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A knock-in mouse model of N-terminal R420W mutation of cardiac ryanodine receptor exhibits arrhythmogenesis with abnormal calcium dynamics in cardiomyocytes

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ABSTRACT

Cardiac ryanodine receptor gene (*RyR2*) mutations cause fatal arrhythmogenic diseases such as catecholaminergic polymorphic ventricular tachycardia and arrhythmogenic right ventricular cardiomyopathy. The N-terminal region of *RyR2* is one of the hot spots for mutations. In this study, we investigated cardiac phenotypes of a knock-in mouse model carrying R420W mutation of *RyR2*. The N-terminal R420W mutation has already been found in juvenile sudden death cadavers of unrelated families. The depolarization-induced Ca^{2+} transient amplitude was significantly lower in cardiomyocytes from $RyR2^{R420W/R420W}$ mice compared with wild-type mice. The time to peak of the Ca^{2+} transient was significantly increased in $RyR2^{R420W/R420W}$ mice. Furthermore, the prolonged decay time from the peak of the Ca^{2+} transient was detected in $RyR2^{R420W/R420W}$ mice. ECG telemetry revealed that various types of arrhythmias were induced in $RyR2^{R420W/R420W}$ mice in response to administration of caffeine and adrenaline. The mutant mice showed high occurrences of arrhythmias in response to heart stimulants compared with wild-type mice. These findings suggest that R420W mutation impairs depolarization-induced Ca^{2+} oscillation in cardiomyocytes, which possibly results in sudden death due to stress-induced arrhythmias.

1. Introduction

RyR2 encodes a Ca^{2+} channel that is localized across the sarcoplasmic reticulum membrane of cardiomyocytes [1]. The channel releases Ca^{2+} from sarcoplasmic reticulum into the cytoplasm in response to action potentials, which regulates intracellular Ca^{2+} concentrations and excitation–contraction coupling in cardiac muscle [2]. Mutations in *RyR2* cause catecholaminergic polymorphic ventricular tachycardia (CPVT), a heritable arrhythmogenic disease resulting in exertional syncope or sudden death [3,4]. *RyR2* mutations can also cause arrhythmogenic right ventricular cardiomyopathy (ARVC) [5,6]. ARVC is characterized by fatty infiltration and fibrosis of the myocardium, and is associated with a high frequency of ventricular arrhythmias and sudden cardiac death especially in the young [7,8]. CPVT and ARVC have been suggested to be different degrees of phenotypic expression of the same disease [9,10].

Mutations of *RyR2* are located in three hot spots: a N-terminal region, a central region, and a C-terminal region containing the pore structure of the channel [11,12]. We previously screened possible *RyR2* mutations for sudden death cases of young cadavers and found two cases in unrelated families with the same R420W mutation [13,14]. R420W is located in the N-terminal region of RyR2 protein. Of the various *RyR2* mutations, R420W has already been identified to be a pathogenic mutation, which causes juvenile sudden death [5]. Autopsy findings showed mild fatty infiltration in the apex of hearts from cadavers with R420W, without any other particular findings [13].

The precise regulatory mechanisms of the N-terminal region of *RyR2* with regard to its channel functions are not fully understood, but the region has been shown to play an essential role in Ca^{2+} release termination [15]. In addition, recently, crystallization experiments have revealed that the N-terminal region contains an anion-binding site, and that mutations at that region destabilize intersubunit interactions of the molecule [16]. The aim of this

Abbreviations: ARVC, arrhythmogenic right ventricular cardiomyopathy; CPVT, catecholaminergic polymorphic ventricular tachycardia; *RyR2*, caridiac ryanodine receptor gene.

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study was to examine cardiac phenotypes of the N-terminal R420W mutation using a knock-in mouse model carrying the mutation.

2. Materials and methods

2.1. Ethics statement

All experiments and procedures were conducted with the approval of the Hyogo College of Medicine Transgenic Committee (No. 212001) and the Animal Research Committee (Nos. 29037 and 12030). All animal experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals of the Hyogo College of Medicine. Mice were housed in a specific pathogen-free room on a 12-h light/dark cycle at 23 °C and 50–60% humidity.

2.2. Animal model with R420W mutation

We generated a knock-in mouse model carrying the R420W mutation for this study. Generation of this model was described previously [17].

