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Original article

Non-ventricular, Clinical, and Functional Features of the RyR2^{R420Q} Mutation Causing Catecholaminergic Polymorphic Ventricular Tachycardia

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ABSTRACT

Introduction and objectives: Catecholaminergic polymorphic ventricular tachycardia is a malignant disease, due to mutations in proteins controlling Ca^{2+} homeostasis. While the phenotype is characterized by polymorphic ventricular arrhythmias under stress, supraventricular arrhythmias may occur and are not fully characterized.

Methods: Twenty-five relatives from a Spanish family with several sudden deaths were evaluated with electrocardiogram, exercise testing, and optional epinephrine challenge. Selective RyR2 sequencing in an affected individual and cascade screening in the rest of the family was offered. The RyR2^{R420Q} mutation was generated in HEK-293 cells using site-directed mutagenesis to conduct *in vitro* functional studies.

Results: The exercise testing unmasked catecholaminergic polymorphic ventricular tachycardia in 8 relatives (sensitivity = 89%; positive predictive value = 100%; negative predictive value = 93%), all of them carrying the heterozygous RyR2^{R420Q} mutation, which was also present in the proband and a young girl without exercise testing, a 91% penetrance at the end of the follow-up. Remarkably, sinus bradycardia, atrial and junctional arrhythmias, and/or giant post-effort U-waves were identified in patients. Upon permeabilization and in intact cells, the RyR2^{R420Q} expressing cells showed a smaller peak of Ca^{2+} release than RyR2 wild-type cells. However, at physiologic intracellular Ca^{2+} concentration, equivalent to the diastolic cytosolic concentration, the RyR2^{R420Q} released more Ca^{2+} and oscillated faster than RyR2 wild-type cells.

Conclusions: The missense RyR2^{R420Q} mutation was identified in the N-terminus of the RyR2 gene in this highly symptomatic family. Remarkably, this mutation is associated with sinus bradycardia, atrial and junctional arrhythmias, and giant U-waves. Collectively, functional heterologous expression studies suggest that the RyR2^{R420Q} behaves as an aberrant channel, as a loss- or gain-of-function mutation depending on cytosolic intracellular Ca^{2+} concentration.

Rasgos no ventriculares, clínicos y funcionales de la mutación RyR2^{R420Q} causante de taquicardia ventricular polimórfica catecolaminérgica

Palabras clave:
Calcio
Muerte súbita
Canales iónicos
Receptor 2 de rianodina
Genética
Electrocardiografía

RESUMEN

Introducción y objetivos: La taquicardia ventricular polimórfica catecolaminérgica es una enfermedad maligna que se debe a mutaciones en las proteínas que controlan la homeostasis del Ca^{2+}. Aunque el fenotipo se caracteriza por arritmias ventriculares polimórficas desencadenadas por el estrés, no se han caracterizado plenamente las arritmias supraventriculares que en ocasiones las acompañan.

Métodos: Veinticinco miembros de una familia española en la que había habido varias muertes súbitas fueron evaluados mediante electrocardiograma, pruebas de esfuerzo y prueba de desenmascaramiento con adrenalina opcionalmente. Se realizó secuenciación selectiva de *RyR2* en un miembro afectado y un cribado en cascada al resto de la familia. Se generó la mutación RyR2^{R420Q} en células HEK-293 mediante mutagénesis dirigida, con objeto de realizar estudios funcionales *in vitro*.

Resultados: Las pruebas de esfuerzo desenmascararon taquicardia ventricular polimórfica catecolaminérgica en 8 familiares (sensibilidad del 89%; valor predictivo positivo del 100%; valor predictivo

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negativo del 93%), todos ellos portadores de una mutación heterocigota RyR2^{R420Q}, que estaba presente también en el caso probando y en una chica joven sin prueba de esfuerzo, lo que corresponde a una penetrancia del 91% al final del seguimiento. Es de destacar que en los pacientes se identificó bradicardia sinusal, arritmias auriculares y de la unión y/u ondas U gigantes tras esfuerzo. Tras la permeabilización y en las células intactas, las células que expresaban RyR2^{R420Q} mostraron un pico de liberación de Ca^{2+} menor que el de las células *RyR2* no mutado o *wild-type*. Sin embargo, a una concentración de Ca^{2+} intracelular fisiológica, equivalente a la concentración citosólica diastólica, las células RyR2^{R420Q} liberaban más Ca^{2+} y oscilaban con mayor rapidez que las células con *RyR2* no mutado o *wild-type*.

Conclusiones: La mutación *missense* RyR2^{R420Q} se identificó en el extremo aminoterminal del gen *RyR2* en esta familia muy sintomática. Es de destacar que esta mutación se asocia a bradicardia sinusal, arritmias auriculares y de la unión y ondas U gigantes. En conjunto, los estudios de expresión heteróloga funcional indican que la mutación RyR2^{R420Q} causa un comportamiento aberrante del canal, con pérdida o ganancia de función, según cuál sea la concentración de Ca^{2+} intracelular citosólica.

Abbreviations

CPVT: catecholaminergic polymorphic ventricular tachycardia
ET: exercise testing
VA: ventricular arrhythmias

INTRODUCTION

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is responsible for 12% to 56% of sudden cardiac deaths or cardiac arrests with structurally normal heart.[1,2] Catecholaminergic polymorphic ventricular tachycardia manifests as syncope or sudden cardiac death triggered by adrenergic states with an estimated 80% penetrance.[3] Resting electrocardiogram is usually normal and patients develop ventricular arrhythmias (VA) during exercise testing (ET) or catecholamine infusion.[1,4] The pathogenesis and real prevalence of non-VA, which have occasionally been reported in CPVT patients remains more elusive, including sinus dysfunction, wandering atrial pacemaker, junctional arrhythmias, and atrial fibrillation and flutter.[3,5–9]

Mutations usually affect the gene encoding the cardiac ryanodine receptor (RyR2), but other genes have also been involved.[10–14] Abnormal intracellular calcium (Ca^{2+}) handling underlies an increased diastolic Ca^{2+} release, delayed after depolarizations and triggered activity, which is the pathophysiological basis of VA in this disease.[3,15] However, the precise mechanisms might differ depending on the specific mutation at the RyR2 protein.[16–18] Herein we present an exhaustive characterization of a large CPVT family, stressing the electrocardiographic features of the disease. Moreover, *in vitro* insights from the mechanism of RyR2^{R420Q} channel dysfunction are provided.

