Protein Kinase A–Dependent Biophysical Phenotype for V227F-KCNJ2 Mutation in Catecholaminergic Polymorphic Ventricular Tachycardia

Amanda L. Vega, PhD; David J. Tester, BS; Michael J. Ackerman, MD, PhD; Jonathan C. Makielski, MD

Background—KCNJ2 encodes Kir2.1, a pore-forming subunit of the cardiac inward rectifier current, I K1. KCNJ2 mutations are associated with Andersen-Tawil syndrome and catecholaminergic polymorphic ventricular tachycardia. The aim of this study was to characterize the biophysical and cellular phenotype of a KCNJ2 missense mutation, V227F, found in a patient with catecholaminergic polymorphic ventricular tachycardia.

Methods and Results—Kir2.1-wild-type (WT) and V227F channels were expressed individually and together in Cos-1 cells to measure I K1 by voltage clamp. Unlike typical Andersen-Tawil syndrome–associated KCNJ2 mutations, which show dominant negative loss of function, Kir2.1WT+V227F coexpression yielded I K1 indistinguishable from Kir2.1-WT under basal conditions. To simulate catecholamine activity, a protein kinase A (PKA)-stimulating cocktail composed of forskolin and 3-isobutyl-1-methylxanthine was used to increase PKA activity. This PKA-simulated catecholaminergic stimulation caused marked reduction of outward I K1 compared with Kir2.1-WT. PKA-induced reduction in I K1 was eliminated by mutating the phosphorylation site at serine 425 (S425N).

Conclusions—Heteromeric Kir2.1-V227F and WT channels showed an unusual latent loss of function biophysical phenotype that depended on PKA-dependent Kir2.1 phosphorylation. This biophysical phenotype, distinct from typical Andersen-Tawil syndrome mutations, suggests a specific mechanism for PKA-dependent I K1 dysfunction for this KCNJ2 mutation, which correlates with adrenergic conditions underlying the clinical arrhythmia. (Circ Arrhythmia Electrophysiol. 2009;2:540-547.)

Key Words: K-channel ■ arrhythmia (mechanisms) ■ long QT syndrome ■ Andersen-Tawil syndrome ■ catecholaminergic polymorphic ventricular tachycardia

The gene KCNJ2 gene encodes the pore-forming subunit of the human inwardly rectifying potassium channel Kir2.1, which underlies the inward rectifier potassium current, I K1.1 Autosomal dominant loss-of-function mutations in KCNJ2 represent the solely identified cause thus far for the heritable arrhythmia syndrome called Andersen-Tawil syndrome (ATS),2 whereas gain-of-function mutations in KCNJ2 have been implicated in the pathogenesis of short-QT syndrome (SQT3)3 and familial atrial fibrillation.4 Classically, ATS presents with a triad of cardiac arrhythmia and prolonged QU intervals, dysmorphic features, and periodic paralysis. Phenotype severity among ATS patients varies,5 however, with some patients presenting with only 1 symptom and other genotype-positive individuals completely nonpenetrant.

Clinical Perspective on p 547

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited arrhythmia syndrome characterized by adrenergically mediated bidirectional or polymorphic ventricular tachycardia that causes syncope and sudden cardiac arrest in the absence of structural heart defects among the young.6 Approximately 50% to 60% of CPVT patients harbor mutations in 1 of 2 critical calcium handling genes: the cardiac ryanodine receptor (RYR2)7 or calsequestrin-2 (CASQ2),8,9 whereas the remaining 40% have no known genetic cause.10 Ventricular arrhythmias in ATS have demonstrated an uncanny similarity to the ventricular arrhythmias observed in CPVT such as bidirectional tachycardia and exercise-induced arrhythmia.5 This can be a source of diagnostic confusion with CPVT. Previously, we identified mu-
tations in KCNJ2 among 3 of 11 unrelated patients that were clinically diagnosed with CPVT and suggested phenotypic mimicry. Two patients had R82W, a mutation previously found in a long-QT syndrome cohort and showing a dominant-negative loss of function typical of ATS. The other was a previously uncharacterized, missense mutation in KCNJ2 causing the valine at position 227 to be substituted by phenylalanine in Kir2.1 (Kir2.1-V227F).