2.3. Adult cardiomyocyte isolation

Mouse ventricular myocytes were isolated from the hearts of adult male mice (6–8 weeks old) using the collagenase type II (Worthington Biochem, Lakewood, NJ, USA) and protease method described previously [18].

2.4. Laser scanning confocal imaging of single cardiomyocytes

The isolated cardiomyocytes were plated on glass-bottom dishes (Matsunami Glass Ind. Ltd, Osaka, Japan) coated with laminin and loaded with 5 μM Quest Fluo-8H AM (AAT Bioquest Inc., Sunnyvale, CA, USA) in Tyrode's solution containing (in mmol/L) 140 NaCl, 4 KCl, 1.1 $MgCl_2$, 10 HEPES, 10 glucose, 1.8 $CaCl_2$ (pH 7.4, with NaOH) for 20 min at room temperature. Following the monitoring of $[Ca^{2+}]_i$ for 3.0 s at rest, cells were excited by field stimulation (30V, 10 ms at 37 °C) using two parallel Pt electrodes. The Ca^{2+} images were obtained with a Zeiss LSM510 confocal microscope (Axiovert 200 M, Carl Zeiss, Jena, Germany) by scanning the cell with an Argon laser every 1.54 ms per line; fluorescence was excited at 488 nm and emissions were collected at >505 nm. Image analyses were performed by ImageJ 1.48k (National Institutes of Health, Bethesda, MD, USA) and OriginPro 9.0 (OriginLab Corp., Northampton, MA, USA) software. The fluorescence values (F) were normalized by the basal fluorescence (F_0) in order to obtain the fluorescence ratio (F/F_0).

2.5. ECG Telemetry

Eighteen male mice (six $RyR2^{R420W/R420W}$, six $RyR2^{R420W/+}$, and six wild-type) (24 weeks old) were studied with ECG telemetry as described previously [19]. Briefly, ECGs using standard limb leads were recorded under pentobarbital anesthesia (30 mg/kg body weight injected intraperitoneally). A heat pad was used during recordings to prevent hypothermia. The telemeter transmitter was embedded, and a control recording was obtained once mice had recovered from the operation. Adrenalin (2 mg/kg), isoproterenol (100 μg/head) and caffeine (120 mg/kg) were injected intraperitoneally for the experiments. Adrenalin (2 mg/kg) and caffeine (120 mg/kg) were administered intraperitoneally at the same time. ECG profiles were recorded for 30 min after administration.

2.6. Statistics

Statistical significance was evaluated using a Student's t test. A P-value of <0.05 was considered statistically significant.

3. Results

3.1. $[Ca^{2+}]_i$ transients in R420W cardiomyocytes

To assess the effects of the R420W mutation on a transient increase in intracellular Ca^{2+} of cardiomyocytes, we examined field stimulation-induced $[Ca^{2+}]_i$ transients in R420W compared with wild-type cardiomyocytes (Fig. 1A).

As shown in Fig. 1B, the depolarization-induced Ca^{2+} transient amplitude was significantly lower in the R420W mutation