METHODS

A family with 4 cases of sudden death underwent familial evaluation with a protocol conforming to the Declaration of Helsinki and previously approved by the local research ethics committee. Informed consent was obtained from each individual.

Clinical Work-up

Electrocardiogram, echocardiography blood sampling, and maximal ET (Bruce protocol) were performed in individual III:10. Once the CPVT phenotype was unmasked during the ET, cascade screening was offered, including an epinephrine challenge to adults with unremarkable ET who were offspring of an affected individual. Maximal U-wave to T-wave amplitude ratio on exertion was recorded. Sinus bradycardia was defined as a heart rate $<$ 60 bpm over 14 years of age or lower than percentile 2 adjusted to age in younger children.[7] Catecholaminergic polymorphic ventricular tachycardia was diagnosed provided sudden cardiac death, polymorphic VA or frequent premature ventricular contractions ($>$ 10/min) during ET, or epinephrine challenge were present (phenotype-positive).[19]

Genetic Work-up

DNA was obtained from whole blood (relatives) or from paraffin-embedded myocardium (proband). Targeted mutational RyR2 analysis (direct sequencing of exons 3, 8, 14, 15, 37, 44-50, 83, 87-105, and adjacent intronic regions, GenBank accession number NM_001035) with an ABI Prism 3100 sequencer (Applied Biosystems) in individual III:10 identified the RyR2^{R420Q} mutation and exon 14 was sequenced in the remaining relatives (mutation carriers were considered genotype+). Since Andersen Tawil syndrome is characterized by a normal or near-normal QTc, giant U waves and polymorphic exercise-related VA due to mutations in *KCNJ2* gene, this gene was also sequenced in CPVT individuals.

Generation of RyR2 Wild Type and RyR2^{R420Q} Constructs

RyR2 site-directed mutagenesis was performed to create RyR2^{R420Q}.[20] Briefly, a plasmid encoding human cardiac N-terminal RyR (Genbank X98330) was used for site-directed mutagenesis (Stratagene, ChickChange kit) using the oligonucleotide 5'-CAGCCCGAGTTATCCAGAGCACAGTCTTCC-3'. The final construct pRyR^{R420Q} was generated by SanDI/SpeI digestion and inserted into a pcDNA3 (Invitrogen) containing full-length, RyR2-enhanced, green fluorescent protein.

Western Blot Analysis

HEK-293 cells (European Collection of Cell Culture Agency, Salisbury, United Kingdom) were transfected.[20–22] Protein fractions (100 μg) were resuspended in SDS-PAGE loading buffer, and proteins were separated in a 4% SDS-PAGE gel strengthened with 0.5% agarose (for RyR).[21] Proteins from 4% gels were electrophoretically transferred to a polyvinylidenedifluoride membrane (Immobilon-P, Millipore) using a semi-dry transfer system (Trans-Blot SD, Bio-Rad) at 22 V for 4 h. Primary antibody specific for the RyR2 isoform (Ab1093, rabbit polyclonal antibody raised to RyR2 residues 4454-4474) was applied at 1:1000 dilution overnight at 4 °C. Immunoreactive protein bands were visualized

by enhanced chemiluminescence detection (ECL, Amersham Biosciences).

Intracellular Ca^{2+} Imaging

HEK-293 cells were plated in polylysineD-coated, glass-bottom Petri dishes (MatTek; Ashland, United States) and transfected with enhanced green fluorescent protein-tagged $RyR2^{R420Q}$ and $RyR2^{WT}$ plasmids.[20,22] Cells were permeabilized to control $[Ca^{2+}]_i$ with saponin (0.01%).[12] The internal solution contained (mmol/L): 120 K-aspartate, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 3 Mg-adenosine triphosphate, 0.5 EGTA (ethylene glycol tetraacetic acid), 10 Na phosphocreatine, 5 U/mL creatine phosphokinase, 0.75 $MgCl_2$ and 8% dextran, saponin (0.01%), pH 7.2. After permeabilization, cells were perfused with the same internal solution without saponin but with 15 μM fluo-4 pentapotassium salt and known $[Ca^{2+}]$ (10^{-1}–10^{5}nM) obtained using different $CaCl_2$:EGTA ratios calculated with Maxchelator.[23] The EGTA concentration was constant at 0.5 mM. Because HEK-293 cells are not excitable, RyR2 was activated by 5 mM caffeine and the resulting $[Ca^{2+}]_i$ transients were recorded using confocal microscopy (Zeiss LSM 510 water-immersion objective ×63; numerical aperture 1.2) at 394 ms/frame. We also performed experiments in intact HEK-293 cells as described for other RyR2 constructs.[24,25] In this case, the cells loaded with fluo-4 AM (acetoxymethyl ester). Afterwards, cells were perfused with external solution containing (Mm): 150 NaCl, 5.4 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 5 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4). Spontaneous $[Ca^{2+}]_i$ oscillations were recorded using confocal microscopy. In both permeabilized and intact cells, fluo-4 fluorescence was excited by an Ar laser (488 nm) and emission fluorescence collected at > 505 nm. Image analyses were performed by Zeiss LSM 510 v2.8 software. Enhanced green fluorescent protein background fluorescence was subtracted. The F (fluorescence) values were normalized by the F_0 (basal fluorescence, determined before the application of the caffeine or in between 2 oscillations) in order to obtain the F/F_0 (fluorescence ratio).

Statistical Analysis

Clinical continuous variables were expressed as mean (standard deviation), laboratory, continuous variables as mean (standard error mean), and Student *t* tests for comparison between groups were applied. Dichotomous variables were expressed as percentages and the chi-square test for comparison between groups was applied (Fisher exact correction, when applicable). A 2-tailed *P* value < .05 was considered statistically significant. In the analyses, SPSS 12.0 software (SPSS, Inc.; Chicago, Illinois, United States) was used.

RESULTS

Family History of Sudden Cardiac Death

The proband (Figure 1, III:8), a 14-year-old male with previous history of unexplained exertion syncopes, suffered a sports-related sudden death with an unremarkable postmortem. Three more relatives had also died suddenly and young (one of them, II:2, during exertion and with a previous unexplained syncope), but no autopsy was performed in those cases.