Here, we characterize the biophysical and cellular phenotypes of Kir2.1-V227F and show that when coexpressed with wild-type channels (Kir2.1-WT), the mutation caused no abnormal phenotype under control conditions. However, protein kinase A (PKA) activation, a cellular consequence of adrenergic stimulation, caused a latent loss of function biophysical phenotype that was abrogated by a mutation that eliminated phosphorylation at S425 on Kir2.1. This phenotype, distinct from typical ATS mutations, provides a new specific mechanism for PKA dependence of I\(_K1\) dysfunction for this KCNJ2 mutation in a CPVT patient.

Methods

Mutagenesis

The Kir2.1-V227F, Kir2.1-S425N, and Kir2.1-AAA (AAA for pore GYG) mutations were introduced into the human Kir2.1 using a Quik Change Site-Directed Mutagenesis kit (Stratagene). The following primer pairs were used to mutate the targeted sites in the cDNA:

- V227F-F, TTGTGGGAAGCTCTTTCGAGACACGCCTCTC;
- V227F-R, CAGAACGCTGTGTGCTCGAAATGAAGCTTCCACCAA;
- S425N-F, CGCCGAGAACGAGATATGAAAGG;
- S425N-R, GCCCTTCTCATATCTGCTTCGC(GCG);
- AAA-F, CAGACAACATAGCCGCTGCCTTCAGATGTGTC;
- AAA-R, GACACATCGAAGCGCAGCGCTATGGTTATGGTCTG.

The S425N mutation was also introduced into the cDNA of both Kir2.1 WT and Kir2.1-V227F. Mutants were generated using the protocol outlined by the manufacturer (Stratagene). DNA integrity was verified by sequencing.

Cos-1 Transfection and Culture

Cos-1 cells were transfected (Supercfect Transfection Kit, Qiagen) with Kir2.1 cDNA and cultured in modified Dulbecco modified Eagle’s medium, as previously described.

Electrophysiology

I\(_{K1}\) was recorded from Cos-1 cells using an Axopatch 200B amplifier (Axon Instruments) as previously described. The protocol for recording I\(_{K1}\) from Cos-1 cells was to hold the cell at a potential of −70 mV and run a step protocol from −140 mV to +40 mV in 20 mV increments for 100 ms. The extracellular solution contained (in mM) 140 NaCl, 5.4 KCl, 1.8 CaCl\(_2\), 0.5 MgCl\(_2\), 5 HEPES, 0.33 NaH\(_2\)PO\(_4\), 5.5 glucose, and the pH adjusted to 7.4. The microelectrodes were created from borosilicate glass capillaries that after fire polishing had a resistance of 1.0 to 2.1 MΩ when filled with internal solution. The internal solution contained (in mM) 30 KCl, 85 K-aspartate, 5 MgCl\(_2\), 10 KH\(_2\)PO\(_4\), 2 K\(_2\)EGTA, 2 K\(_2\)ATP, 5 HEPES, 0.33 NaH\(_2\)PO\(_4\), 5.5 glucose, and the pH adjusted to 7.2. All recordings were performed at room temperature.

A PKA cocktail (100 μmol/L forskolin + 10 μmol/L 3-isobutyl-1-methylxanthine; Sigma-Aldrich) was prepared in DMSO and diluted in bath solution. After successful whole-cell access I\(_{K1}\) was recorded in control bath solution and the bath was immediately superfused with PKA cocktail bath solution for 5 minutes. I\(_{K1}\) was recorded again in the presence of the PKA cocktail and after the PKA cocktail was washed out over a 10-minute period. All currents returned to basal levels after complete washout of PKA cocktail. In a set of parallel experiments, the cells were perfused for 2 hours with the PKA cocktail and compared with cells in control media.

