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Bidirectional Ventricular Tachycardia and Fibrillation Elicited in a Knock-In Mouse Model Carrier of a Mutation in the Cardiac Ryanodine Receptor

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Abstract—Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited disease characterized by adrenergically mediated polymorphic ventricular tachycardia leading to syncope and sudden cardiac death. The autosomal dominant form of CPVT is caused by mutations in the RyR2 gene encoding the cardiac isoform of the ryanodine receptor. In vitro functional characterization of mutant RyR2 channels showed altered behavior on adrenergic stimulation and caffeine administration with enhanced calcium release from the sarcoplasmic reticulum. As of today no experimental evidence is available to demonstrate that RyR2 mutations can reproduce the arrhythmias observed in CPVT patients. We developed a conditional knock-in mouse model carrier of the R4496C mutation, the mouse equivalent to the R4497C mutations identified in CPVT families, to evaluate if the animals would develop a CPVT phenotype and if beta blockers would prevent arrhythmias. Twenty-six mice (12 wild-type (WT) and 14RyR4496C) underwent exercise stress testing followed by epinephrine administration: none of the WT developed ventricular tachycardia (VT) versus 5/14 RyR4496C mice (P=0.02). Twenty-one mice (8 WT, 8 RyR4496C, and 5 RyR4496C pretreated with beta-blockers) received epinephrine and caffeine: 4/8 (50%) RyR4496C mice but none of the WT developed VT (P=0.02); 4/5 RyR4496C mice pretreated with propranolol developed VT (P=0.56 nonsignificant versus RyR4496C mice). These data provide the first experimental demonstration that the R4496C RyR2 mutation predisposes the murine heart to VT and VF in response caffeine and/or adrenergic stimulation. Furthermore, the results show that analogous to what is observed in patients, beta adrenergic stimulation seems ineffective in preventing life-threatening arrhythmias.

Key Words: arrhythmias ■ genetics ■ ion channels ■ transgenic mice ■ calcium ■ catecholamine

Catecholaminergic polymorphic ventricular tachycardia (CPVT; OMIM: 604772) is a highly malignant cardiac disease manifesting in childhood and adolescence. It is characterized by adrenergically-mediated bidirectional or polymorphic ventricular tachycardia leading to syncope and/or sudden cardiac death. Based on previously reported linkage data that had mapped the disease to chromosome 1q42–43, we reported that the gene for the autosomal dominant variant of CPVT was RyR2; i.e., the gene encoding for the cardiac isoform of the ryanodine receptor. The first family in which a RyR2 mutation was identified was affected by a highly malignant form of the disease that was resistant to beta blockers; the mutation present in the family (R4497C) is a hot spot that we subsequently identified in other CPVT patients unrelated to the first kindred. The R4497C mutation has been extensively investigated in different in vitro models that demonstrated that it enhances the release of calcium from the sarcoplasmic reticulum. It has therefore been inferred that arrhythmias may develop as a consequence of abnormal calcium release from intracellular stores. However, experimental evidence linking this mutation to the development of life-threatening arrhythmias is still lacking. Here we report on a conditional knock-in mouse-model carrier of the R4496C mutation that is the mouse equivalent of the human mutation R4497C. The objectives of the present study were to evaluate if the R4496C mutation results in a CPVT phenotype in the mouse and thus to provide experimental proof to the concept that abnormalities in the ryanodine receptor cause a severe form of polymorphic ventricular tachycardia.

Materials and Methods

Generation of Conditional Knock-In of RyR2 in Mouse Model

We amplified by PCR a 900 bp segment encompassing exons 94 and 95 of the RyR2 gene using exonuc primers identified on mouse RyR2 cDNA. This fragment was used as a probe to screen a 129-SV/J lambda mouse genomic library (Stratagene): 800,000 phages were screened and 2 positive plaques were isolated. Through Southern blot hybridization we identified one 3850 bp fragment (BamHI) and one 3500 bp fragment (XbaI) that were cloned into pBluescript (S.G.P.), Italy; and the Pathology Division (M.S., L.V.), IRCCS Fondazione S. Maugeri, Pavia, Italy.