Familial Evaluation: Cathecholaminergic Polymorphyc Ventricular Tachycardia Phenotype and RyR2 Genotype

Fifteen years following the index event, 25 living proband relatives underwent evaluation (Table). Structural heart disease was ruled out with routine echocardiography and, for patient III:10, also with cardiac magnetic resonance imaging. Three relatives could not be evaluated with a routine ET because of their young age (unknown phenotype). Among them, only one (IV:4) was proven to be genotype+ and electrocardiogram monitoring did not show VA (either without drugs at the initial evaluation at 2 years old or on weight-adjusted beta-blockers at 4 years old). In the remaining 22 relatives, ET allowed the clinical diagnosis of CPVT in 8 patients (phenotype+). Typically, a variable increase in heart rate was followed by a progressive appearance of premature ventricular contractions at a mean heart rate threshold of 103 (24) [62-130] bpm, first isolated and monomorphic and then in bigeminy, polymorphic, in couplets, and also in bursts of nonsustained polymorphic ventricular tachycardia in 50% of phenotype+ individuals at a mean heart rate threshold of 121 (10) [109-131] bpm. Finally, premature ventricular contractions progressively disappeared during the recovery period. Only one individual showed the bidirectional ventricular tachycardia. Of note, these arrhythmias were neither sustained nor syncopal

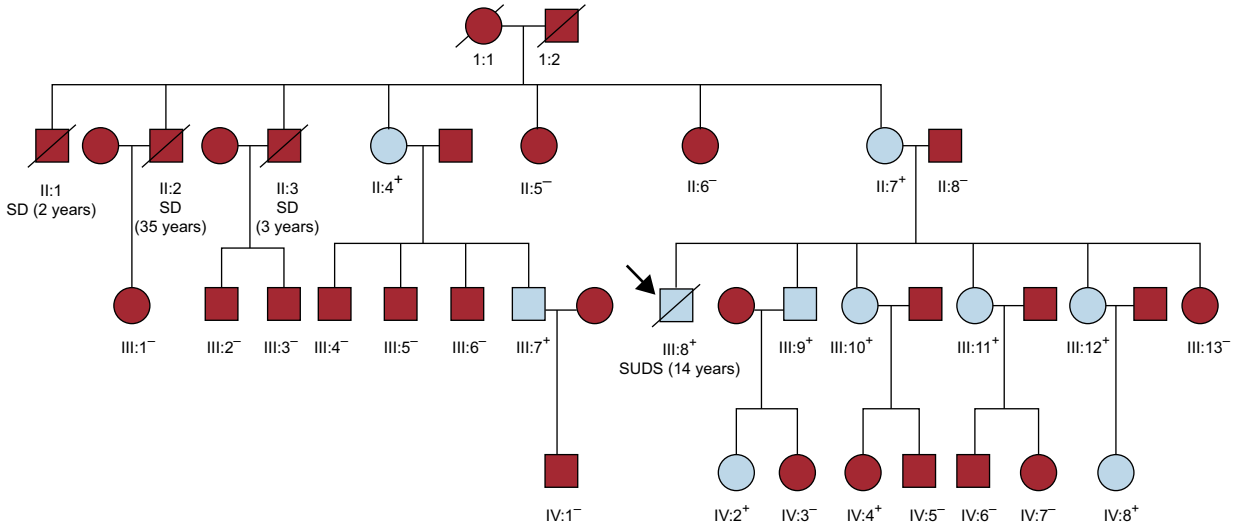

Figure 1. Family pedigree. Squares, males. Circles, females. Crossed symbols, deceased individuals. Blue symbols, catecholaminergic polymorphic ventricular tachycardia phenotype+ (with sudden cardiac death or ventricular effort-related arrhythmias) at the end of the follow-up. The arrow points to the proband. Plus and minus signs depict mutation-positive and mutation-negative individuals, respectively. SCD: sudden cardiac death with postmortem without structural heart disease; SD: sudden death, no autopsy.

Table
Initial Familial Work-up

Family member	Age at first evaluation	BB at first evaluation	Age first-last syncope, triggers	Arrhythmias	U/Tex	EPI	Group
II:4	57 y	Yes	12-47 y, exercise and emotions	SB, VA, AT/JT	0.13	NP	P+G+
II:5	64 y	No	No syncope	No	0.00	NP	P-G-
II:6	51 y	No	No syncope	No	0.00	NP	P-G-
II:7	68 y	Yes	9-55 y, exercise and emotions	SB, VA, AT/JT	0.14	NP	P+G+
II:8	74 y	No	No syncope	AF	0.00	NP	P-G-
III:1	34 y	No	12-30 y, no triggers	No	0.00	–	P-G-
III:2	24 y	No	No syncope	No	0.00	–	P-G-
III:3	30 y	No	No syncope	No	0.00	–	P-G-
III:4	36 y	No	No syncope	SB	0.32	–*	P-G-
III:5	33 y	No	No syncope	SB	0.28	–	P-G-
III:6	23 y	No	No syncope	SB	0.15	–	P-G-
III:7	29 y	No	28 y, emotions	SB, VA, AT/JT	0.33	NP	P+G+
III:8	14 y at SCD	No	6 y, exercise	NP	NP	NP	P+G+
III:9	49 y	No	No syncope	SB, VA, AT/JT	0.28	NP	P+G+
III:10	35 y	No	11 y, exercise and emotions	SB, VA, AT/JT	0.31	NP	P+G+
III:11	44 y	No	30 y, exercise	SB, VA, AT/JT	0.35	NP	P+G+
III:12	39 y	No	20 y, exercise and emotions	VA	0.21	NP	P+G+
III:13	47 y	No	No syncope	No	0.14	-	P-G-
IV:1	Unknown/3 months	No	No syncope	NP	0.00	NP	P?G-
IV:2	21 y	No	18 y, exercise and emotions	SB, VA, AT/JT	0.26	NP	P+G+
IV:3	12 y	No	No syncope	No	0.28	NP	P-G-
IV:4	Unknown/2 y	No	No syncope	NP	0.00	NP	P?G+
IV:5	Unknown/2 months	No	No syncope	NP	NP	NP	P?G-
IV:6	17 y	No	No syncope	No	0.14	NP	P-G-
IV:7	12 y	No	No syncope	No	0.05	NP	P-G-
IV:8	7 y	No	No syncope	No*	0.00	NP	P-G+

+, positive; –, negative; ?, unknown; AT/JT, atrial and/or junctional tachyarrhythmias; BB, beta-blockers; EPI, epinephrine challenge; ET, exercise testing; P, catecholaminergic polymorphic ventricular tachycardia phenotype; G, $RyR2^{R420Q}$ genotype; NP, not performed; SB, sinus bradycardia; SCD, sudden cardiac death; U/Tex, maximal U-wave to T-wave amplitude ratio on exertion; VA, ventricular arrhythmias.