Statistical Analysis

The key biological questions asked is whether current density at −40 mV and −60 mV (the physiological range of importance) were affected by the mutation or by exposure to PKA. Data are expressed as mean±SEM and analyzed using an unpaired Student t-test. Where indicated, a 1-way ANOVA was used to compare 3 means among different channel types. Values of P≤0.05 were considered significant.

Results

A CPVT Patient With Kir2.1-V227F

Previously, we identified the mutation Kir2.1-V227F in a female patient referred for CPVT genetic testing. The 32-year-old white female was genotype-negative for mutations in the 2 CPVT-susceptibility genes (RYR2 and CASQ2) and also negative for the principal long-QT syndrome susceptibility genes (LQT1-6). The patient was heterozygous for a missense mutation in the ATS-susceptibility gene KCNJ2 annotated as Kir2.1-V227F. The proband had no evidence of periodic paralysis or dysmorphism to suggest ATS. Instead, the proband had a history of exertion/emotion-triggered syncope and presyncope that had been documented since the age of 2 years. The subject also had palpitations, ventricular bigeminy, ventricular couplets, 3-beat runs of polymorphic ventricular tachycardia suggestive of bidirectional ventricular tachycardia, and nonsustained ventricular
tachycardia (Figure 1B). The proband had a defibrillator implanted and at last follow-up was taking oral bisoprolol (5 mg) to treat cardiac symptoms. The maternal family history and progeny is unremarkable, but they have not been genotyped. Paternal history is unavailable.

Kir2.1-V227F Mutation Has a Distinct Molecular Phenotype for IK1
Expression of Kir2.1-V227F in Cos-1 cells formed functional pores in contrast to the majority of ATS-associated Kir2.1 mutant channels that show negligible current. Inward $I_{K1}$ for Kir2.1-V227F was significantly reduced by $\approx 40\%$ in comparison to Kir2.1-WT, as shown by the current traces (Figure 2A) and summary peak current-voltage plots (Figure 2B). Over the physiological range of terminal repolarization ($\approx -40mV$ to $-60mV$), outward $I_{K1}$ was decreased 85% to 99% in comparison to Kir2.1-WT (Figure 2B inset).

Kir2.1-V227F and Kir2.1-WT Interact to Form Multimeric Pore
Inward rectifier potassium channels are formed by the assembly of 4 subunits allowing for heteromeric pores containing both WT and mutant subunits. The patient was heterozygous with both a mutant V227F allele and a WT allele, so we examined heteromeric channels by coexpression studies. In contrast to most ATS-associated Kir2.1 mutations that show a dominant negative decrease in $I_{K1}$, this mutation reduced $I_{K1}$ from heteromeric channels produced by coexpression of Kir2.1-V227F with Kir2.1-WT was statistically indistinguishable from Kir2.1-WT (Figure 2). To exclude the possibility that this normal appearing $I_{K1}$ was caused by the failure of Kir2.1-V227F to interact with Kir2.1-WT, we used a dominant-negative approach as previously described. We introduced the dominant-negative mutation, AAA, to remove the potassium selectivity filter (GYG) in both WT and mutant cDNA. Expression of Kir2.1-AAA alone yielded no potassium currents (Figure 3), as expected. Expression of Kir2.1-AAA with Kir2.1-WT or Kir2.1-V227F both caused a strong dominant-negative reduction of $I_{K1}$. These results support the idea that Kir2.1-V227F interacted with Kir2.1-WT to form a multimeric pore.

Abnormal Phenotype Depends on PKA Activation
The normal $I_{K1}$ phenotype of Kir2.1-WT+Kir2.1-V227F under basal conditions did not leave a plausible explanation for the arrhythmia phenotype observed in the patient. The possibility of V227F being a rare variant was considered, but the mutant was absent from more than 800 reference alleles.10

Kir2.1-WT or Kir2.1-V227F both caused a strong dominant-negative reduction of $I_{K1}$. These results support the idea that Kir2.1-V227F interacted with Kir2.1-WT to form a multimeric pore.