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Facility for Conditional Mutagenesis, Dibit- San Raffaele Scientific Laboratories in Calco, Italy, and transferred to the Maugeri Foundation for characterization of the phenotype. Animals were maintained and studied according to the protocols approved by the Animal Care and Use facility at the Maugeri Foundation.

**Figure 1.** Schematic representation of the genomic structure of mouse RyR2 (a); the targeting vector used to generate the knock-in RyR<sup>R4496C</sup> mouse strain (b); the recombinating genomic structure of RyR<sup>R4496C</sup> (c). Ex=exon; For abbreviations see methods.

**ECG Monitoring, Exercise Stress Testing, and Drug Testing**

ECG radiotelemetry monitors (Data Sciences International) were implanted intra-abdominally under general anesthesia (Avertin 0.025 mg/kg). Body temperature was maintained at 37°C by use of a thermally controlled heating pad (Harvard Apparatus). ECG was continuously monitored starting 48 hours after surgery. After 72 hours of recovery from surgery, phenotype characterization was performed.

One group (Group 1) of animals exercised on a treadmill until exhaustion. The animals were then injected with epinephrine 2 mg/kg i.p. (WT n=12, RyR2<sup>/RyR<sub>R4496C</sub> n=14). A second group of animals (Group 2) was injected with epinephrine (2 mg/kg ip) and caffeine (120 mg/kg i.p.) (WT, n=8, RyR2<sup>/RyR<sub>R4496C</sub> n=7). ECG monitoring was performed continuously during both exercise and drug testing protocols. Five additional RyR2<sup>/RyR<sub>R4496C</sub> animals were treated for 24 hours with propranolol i.p. (2 mg/Kg) every 12 hours before being exposed to the epinephrine and caffeine protocol.

**Analysis of the Allele-Specific Transcription of the RyR2 Gene**

At the end of the experimental protocols, animals were anesthetized and sacrificed by cervical dislocation and the heart was immediately excised. Total mRNA was extracted using a RNAEasy Fibrous Tissue Midi Kit (Qiagen). cDNA was generated using random examer from 2.5 μg of mRNA using a Thermoscript RT-PCR system (Invitrogen). PCR fragments encompassing the mutation were amplified from cDNAs using p93F and p94R primers (5' CACATCGCTATGGGGAGCCGAAG 3' and exon primer P-96R (5' - GATCA- CAAAGTCTGTCCCACTGGCC -3')) and exon primer P-96R (5' - GATCA- CAAAGTCTGTCCCACTGGCC -3'). One positive clone was isolated and confirmed by Southern blot using an external probe to the target sequence of homology. The positive clone was injected into the blastocysts of C57BL/6 mice and chimeric animals were obtained (Core Facility for Conditional Mutagenesis, Dibit- San Raffaele Scientific Institute, Milan). Chimeric male mice were bred to C57BL/6 female mice to establish a hybrid line. In fact, germ-line transmission has been observed by breeding a male chimera to a C57BL/6 female. In fact, germ-line transmission has been observed by breeding a male chimera to a C57BL/6 female.
Statistical Analysis and Definitions
Statistical analysis was performed using the SPSS statistical package (v. 12.01). Parametric tests were used to compare normally distributed variables (unpaired t-test and ANOVA with Bonferroni correction for multiple comparisons). Cross tabulations with chi-square or Fisher’s exact test were used as appropriate for categorical variables. Data are expressed as mean±SD.

Arrhythmias were defined as follows: non-sustained ventricular tachycardia (VTns) was defined as a series of 4 to 10 consecutive repetitive ventricular ectopic beats (VEBs), sustained VT (VTsust) was defined as a run of >10 consecutive VEBs, ventricular fibrillation (VF) was defined as a VT sus degenerating into ventricular fibrillation leading to sudden death.