* Developed ventricular arrhythmias at 2-years follow-up (female, catecholaminergic polymorphic ventricular tachycardia phenotype positive, $RyR2^{R420Q}$ genotype positive).

(Figure 2). Among the 13 individuals at risk, the epinephrine challenge was negative in 7 individuals, in keeping with the previous negative ETs, and was declined in 4 children (IV:3 and IV:6-8) and 2 adults (II:5 and II:6), awaiting the genetic results.

A heterozygous missense mutation in RyR2 (1259 G>A, R420Q) was identified in individual III:10. Cascade screening for this mutation rendered a total of 11 genotype+ individuals: the 8 phenotype+ individuals, 1 young phenotype – girl (IV:8), 1 young unknown phenotype girl (IV:4), and also the proband (III:8). All family members were classified according to their phenotype and genotype (+, –, or unknown) (Table). No *KCNJ2* mutations were found.

Considering patient status as a mutation carrier, ET at first evaluation showed a sensitivity of 89%, a positive predictive value of 100%, and a negative predictive value of 93%. The only false negative ET was obtained in a young asymptomatic genotype+ girl (IV:8) aged 7 years who converted into phenotype+ despite beta-blockers 2 years thereafter. Consequently, overall performance of the ET increased up to 100%. Disease penetrance increased from 82% to 91% at the end of follow-up, when clinical (syncope and/or sudden cardiac death) and ET results were considered in genotype+ patients. Notably, syncope occurrence was associated with mutation carrier status in assessable individuals > 6 years (8/10 genotype+ with stress-triggers vs 1/13 genotype–individuals without stress-triggers; $P = .001$) (Table, Figure 3A).

Other Electrocardiographic Features

Sinus, atrial, junctional, and VA were identified in CPVT patients both in resting and exercise electrocardiograms. Assessable individuals (> 6 years old, in sinus rhythm, and with available ET) were evaluated with respect to non-VA (N = 21; 9 genotype+, 12 genotype-). A higher incidence of sinus bradycardia (Table, Figure 3B) was observed in genotype+ patients when compared with genotype– individuals (78% vs 25%, odds ratio = 10.5; $P = .030$). Atrial and/or junctional tachyarrhythmias included atrial premature beats, atrial bigeminy, nonsustained atrial tachycardia, atrial and junctional accelerated rhythm, and junctional escapes. Atrial and/or junctional tachyarrhythmias were detected in 7/9 genotype+ patients and in none of the genotype– individuals (Table, Figure 3C). Maximal U-wave to T-wave amplitude ratio on exertion was significantly higher in phenotype+ than in phenotype– individuals (Figure 3D), although

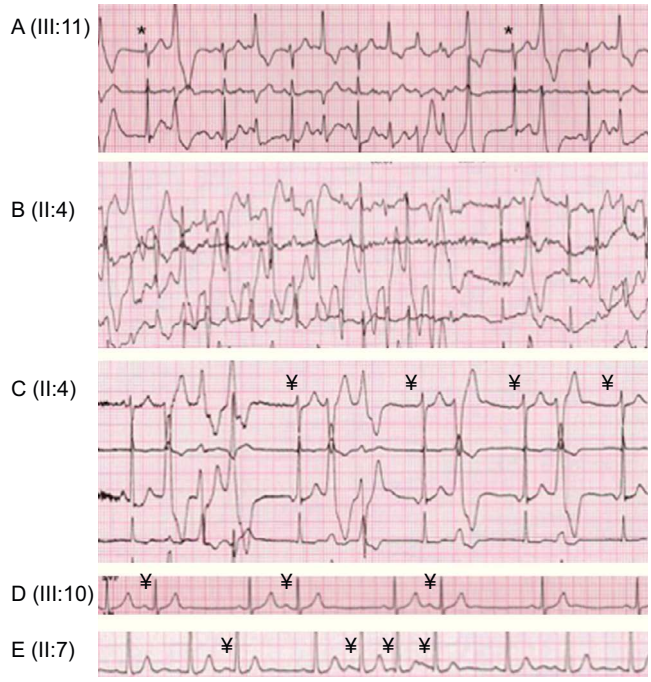

Figure 2. Ventricular and nonventricular arrhythmias during exercise testing. A: ventricular monomorphic bigeminy and bursts of nonsustained polymorphic ventricular tachycardia (*junctional escape). B: bidirectional nonsustained ventricular tachycardia. C: ventricular premature beats, nonsustained polymorphic ventricular tachycardia, polymorphic ventricular couplet, monomorphic ventricular bigeminy, and atrial ectopic rhythm (¥). D: atrial bigeminy (¥). E: atrial premature beats isolated and in salvos (¥).

data overlap precluded the identification of an accurate cut-off point. Remarkably, giant postexercise U-waves were seen in several CPVT patients (Figure 4). Neither U-wave alternans nor post-extrasystolic U-wave polarity changes were detected.

Management and Follow-up

Maximal tolerated dose of beta-blockers was achieved in genotype+ individuals. Five implantable cardioverter defibrillators were placed in phenotype+ patients with frequent VA despite maximal treatment with beta-blockers (one of them, IV:2, also reported stress-triggered presyncopes). At that time, reports concerning the role of flecainide in CPVT had not yet been published[4]. No implantable cardioverter defibrillator-shock has been registered yet (mean follow-up 23.3 months).