Abnormal Phenotype Depends on PKA Activation
The normal $I_{K1}$ phenotype of Kir2.1-WT+Kir2.1-V227F under basal conditions did not leave a plausible explanation for the arrhythmia phenotype observed in the patient. The possibility of V227F being a rare variant was considered, but the mutant was absent from more than 800 reference alleles.10

Kir2.1-WT or Kir2.1-V227F both caused a strong dominant-negative reduction of $I_{K1}$. These results support the idea that Kir2.1-V227F interacted with Kir2.1-WT to form a multimeric pore.
Also, the homomeric V227F currents were reduced (Figure 2), supporting the possibility of a latent pathological defect. For CPVT, increased catecholamine activity is a characteristic clinical feature for arrhythmia. Therefore, we used a PKA-activating cocktail (100 μmol/L forskolin + 10 μmol/L 3-isobutyl-1-methylxanthine) in the Cos-1 cells to mimic adrenergic stimulation. IK1 was measured before and after 5 minutes of exposure to PKA activation. PKA activation caused a significant reduction \((P<0.05)\) of inward \(I_{K1}\) for Kir2.1-WT (35%), Kir2.1-V227F (35%), and Kir2.1-WT+Kir2.1-V227F (56%) in comparison to basal conditions (Figure 4). Outward \(I_{K1}\) in the physiological range is of particular interest. In WT channels, PKA caused an increase in outward \(I_{K1}\) (Figure 4B inset), whereas in the heteromeric channels outward \(I_{K1}\) was decreased by 73% and 68% (at -60 mV and -40 mV respectively; Figure 4D inset). The vehicle control solution (DMSO 1%) and timed control experiments (where no solution was added) showed no significant effects on \(I_{K1}\) (data not shown). The effects on \(I_{K1}\) were reversible after a 10-minute washout (data not shown). After longer exposure to PKA stimulation (2 hours) the outward \(I_{K1}\) of Kir2.1-WT channels was not affected, but heteromeric channels showed (Figure 5) a significant decrease in agreement with experiments after 5 minutes of exposure.

**Mutation of Serine 425 on Kir2.1 Abrogates PKA Effect**

We hypothesized that the PKA-induced effects on \(I_{K1}\) density were mediated by a direct phosphorylation of the channel. The single known PKA consensus motif, S425 on Kir2.1, has been shown to regulate PKA effects on \(I_{K1}\). We mutated S425 to an asparagine (S425N) in both Kir2.1-WT and Kir2.1-V227F to prevent phosphorylation. The introduced mutation had no impact on Kir2.1-WT function in the absence of PKA (data not shown) and prevented PKA-mediated effects on homomeric Kir2.1-WT channels (Figure 6A and 6B) in agreement with previously published results. The S425N mutation abrogated the PKA-mediated inhibition of \(I_{K1}\) for heteromeric WT and V227F channels when it was...
IK1 depended on PKA activation (Figure 4).

A “Latent” PKA-Dependent Dysfunctional Biophysical Phenotype for Kir2.1-V227F

The predominant molecular phenotype for KCNJ2 mutations associated with ATS is a dominant-negative decrease in IK1 when expressed with WT subunits (see References 5 and 12 for reviews). Kir2.1-V227F showed no effect on IK1 when expressed with WT subunits (Figure 2) and joins only R67Q12 and P351S15 as examples of KCNJ2 mutations identified from cardiac arrhythmia patients that have a “WT-like phenotype” when expressed in heterologous systems (Figure 2). Mindful of the clinical CPVT presentation, we tested the hypothesis that a β-adrenergic input as mimicked by PKA stimulation would induce channel dysfunction of heteromeric Kir2.1-WT and V227F channels. We found a latent PKA-dependent pathological phenotype in which a profound loss of outward IK1 depended on PKA activation (Figure 4).

Environmental and regulatory factors have been previously reported to trigger or exacerbate inherited arrhythmia. For example, increased temperature exacerbated an already abnormal biophysical phenotype in Brugada syndrome16,17 and in LQT2.18 More germane to our case, PKA stimulation has been reported to worsen the phenotype for mutant CPVT RYR2 channels.19 It is unusual, however, for a channel with an arrhythmia causing mutation to exhibit a completely WT phenotype. Such a latent pathogenic biophysical phenotype has a precedent in the cardiac sodium channel, where low pH was required to elicit an abnormal late current in sudden infant death syndrome.20 Kir2.1-V227F provides an unusual example of a mutation that depends on PKA-dependent phosphorylation to manifest a latent and potentially pathogenic phenotype.