Results
Development and Pathology of WT and RyR2+/RyR R4496C Mice
No difference between the WT versus RyR2+/RyR R4496C animals was present in the duration of the pregnancy, delivery, size and survival of litters, development, and behavior. Young adult mice of both genders entered the experimental protocol: no difference in the weight between WT and RyR2+/RyR R4496C mice was observed (mean weight WT 25.6±3.6 gr; RyR2+/RyR R4496C 27.3±4.9 gr; P=0.189).

Gross inspection did not show any macroscopic alteration of the heart and vessels. Histological examination did not show any tissue abnormalities, no fibrous-fatty infiltration was observed, no signs suggestive of right ventricular cardiomyopathy were identified (Figure 2).

Phenotype Characterization
Continuous ECG monitoring revealed the absence of spontaneous ventricular arrhythmias both in WT and RyR2+/RyR R4496C mice. Interestingly the RyR2+/RyR R4496C at variance with CPVT patients, did not manifest supraventricular arrhythmias during EG monitoring. The QT interval and RR interval of the WT and of the mutant mice did not present significant differences (Table 1). None of the 12 WT mice developed repetitive ventricular arrhythmias although 5/14 RyR2+/RyR R4496C mice developed VTns (n=3) or VTns (n=2; Figure 3B) (WT versus RyR2+/RyR R4496C; P=0.02; Table 1).

Group 2 included 21 mice (8 WT, 8 RyR2+/RyR R4496C, and 5 RyR2+/RyR R4496C pretreated with beta-blockers) that received epinephrine and caffeine i.p. The QT interval (measured using the tangent method9 and RR interval of the WT and of the mutant mice did not present significant differences (Table 1), the RR interval was significantly prolonged in the RyR2+/RyR R4496C animals pretreated with beta blockers (Table 1). The epinephrine and caffeine test induced more severe arrhythmias than the exercise protocol. Two WT mice developed VTns 6 had no arrhythmias; thus none of the WT animals experienced sustained cardiac arrhythmias. Among the RyR2+/RyR R4496C mice 4/8 or 50% developed sustained arrhythmias (VTsust n=2; Figure 4B and VF n=2; Figure 5B P=0.02 versus WT; Table 1).

It is remarkable that VT in the RyR2+/RyR R4496C mice had the typical bidirectional morphology that is considered the most distinguishing characteristics of CPVT patients (Figure 3, 4, and 5A).

The coupling interval of the beats initiating VTs was only slightly shorter than the preceding RR interval (Table 1), R-on T phenomenon was never observed: these features are similar to those observed in CPVT patients (Figure 6). Panel B of Figure 3, 4, and 5 show examples of a non-sustained VT, of a sustained VT that spontaneously terminates and of a sustained VT degenerating into VF respectively. In panel A of the same figure similar arrhythmias recorded in CPVT patients are shown.

Five RyR2+/RyR R4496C mice were treated with propranolol to evaluate if antiadrenergic compounds could prevent induction of arrhythmias. After drug administration we observed a prolongation of RR interval from 94.5±13 to 118±13 ms beats per minute (P<0.001). Interestingly, the effect of beta blockers on heart rate in RyR2+/RyR R4496C mice was not different from the effect on WT mice (mean prolongation or RR interval was as follows: RyR2+/RyR R4496C mice 23.62±0.14 ms versus WT mice 22.92+3.6 ms P=0.69; the percentage of RR change after beta blockers (Δ% = ΔRR*
100/RR baseline) was RyR2\textsuperscript{+/−}/RyR4496C mice 25±3% versus WT mice 27±5% \(P=0.52\). Despite pretreatment with beta blockers, the administration of caffeine and epinephrine elicited VTsust in 2 and VF in 2 (sustained arrhythmias in 4 mice); only 1 mouse had no arrhythmias \(P=0.56;\) ie, nonsignificant versus RyR2\textsuperscript{+/−}/RyR4496C mice.

