We propose that this detailed investigation will lead to improved classification, diagnosis, and treatment of these and other inherited arrhythmic syndromes.

Limitations

All experiments were performed as heterologous expression, in order to compare to previously published work with *KCNJ2* mutations. We recognize the challenges of translating these findings from a non-myocyte model to a human myocytes. Currently, we are studying these mutations in human induced-pluripotent cardio myocytes (iPS-CM), which may allow for additional physiologic relevance. However, given that the majority of the work in this field has been with heterologous expression, interesting comparisons are more easily made with the heterologous model. We look forward to expanding this exciting work into iPS-CMs to further our understanding of this disease mechanism.

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List of Abbreviations

CPVT	Catecholaminergic Polymorphic VT
LQTS	LQTS Long QT Syndrome
ATS	Andersen-Tawil Syndrome
PMVT	Polymorphic VT
BiVT	Bidirectional VT
RI	Rectification Index
WT	Wild type
PKA-CT	Protein kinase A stimulating cocktail
OSH	Outside Hospital

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Figure 1. ECG and Holter recordings from Proband

A. Baseline ECG from a 33-year-old female harboring *KCNJ2* R67Q mutation. The ECG shows a normal QTc (430 msec). There are prominent U waves, seen best in lead V2, with a QUc 620 msec. **B.** Holter monitor from the same patient demonstrates frequent polymorphic ventricular ectopy (top panel) and ventricular ectopy with bi-directional and polymorphic qualities (lower panel) both occurring during exercise.

Figure 2. R67Q-Kir2.1 lacks dominant negative effect when co-expressed with WT-Kir2.1
 Schematic of protocol used to measure currents and representative Kir2.1 current density traces are illustrated in the left panel: WT-Kir2.1 (top), R67Q-Kir2.1 (middle), R67Q/WT-Kir2.1 (bottom). Dotted line indicates 0 pA, scale bar represents 50 ms and 10 pA/pF. The right panel shows baseline current voltage (I-V) relationship for WT-Kir2.1 (filled squares; n=9), R67Q-Kir2.1 alone (filled triangles, n=3), and co-expressed R67Q/WT-Kir2.1 (filled triangles, n=10). Homomeric R67Q-Kir2.1 channels do not produce current while co-expressed WT and R67Q-Kir2.1 channels produce typical I_{K1} . The current reduction is as expected as the total WT-Kir2.1 DNA is 50% less in the co-expression experiments. * denotes $p < 0.05$ by Student's t -test.

Figure 3. Response of R67Q/WT-Kir2.1 to PKA-cocktail differs from WT-Kir2.1

A. Acute application of PKA-CT to WT-Kir2.1. For each cell, I_{K1} was recorded after 8 minutes of perfusion with control bath solution (filled squares, $n=5$). PKA-CT was then perfused and Kir2.1 was recorded again after another 8 minutes (open squares, $n=5$). B. Contemporaneously, this experiment was repeated with co-expressed R67Q/WT-Kir2.1 (control filled triangles, $n=6$; PKA-CT open triangles, $n=6$). After application of PKA-CT, outward currents in the physiologically significant range (see inset) increased in WT cells ($p=0.06$ at -50 mV) but decreased in cells with co-expressed Kir2.1. Insets highlight the current at physiologically significant potentials. C. Example current density traces in all experiment conditions are shown below. Dotted line indicates 0 pA, scale bar represents 50 ms and 10 pA/pF.

Figure 4.

A,B. R67Q-Kir2.1/WT-Kir2.1 fails to have a typical WT-Kir2.1 outward current increase following incubation with PKA-CT. I-V plots obtained under control conditions or after 2 hour incubation with PKA-CT. A. WT-Kir2.1 (control filled squares, n=9; PKA-CT open squares, n=6). B. R67Q/WT-Kir2.1 (control filled triangles, n=10; PKA-CT open triangles, n=6). **C and D. The increase in outward currents with PKA-CT is phosphorylation dependent.** I-V plots obtained under control conditions or after 2 hours of incubation with PKA-CT using phosphorylation deficient constructs (S425N mutants). C. S425N-Kir2.1 (control filled squares, n=7; PKA-CT open squares, n=5). D. R67Q-S425N-Kir2.1/S425N-Kir2.1 (control filled triangles, n=7; PKA-CT open triangles, n=5). Insets highlight the current at physiologically significant potentials.

Figure 5. R67Q-Kir2.1 affects channel rectification index and may alter channel sensitivity to calcium

COS-1 cells expressing either WT-Kir2.1 (left hand portion of figure) or R67Q/WT-Kir2.1 (right hand portion) were incubated for 2 hours in control bath solution or PKA-CT containing solution. I_{K1} was then measured using a pipette solution with no added Ca^{2+} (low Ca^{2+}) or a pipette solution containing 4.2 mmol Ca^{2+} (high Ca^{2+}). A rectification index (RI) was then calculated by dividing the value of the outward current at -60 mV by the absolute value of the current at -100 mV then multiplying by 100(14). * denotes $p < 0.05$ by Student's t -test.

Figure 6. Pattern of localization of Myc-WT-Kir2.1 (red), HA-R67Q-Kir2.1 (green) and HA-R67W-Kir2.1 (green) transiently transfected in COS-1 cells
 Wild-type (a, b and c) and mutant (R67Q (d, e and f); R67W (g, h and i)) Kir2.1 both show very similar pattern of localization 1) punctuate pattern and 2) edge of the cell. DAPI staining was used to identify the nucleus. Scale bar = 25 μm .

Scale bar = 25 μm

Fig 7. Pattern of co-localization of Myc-WT-Kir2.1 (red) with HA-WT-Kir2.1 or HA-R67Q-Kir2.1 (green) or HA-R67W-Kir2.1 (green) in COS-1 cells

COS-1 cells show very similar pattern of co-localization of Myc-WT-Kir2.1 (c, g and h) with that of HA-WT-Kir2.1 (b) or HA-R67Q-Kir2.1 (f) or HA-R67W-Kir2.1 (j) in the staining pattern. Co-localization was confirmed by Pearson's correlation coefficient of >0.5 indicating significant co-localization, also evident from the merged panels (d, h and l). DAPI staining was used to identify the nucleus. Scale bar = 25 μm .