β-Adrenergic Modulation of IK1 in Heart

Heteromeric Kir2.1-WT+Kir2.1-V227F channels expressed in Cos-1 had significantly reduced function in the presence of PKA (Figure 4) that depended on phosphorylation at S425 (Figure 6). The few reports available for PKA effects on Kir2.1-WT channels have contradictory results including activation after application of PKA in Xenopus oocytes22 and CHO cells,23 no effect in studies of bovine pulmonary artery endothelial cells,24 and inhibition in Cos-7 cells.14 Our results support observations made by Wischmeyer et al,14 possibly because the cell model and experimental approach were similar. Of the 4 members of the Kir2.x family, 3 (Kir2.1, Kir2.2, Kir2.3) are known to be expressed in the human heart25 and underlie cardiac ECG interval34 but no transmural dispersion of repolarization. In native tissue, isoproterenol or PKA has been reported to decrease IK1 in canine Purkinje fibers27 and guinea pig28 and human29 ventricular myocytes and to have no effect on rat30 and bull-frog atrial cells.31

I-K1 Adrenergic/Ca2+-Mediated Arrhythmia and CPVT

Decreased I-K1 is postulated to play a role in Ca2+-dependent and triggered arrhythmia, based on studies in a rabbit model of heart failure.32 Loss of I-K1 results in membrane “destabilization” caused by a reduction in outward current opposing pathogenic transient inward currents. A computer simulation study of decreased I-K1 showed prolonged action potentials, depolarized resting membrane potential, and early afterdepolarizations, with delayed afterdepolarizations emerging after simulated β-adrenergic activity.33 A computer simulation study of the ATS mutation D71V in KCNJ2 showed prolonged QT interval36 but no transmural dispersion of repolarization. In addition, the canine arterial wedge preparation model using barium to block I-K1 failed to generate early afterdepolarizations, dispersion of repolarization, or sustained arrhythmia despite provocation with isoproterenol and low K+.35 but, in a similar model, I-K1 block by Cs+ caused delayed afterdepolarizations and ventricular tachycardia was eliminated by verapamil.36 Together these results support a role generally for I-K1 in adrenergically mediated Ca2+-dependent arrhyth-
mia. Our results with V227F suggest a special direct role of adrenergic stimulation for this mutation. We cannot, however, on the basis of this single patient, say how this novel biophysical phenotype affects the clinical phenotype. For example, other mutations that show a dominant negative pattern such as R82W also are adrenergic dependent. Adrenergic effects outside of the channel (eg, on calcium handling) may in combination with a fixed Kir2.1 deficit make the arrhythmia adrenergic dependent. The adrenergic dependence of the clinical phenotype of Kir2.1 mutations is likely to represent a spectrum.

**Kir-V227F: ATS1 or CPVT3?**

The patient presented with a diagnosis of CPVT and had clinical features of CPVT and none for ATS aside from arrhythmia. Approximately 50% to 60% of CPVT cases are caused by mutations in either RYR2 or CASQ2, whereas the remaining 40% have are genotype negative. Genetic screening of patients diagnosed with CPVT and genotype negative for RYR2 or CASQ2 have identified mutations in KCNJ2, a gene that has been previously well established as a cause of the pleiotropic ATS. The discovery of KCNJ2 mutations in CPVT patients has been described as phenotypic mimicry and considered within the variable presentation of ATS. The latent biophysical phenotype of Kir2.1-V227F, however, poses a possibly distinct mechanistic classification. Unlike other ATS mutations in which the defect is always present, the abnormal phenotype would be predicted to be present only transiently under conditions of adrenergic stress. It can be speculated that a transient defect might preclude development of dysmorphic features characteristic of ATS that presumably requires a fixed loss of function during development. Moreover, a permanent defect might be more likely to promote compensatory mechanisms that would ameliorate the defect. Resolving these questions of mechanism and classification will require additional clinical evidence.