We used a single nucleotide primer extension method to perform a relative quantification of allele-specific transcripts\textsuperscript{10} of the RyR2 gene in the heterozygous mice. Total mRNA was available for 24/27 RyR2\textsuperscript{+/−}/RyR4496C mice from Group 1 and Group 2. 10/24 of the mice developed either VTs or VTs whereas 14 had no arrhythmias. The WT-to-R4496C mRNA ratio was similar in the 2 groups (R4496C: mRNA=0.66±0.14 in the heart of the mice without arrhythmias and 0.67±0.02 in the mice with arrhythmias; NS).

Discussion

Our data provide the previously missing demonstration that the presence of the R4496C mutation predisposes the murine heart to the development of bidirectional and polymorphic VT and to ventricular fibrillation on administration of caffeine and of adrenergic agonists. Combined with the evidence provided by in vitro characterization of the same RyR2 mutant\textsuperscript{6–8} it seems plausible to suggest that arrhythmias in the RyR2\textsuperscript{+/−}/RyR4496C mice are caused by enhanced calcium release from the sarcoplasmic reticulum through the defective RyR2 channels.

The cardiac ryanodine receptor (RyR2) is a tetrameric intracellular calcium release channel located in the sarcoplasmic reticulum (SR) that has a pivotal role in cardiac excitation-contraction coupling. In response to a small intracellular calcium influx through the L-type voltage dependent calcium channels, RyR2 releases calcium from the SR which then may be triggered by the SR large amount of calcium that is needed to elicit contraction of the cardiac cell. However, in addition to such a tightly regulated physiological process, RyR2 may also release calcium in response to SR calcium overload, which may occur under pathological conditions such as physical and emotional stress and digitalis toxicity and may be arrhythmogenic.

Previously, we reported that mutations in the gene encoding for RyR2 cause the autosomal dominant form of catecholaminergic polymorphic ventricular tachycardia (CPVT).\textsuperscript{4} Shortly after our report, other groups identified novel RyR2 mutations in patients affected by CPVT.\textsuperscript{11} To date 34 RyR2 mutations have been reported in the “Gene Connection for the Heart” mutation database for inherited arrhythmogenic diseases (http://pc4.fsm.it:81/cardiomoc).

The first mutation that we identified in an Italian CPVT family lead to the replacement of arginine at position 4497 with a cysteine. Because this mutation was associated with a highly malignant phenotype it has been selected by several authors for their in vitro studies aimed at the functional characterization of RyR2 mutants. Jiang et al\textsuperscript{6} were the first to investigate the R4496C mouse equivalent of the R4497C human mutation. They suggested that when expressed in HEK293 cells, the mutation enhances the basal channel activity and the propensity for spontaneous calcium release at rest and in response to caffeine. More recently, the same authors further elaborated their results and proposed that the R4496C, as well as other RyR2 mutations identified in CPVT families, increase the sensitivity of RyR2 channels to luminal calcium thus facilitating the spontaneous release of calcium from the SR.\textsuperscript{5} George et al\textsuperscript{5} investigated the same mutation by expression in HL-1 cardiac myocytes. At variance with what was suggested by Jiang et al\textsuperscript{6}
based on their studies in HEK 293 cells, George et al\textsuperscript{7} reported that the R4496C mutant presents no enhancement of basal activity, but they confirmed that after exposure to the RyR agonist caffeine or to beta adrenergic stimulation, calcium release was significantly augmented in the mutant channels. George et al also showed that the dissociation of the FKBP12.6 protein from the mutant was similar to that observed in the WT RyR2 thus challenging the hypothesis advanced by Wehrens et al\textsuperscript{8} who proposed that the enhanced calcium release observed in the mutant during beta adrenergic stimulation was caused by the excessive dissociation of the RyR2-FKBP12.6 complex. Overall, although disagreement exists on the mechanisms by which the R4496C mutation sensitizes the RyR2 channel to agonists, three independent groups have confirmed that on caffeine and beta adrenergic stimulation RyR2\textsuperscript{R4496C} channels respond with an augmented calcium release.