In Vitro RyR2^{R420Q} Cell Model

To test whether RyR2^{R420Q} channel function was altered, we generated the mutation and expressed it in HEK-293 cells (Figure 5A). The expressed RyR2-specific immunoreactive proteins were located at the sarcoplasmic reticulum membrane (Figure 5B). Figure 5C depicts examples of confocal images in permeabilized HEK-293 cells expressing RyR2^{WT} and RyR2^{R420Q} under caffeine addition at $[Ca^{2+}]_i 10^{-7.5}$ M. Additionally, pictures of fluorescence profiles recorded at various $[Ca^{2+}]_i$ are plotted in Figure 5D. Both RyR2^{WT} and RyR2^{R420Q} Ca^{2+} releases displayed a bell-shaped curve when plotted as a function of cytosolic $[Ca^{2+}]_i$ with a 34% reduction in the peak of Ca^{2+} release in RyR2^{R420Q} expressing cells (Figure 5E) at pCa 7.5. A different degree of RyR2 expression in each cell group was ruled out as an explanation for this finding when a similar enhanced green fluorescent protein-RyR2 fluorescence was measured in RyR2^{WT} and RyR2^{R420Q} cells (F, in arbitrary units, 50.4 (1.6) in 195 RyR2^{WT}cells vs 50.1 (1.5) in 241 RyR2^{R420Q} expressing cells; no significant difference). However, at the lowest pCa tested, a significant increase in caffeine-evoked Ca^{2+} release was observed in RyR2^{R420Q} cells (pCa10.5, Figures 5D and 5E). In order to calculate the EC_{50} for the Ca^{2+} activating curve and the IC_{50} (half maximal inhibitory concentration) for the Ca^{2+} inactivating curve, we normalized the caffeine-evoked fluorescence by its peak, which happened in both groups at the same $[Ca^{2+}]_i$ (pCa = 7.5)

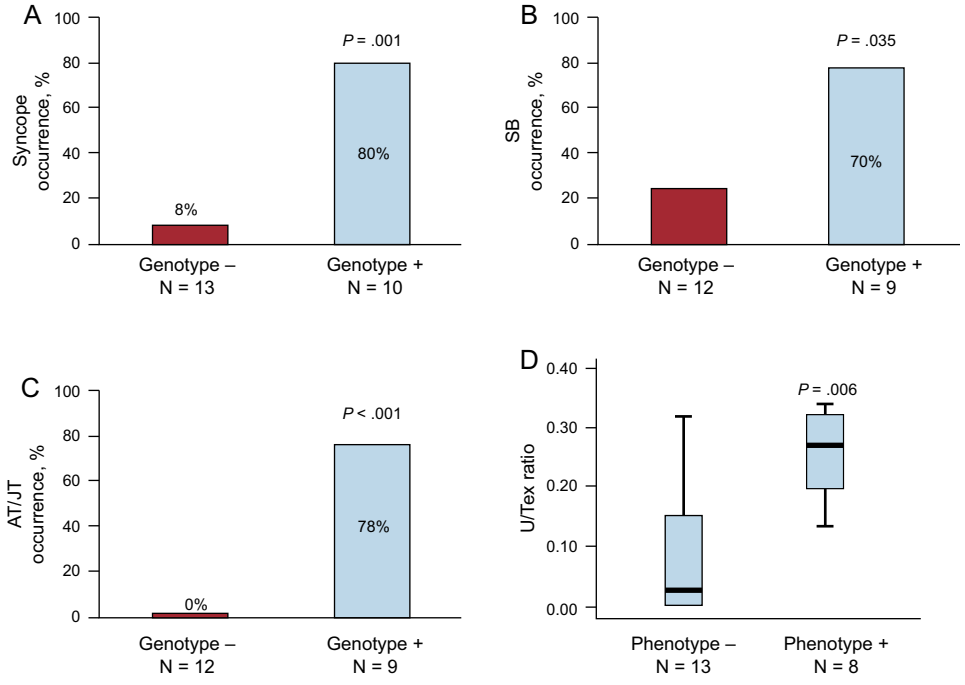

Figure 3. Clinical features. A: syncope occurrence. Children under the age of the first syncope in the family, 6 years old, were not considered. B: sinus bradycardia occurrence. C: atrial and junctional arrhythmias. D: maximal U-wave to T-wave amplitude ratio on exertion comparison attending to the phenotype. For B-D, only individuals in sinus rhythm and with available exercise testing were considered. +, positive; –, negative; AT/JT, atrial and/or junctional tachyarrhythmias; SB, sinus bradycardia; U/Tex, maximal U-wave to T-wave amplitude ratio on exertion.