---

**Figure 6.** PKA consensus site S425 required for PKA-mediated effects on Kir2.1 channels. A, Representative I_M traces of Kir2.1-S425N, Kir2.1-S425N+V227F, and Kir2.1-WT+V227F in the presence or absence of the PKA activation. B, IV plot for Kir2.1-S425N with PKA activation (open squares; n=5) or without PKA activation (closed squares; n=8) shows nearly complete abrogation of inhibition seen in WT (Figure 4B). C, IV plot for Kir2.1-S425N+V227F with PKA activation (open triangle; n=5), and without PKA activation (closed triangle; n=11) shows abrogation of the inhibitory effect seen in heteromeric channels (Figure 4C). D, IV plot for Kir2.1-WT+V227F with PKA activation (open diamond; n=8) and without PKA activation (closed diamond; n=11) shows that the inhibitory effect remains with a WT subunit that can be phosphorylated. *P<0.05.
and basic information on additional mutations showing this mechanism.

Caveats and Summary
The Kir2.1-V227F mutation found in a patient with CPVT shows an unusual latent biophysical phenotype where loss of function occurred only with PKA stimulation, suggesting a direct link with the adrenergic dependence of the clinical phenotype. We showed that phosphorylation on S425 of 1 subunit of Kir2.1 was both necessary and sufficient for the effect. Although this new biophysical phenotype is of interest, the effects of this mutation on action potential and arrhythmogenesis in myocardial cells and transgenic animals will be required to further confirm and elucidate the mechanism. Also, this is a single case, and the importance and implications for the classification of KCNJ2 mutations in CPVT are not clear.

Acknowledgments
We thank Drs Lee Eckhardt and Carmen Valdivia for valuable discussion and technical assistance.

Sources of Funding
Support was provided by the University of Wisconsin Cellular and Molecular Arrhythmia Research Program and the Mayo Clinic Windland Smith Rice Comprehensive Sudden Cardiac Death Research program. Dr Vega received support from National Heart, Lung, and Blood Institute grant T32 HL07936 (principal investigator, J.C.M.) and the Graduate School of the University of Wisconsin.

Disclosures
Dr Ackerman is a consultant to PGx Health.

References
KCNJ2 encodes Kir2.1, a pore-forming subunit of the cardiac inward rectifier current \( I_{K1} \) that contributes to maintenance of the resting potential and to termination of the action potential. \( KCNJ2 \) mutations are associated with Andersen-Tawil syndrome and catecholaminergic polymorphic ventricular tachycardia. This study characterized the \( I_{K1} \) currents of a particular novel \( KCNJ2 \) missense mutation found in a patient with catecholaminergic polymorphic ventricular tachycardia and showed that unlike previously characterized \( KCNJ2 \) arrhythmia mutations, this mutation required protein kinase A stimulation (a downstream effect of adrenergic stimulation) to show the biophysical phenotype of \( I_{K1} \) abnormality usually associated with arrhythmia. This protein kinase A dependence of the biophysical phenotype directly correlates with the adrenergic dependence of the catecholaminergic polymorphic ventricular tachycardia clinical phenotype and has implications for the classification and pathophysiology of these inherited arrhythmias.
Protein Kinase A-Dependent Biophysical Phenotype for V227F-KCNJ2 Mutation in Catecholaminergic Polymorphic Ventricular Tachycardia
Amanda L. Vega, David J. Tester, Michael J. Ackerman and Jonathan C. Makielski

_Circ Arrhythm Electrophysiol_. 2009;2:540-547; originally published online August 25, 2009; doi: 10.1161/CIRCEP.109.872309

_Circulation: Arrhythmia and Electrophysiology_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 1941-3149. Online ISSN: 1941-3084

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circep.ahajournals.org/content/2/5/540

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation: Arrhythmia and Electrophysiology_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation: Arrhythmia and Electrophysiology_ is online at:
http://circep.ahajournals.org/subscriptions/