None of the functional studies performed so far proved that the presence of RyR2\textsuperscript{R4496C} channels is able to induce sustained ectopic activity leading to VT and VF on exposure to the RyR-agonist caffeine or during adrenergic stimulation. The present study fills that gap by showing that polymorphic and even bidirectional VT may be elicited in the RyR2\textsuperscript{−/−}RyR\textsuperscript{R4496C} mice under conditions that strictly resemble those eliciting cardiac arrhythmias in CPVT patients. Consistent with the incomplete penetrance of the CPVT phenotype in humans, not all RyR2\textsuperscript{−/−}RyR\textsuperscript{R4496C} mice developed arrhythmias. The cause for incomplete penetrance is today the most puzzling aspect of inherited arrhythmogenic diseases and no satisfactory explanation has been provided to account for the major differences in the clinical manifestations observed among affected patients, even when they are members of the

Figure 4. A, ECG recording of sustained and self terminating bidirectional VT in a CPVT patient. B, ECG recording of a sustained and self terminating bidirectional VT in a RyR2\textsuperscript{−/−}RyR\textsuperscript{R4496C} mouse. msec=milliseconds; sec=seconds

Figure 5. A, ECG recording of a bidirectional VT degenerating into VF in a CPVT patient. B, ECG recording of a bidirectional VT degenerating into VF in a RyR2\textsuperscript{−/−}RyR\textsuperscript{R4496C} mouse. msec=milliseconds; min=minutes
same family. Both genetic and environmental factors have been advocated to account for this variability but a robust hypothesis supported by experimental data is missing.

We used a single nucleotide primer extension method to perform a relative quantification of allele-specific transcription of the RyR2 gene in the heterozygous mice to investigate if the variability in the pattern of arrhythmias elicited in response to exercise and/or pharmacological challenges correlates with the allele specific transcription of RyR2. When we measured the relative transcription of WT and mutant mRNA in the hearts of the RyR2+/RyR\textsuperscript{R4496C} mice and showed that the mutant mRNA was slightly underrepresented as compared with the WT mRNA, but no differences in the levels of the mutant mRNA were present between the animals that developed arrhythmias and those that remained asymptomatic throughout the provocative tests. It is concluded that the severity of the arrhythmias is not related to the allele-specific transcription of RyR2.

Patients affected by the R4497C mutation appear to have an extremely lethal form of the disease. Cardiac arrest occurred in 7/13 (53%) carriers of the mutation and in 4 patients it was a lethal event. Furthermore VT or VF occurred in 5 patients also during beta-blocker therapy suggesting that the protection afforded by these agents may not be sufficient to prevent life-threatening events in CPVT. Treatment with propranolol was not effective in preventing arrhythmias in RyR2+/RyR\textsuperscript{R4496C} mice. CI= Coupling Interval; interval in msec between an extrasystolic beat and the preceding sinus beat; RR= interval between 2 consecutive sinus beats; CI/RR= ratio between the coupling interval of an extrasystolic beat and the preceding RR interval; msec= milliseconds.

![Figure 6. A, Coupling interval of the initiating beat of polymorphic ventricular tachycardia in CPVT patients. B, Coupling interval of the initiating beat of polymorphic ventricular tachycardia in RyR2+/RyR\textsuperscript{R4496C} mice. CI= Coupling Interval; interval in msec between an extrasystolic beat and the preceding sinus beat; RR= interval between 2 consecutive sinus beats; CI/RR= ratio between the coupling interval of an extrasystolic beat and the preceding RR interval; msec= milliseconds.](http://circres.ahajournals.org/)

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