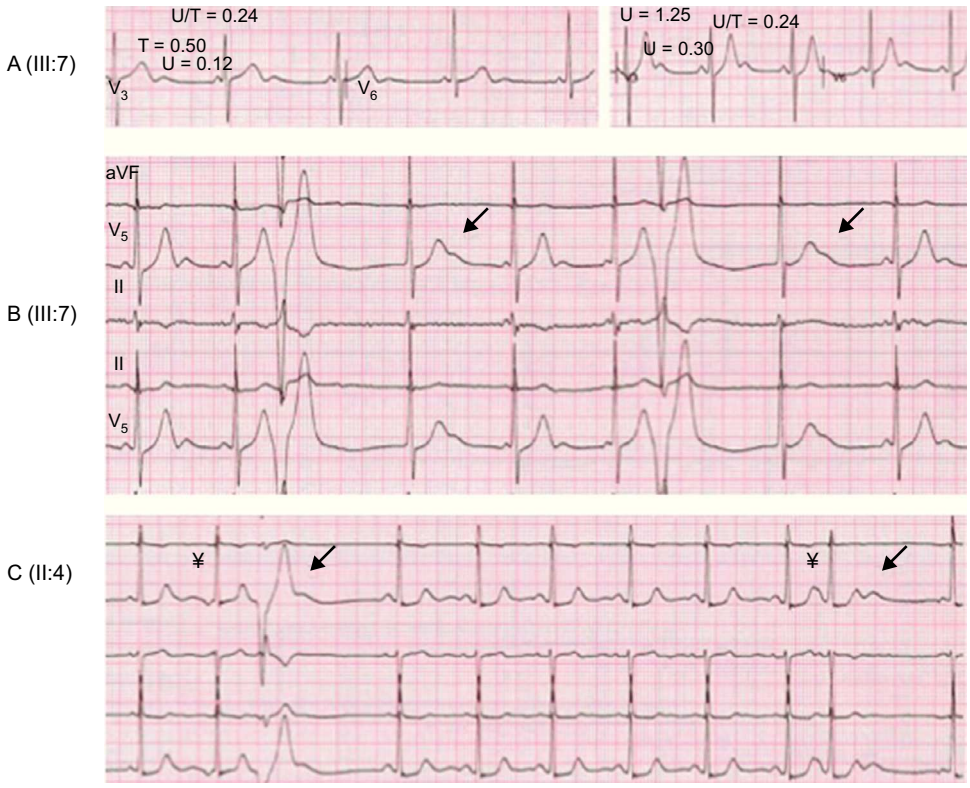

Figure 4. U-wave features in catecholaminergic polymorphic ventricular tachycardia patients. A: resting U-wave (left) and on exertion (right). A fixed, short PR interval suggests an accelerated atrioventricular conduction (left). B: transient increase in U-wave amplitude in the first beat after a ventricular premature beat (arrows) on exertion. A junctional rhythm competes with sinus rhythm. C: transient increase in U-wave amplitude on exertion associated to premature beats, either atrial (right arrow) or ventricular (left arrow).

(Figure 5F). The EC_{50} (half maximal effective concentration) was lower in $RyR2^{R420Q}$ when compared to $RyR2^{WT}$ (2.66 [0.13] in $RyR2^{R420Q}$ vs 4.98 [5.15] nM in $RyR2^{WT}$; no significant difference). At higher $[Ca^{2+}]_i$ the RyR2 was inactivated with a similar pattern, as observed in the descending portion of the fitted curves (IC_{50}, 365.5 (50.5) in $RyR2^{WT}$ vs 339.5 (58.4) nM in $RyR2^{R420Q}$; no significant difference).

Intact HEK-293 cells transfected with $RyR2^{WT}$ or $RyR2^{R420Q}$ plasmids were used to analyze spontaneous $[Ca^{2+}]_i$ oscillations (Figures 6A and 6B). Interestingly, at physiological $[Ca^{2+}]_o$ (1.5 mM) $[Ca^{2+}]_i$ oscillations recorded in $RyR2^{R420Q}$ cells had lower amplitude (Figure 6C), shorter duration (7.6 [0.5] ms in 51 $RyR2^{WT}$ cells, vs 5.9 [0.5] ms in 40 $RyR2^{R420Q}$ cells; $P < .05$), and longer cycle length than in $RyR2^{WT}$ cells (Figure 6D). Notably, these alterations were not due to a decrease in the sarcoplasmic reticulum Ca^{2+} load, which was in fact similar between both cell groups (F/F_0, 3.8 [0.3] in 15 $RyR2^{WT}$cells vs 3.6 [0.3] in 13 $RyR2^{R420Q}$ cells, no significant difference). In a group of cells, we first recorded spontaneous oscillations at 1.5 mM $[Ca^{2+}]_o$ and then lowered $[Ca^{2+}]_o$ to 0.1 mM $[Ca^{2+}]_o$. This maneuver suppressed the automatic activity in a greater proportion of $RyR2^{WT}$ than of $RyR2^{R420Q}$ HEK-293 cells, as evidenced by a higher frequency (or shorter cycle length) of spontaneous oscillations (Figure 6E) and a higher proportion of oscillating $RyR2^{R420Q}$ expressing cells (Figure 6F), in keeping with our findings in permeabilized cells at the lowest $[Ca^{2+}]_i$ analyzed.

DISCUSSION

The present report on a highly symptomatic CPVT family describes for the first time the phenotype associated with the $RyR2^{R420Q}$ mutation, highlighting a high incidence of non-VA. Our *in vitro* results confirm a dysfunction of the mutant channel.

The global yield of the initial ET for CPVT diagnosis (sensitivity, 89%; positive predictive value, 100%, and negative predictive value, 93%) was higher than that reported in other series.[26] The behavior of two young genotype+ patients (IV:4 and IV:8) suggests that penetrance could be age-related. Additionally, sinus bradycardia, atrial and/or junctional tachyarrhythmias and postexercise giant U-waves were present in $RyR2^{R420Q}$ carriers (Figures 3 and 4). U-wave alternans and post-extrasystolic U-wave polarity changes[27] (as a result of an altered Ca^{2+}cycling), and also increased U/T[28,29] (regarded as the electrocardiographic counterpart of delayed after depolarizations), have occasionally been described in CPVT individuals. Of note, our CPVT patients exhibited post-extrasystolic U-wave augmentation and increased U/T *on exertion*, precisely under catecholaminergic drive. The origin of supraventricular arrhythmias could also be ascribed to the RyR2 mutation, as RyR2 is expressed in all cardiomyocytes, including pacemaker cells and atrial myocytes.[30,31] In fact, we recently found that the $RyR2^{R4496C}$ mutation promotes an increased automatism in ventricular cells[15] but a decreased automatism in sinoatrial nodal cells.[9] Thus, mutated RyR2 channels appear to exhibit cell–type-dependent dysfunctional activity.

The $RyR2^{R420Q}$ belongs to the N-terminal cluster and provokes the substitution of the most strongly charged basic amino acid (arginine) by a polar uncharged residue (glutamine). This mutation, recently reported in 4 unrelated patients, lacks clinical and functional characterization.[14,32] Remarkably, the residue 420 is highly conserved across species[32] and behaves as a hot spot, given that different substitutions have been described at the same point and near it (R420W[3,33-35] and I419F[36,37]). Notably, the

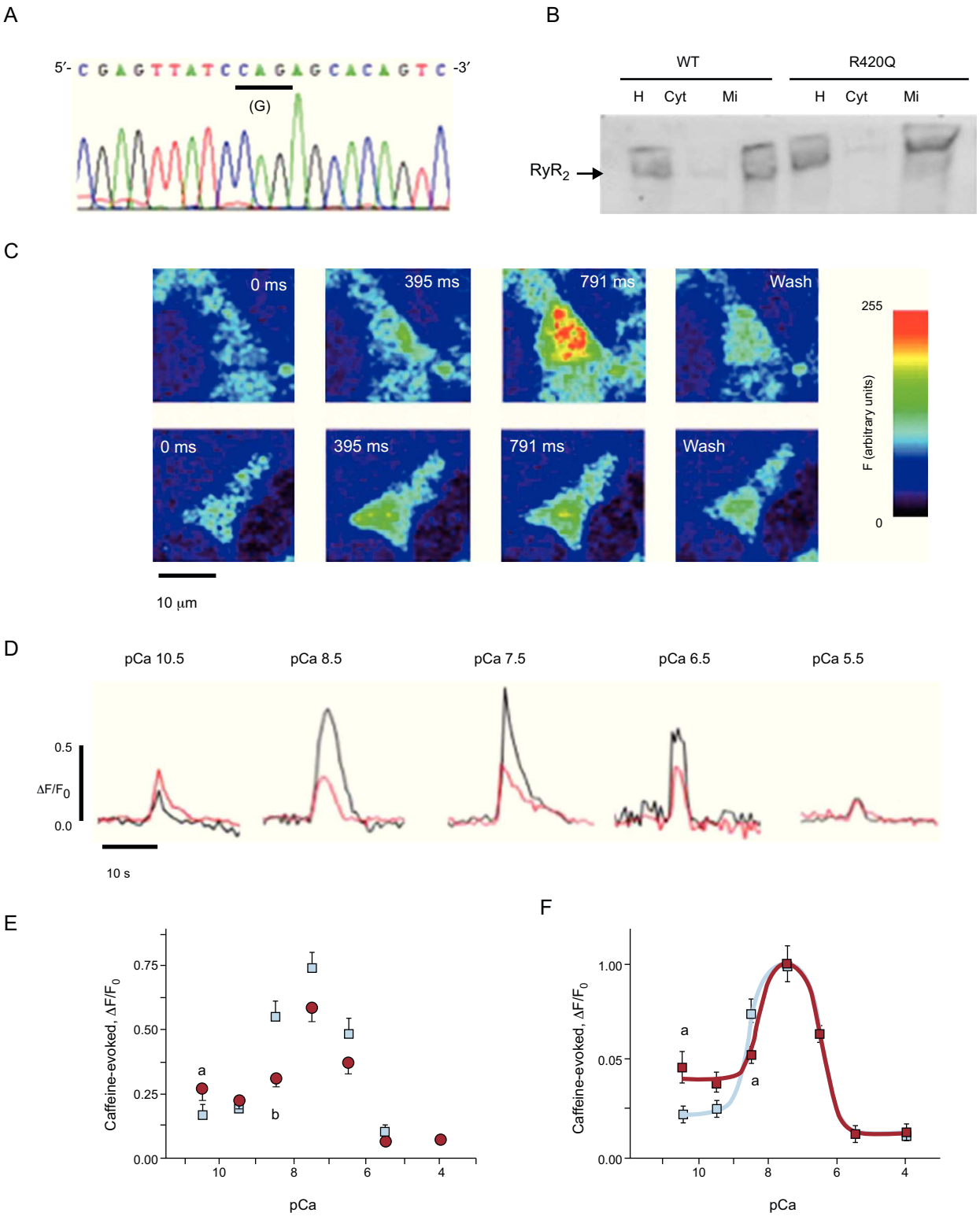

Figure 5. Functional *in vitro* assessment of the RyR2^{R420Q}mutation. A: electropherogram confirming the introduction of the point mutation G1380A that results in conversion of arginine (A) to glutamine (G), RyR2R420Q, in the plasmid contruct. B: Western blot analysis of RyR2 from transfected HEK-293 cells confirming that the RyR2^{RWT} and RyR2^{R420Q} proteins are expressed in the homogenate and the microsomal fractions, but not in the cytosol. The arrow points the 595 kDa band (RyR2-enhanced green fluorescent protein). C: confocal images of calcium changes in permeabilized HEK-293 cells expressing RyR2^{WT} (top) and RyR2^{R420Q} (bottom) and superfused with internal solution containing $[Ca^{2+}]_I = 10^{-7.5}$ M and 5 mM caffeine. D: representative examples of fluorescence profiles obtained from permeabilized HEK-293 cells expressing RyR2^{WT} (black) and RyR2^{R420Q} (red) and superfused with solutions containing different $[Ca^{2+}]_i$ and stimulated with 5 mM caffeine. E: averaged caffeine-induced calcium release (peak values normalized for the basal fluorescence ratio) in HEK-293 cells expressing RyR2^{WT} (squares) and RyR2^{R420Q} (circles) at various $[Ca^{2+}]_i$. Data are expressed as mean (standard error of the mean), N = 20-50 cells per group. F: averaged enhanced green fluorescent protein-RyR2 fluorescence observed in HEK-293 cells expressing RyR2^{WT} (N = 195) and RyR2^{R420Q} (N = 241). △, increase; Cyt, cytosol; eGFP, enhanced green fluorescent protein; F, fluorescence; F_0, basal fluorescence; H, homogenate; Mi, microsomal fractions. $^{a}P < .05$, RyR2^{R420Q} vs RyR2^{WT}. $^{b}P < .001$.

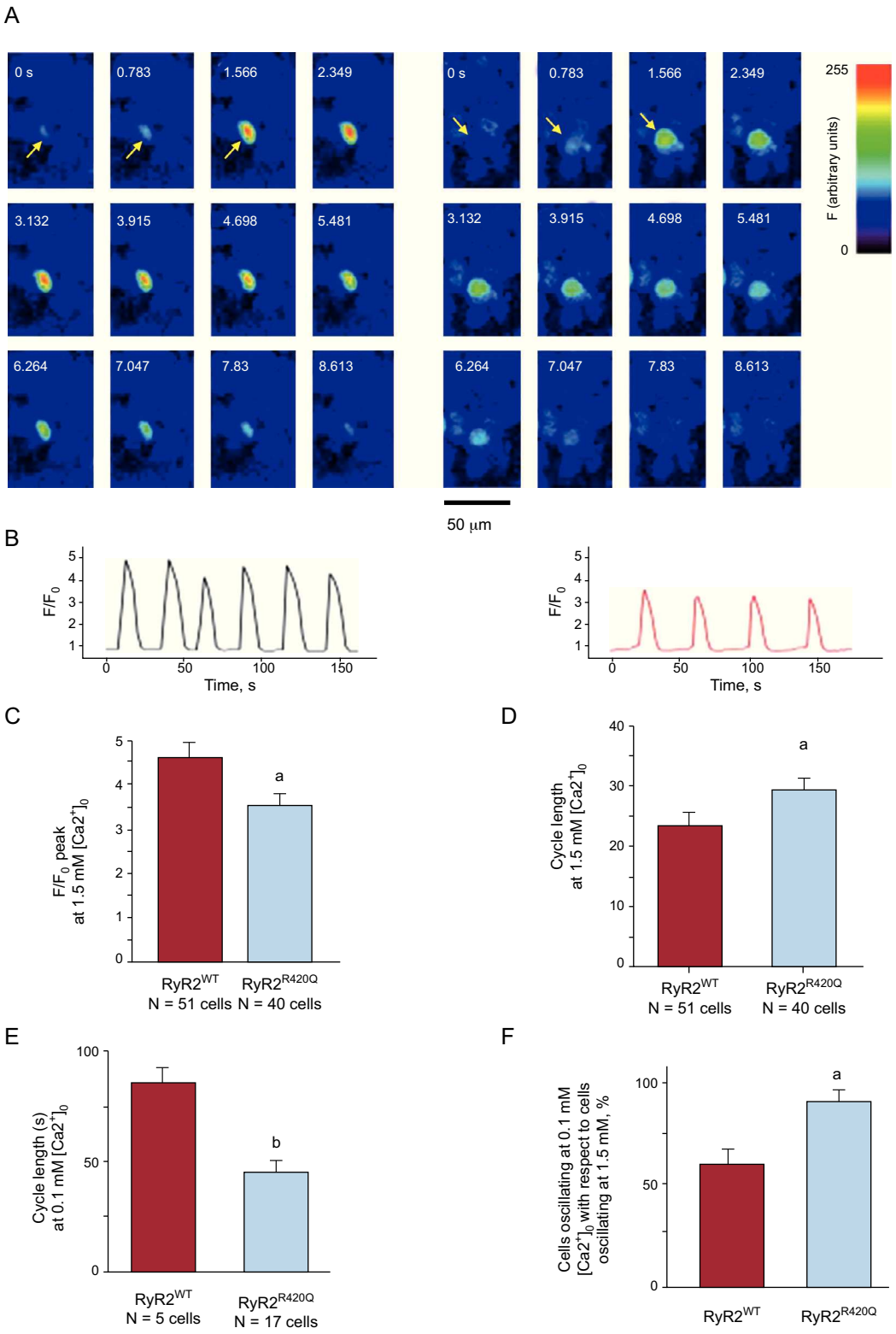

Figure 6. Spontaneous Ca^{2+} oscillations in HEK-293 cells expressing $RyR2^{R420Q}$ are smaller and slower but change to faster than $RyR2^{WT}$ at lower $[Ca2+]_o$. A: time series of confocal images in intact HEK-293 cells expressing $RyR2^{WT}$ (left) and $RyR2^{R420Q}$ (right). B: representative fluorescence profiles in intact HEK-293 cells expressing $RyR2^{WT}$ (left) and $RyR2^{R420Q}$ (right), at 1.5 mM $[Ca2+]_o$. C: amplitude of Ca^{2+} oscillations at 1.5 mM $[Ca2+]_o$. D: as in C but for the cycle length between consecutive Ca^{2+} oscillations. E: as in D but at 0.1 mM external $[Ca^{2+}]_o$. F: percentage of cells that oscillate at 0.1 mM $[Ca^{2+}]_o$ with respect to cells that oscillate at 1.5 mM $[Ca^{2+}]_o$. $RyR2^{R420Q}$ vs $RyR2^{WT}$. $^aP < .05$. $^bP < .01$.

$RyR2^{R420Q}$ shows a higher penetrance than the $RyR2^{R420W}$ (91% vs 25%), and does not display any feature of arrhythmogenic cardiomyopathy.[33,34]

So far, most CPVT mutations in which functional studies have been performed behave as gain-of-function.[35,38] Increased Ca^{2+} sensitivity (luminal or cytosolic),[15,24,38,39] an altered interdomain interaction between the N-terminal and central domains,[40,41] and diminished FKBP12.6 binding[38,39] account for the most accepted mechanisms. Very recently, a disrupting effect of the $RyR2^{R420Q}$ has been observed in the crystal structure of the N-terminal region, since it ablates chloride binding, disturbing its domain folding[42,43] even though, in linear terms, the 420 residue is very far from the proposed molecular region involved in Ca^{2+}-dependent activation (residues 4485-4494).[40] The N-terminus has also been shown to be an important structural region that has the ability to self-tetramerise, and may be involved in regulation of native channel function.[44] Thus, it appears clear from a *static structural* standpoint that the $RyR2^{R420Q}$ destabilizes the inter-subunit interface, probably facilitating the channel opening.[42-45]

Herein, we offer the first initial assessment of the $RyR2^{R420Q}$ mutation from a *functional* perspective. First, our findings clearly reflect that the $RyR2^{R420Q}$ mutation behaves as a gain-of-function both in permeabilized and intact cells (Figures 5D, 5F, 6E and 6F) at very low $[Ca^{2+}]_i$, similar to that measured in rat and human cardiomyocytes.[46,47] Second, the reason for the reduced peak of caffeine-evoked $[Ca^{2+}]$ release in $RyR2^{R420Q}$ cells (Figure 5E) does not have a straightforward interpretation. It could be related to a hypoactive channel at higher cytosolic Ca^{2+} but without significant shift in EC_{50}. Finally, in intact $RyR2^{R420Q}$ cells, spontanous oscillations had decreased amplitude and frequency (Figures 6B and 6D). This decrease could be reminiscent of the sinus bradycardia found in CPVT patients (Figure 3B), and it is not dependent of lower sarcoplasmic reticulum Ca^{2+} load, since this was normal (see "Results").

Study Limitations

Heterologous cell expression systems are useful to characterize RyR2 mutations, although they may present limitations because they cannot replicate the electrical behavior *in vivo*, where other proteins and the autonomic tone may interact with the dysfunctional channel. Thus, experiments in either transgenic mice or iPS-CM (induced pluripotent stem cell-derived cardiomyocytes) will provide more definitive conclusions.

CONCLUSIONS

This work provides a novel and detailed clinical characterization of the $RyR2^{R420Q}$ mutation. Our analyses highlight that non-VA are common and might reinforce the suspicion of CPVT in an appropriate scenario. Furthermore, *in vitro* analyses suggest that the $RyR2^{R420Q}$ results in an aberrant channel.

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CONFLICTS OF INTEREST

None declared.

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