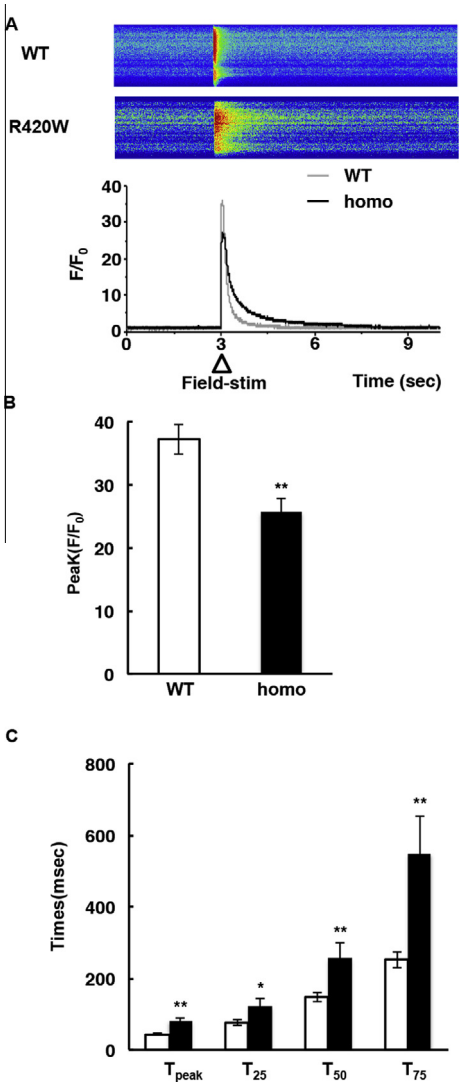

Fig. 1. Confocal imaging of Ca^{2+} transients in ventricular myocytes. (A) Line-scan confocal imaging of field-stimulated (stim) Ca^{2+} transients in isolated wild-type (WT) and R420W ventricular myocytes loaded with the Ca^{2+} indicator Fluo-8 AM. Ca^{2+} signal is shown as fluorescence ratio (F/F_0), with the fluorescence intensity (F) normalized to intensity at rest before stimulation (F_0). (B) Field-stimulated Ca^{2+} transient amplitudes in WT ($n = 22$) and R420W ($n = 17$) myocytes. Data are mean ± s.e.m. (C) The kinetics of the field-stimulated Ca^{2+} transient in WT and R420W ventricular myocytes (Tpeak, time to peak: T25, T50 and T75, time from peak to 25%, 50% and 75% decay in the Ca^{2+} transient, respectively). Data are mean ± s.e.m. from 22 WT and 18 R420W myocytes (Student's t-test; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with WT).

Table 1
Comparison of ECG parameters in wild type and *RyR2* $^{R420W/R420W}$ mice.

	RR (msec)	*P* (1/100 mV)	*R* (1/100 mV)	*S* (1/100 mV)	*T* (1/100 mV)	Pd (msec)	PQ (msec)	QRS (msec)	QT (msec)
Wild	120.2 ± 7.9	4.1 ± 1.8	50.3 ± 7.6	32.3 ± 5.9	4.7 ± 0.9	8.3 ± 0.9	36.2 ± 2.4	9.7 ± 1.3	28.7 ± 3.9
Homo	107.5 ± 24.9	4.7 ± 2.8	38.2 ± 7.3	34.9 ± 7.9	0.8 ± 5.8	10.9 ± 2.6	38.6 ± 2.9	10.6 ± 2.5	34.7 ± 8.3

(F/F_0: 25.71 ± 2.26, $P = 0.00143$) compared with the wild-type (F/F_0: 37.26 ± 2.37). Furthermore, the time to the peak of the Ca^{2+} transient was significantly increased in R420W (Tpeak: 81.62 ± 8.84 ms, $P = 0.00233$) compared with wild-type (44.06 ± 3.86 ms) cardiomyocytes. The T75 decay time from peak to 75% decay of the Ca^{2+} transient in the R420W (122.13 ± 19.22 ms, $P = 0.03661$) was significantly longer than in the wild-type (76.30 ± 8.21 ms). Also, there were significant increases in the decay time T50 (R420W: 254.87 ± 43.05 ms *vs.* wild-type: 145.99 ± 12.69 ms, $P = 0.01227$) and T25 (R420W: 548.87 ± 103.95 ms *vs.* wild-type: 251.70 ± 22.32 ms, $P = 0.00407$) (Fig. 1C).

3.2. Induction of various types of arrhythmias in RyR2 mutant mice

ECG profiles from *RyR2* mutant mice were compared with those from wild-type mice. Spontaneous arrhythmias were not observed in every mouse examined. ECG parameters at a resting condition did not change significantly between *RyR2*$^{R420W/R420W}$ mice and wild-type mice (Table 1). The administration of heart stimulants induced various types of arrhythmias in *RyR2*$^{R420W/R420W}$ mice. Representations of ECG traces of arrhythmias are shown in Fig. 2. Bradycardia and irregularity in RR intervals were induced in *RyR2*$^{R420W/R420W}$ mice by isoproterenol treatment (Fig. 2A). VT-like arrhythmia was induced by administration of adrenalin (2 mg/kg) and caffeine (120 mg/kg) (Fig. 2B). Fig. 2C shows trigeminy arrhythmias, which appeared at the time of adrenalin and caffeine administration. The occurrences of arrhythmias in all mice were examined and mutant mice showed higher occurrences of arrhythmias compared with the wild-type (Fig. 2D). The types of arrhythmias in response to adrenergic stimulation in each mouse examined are shown in Table 2. Not every mutant mouse showed arrhythmias, but mutant mice showed susceptibility to arrhythmias in response to heart stimulants when compared with wild-type mice.

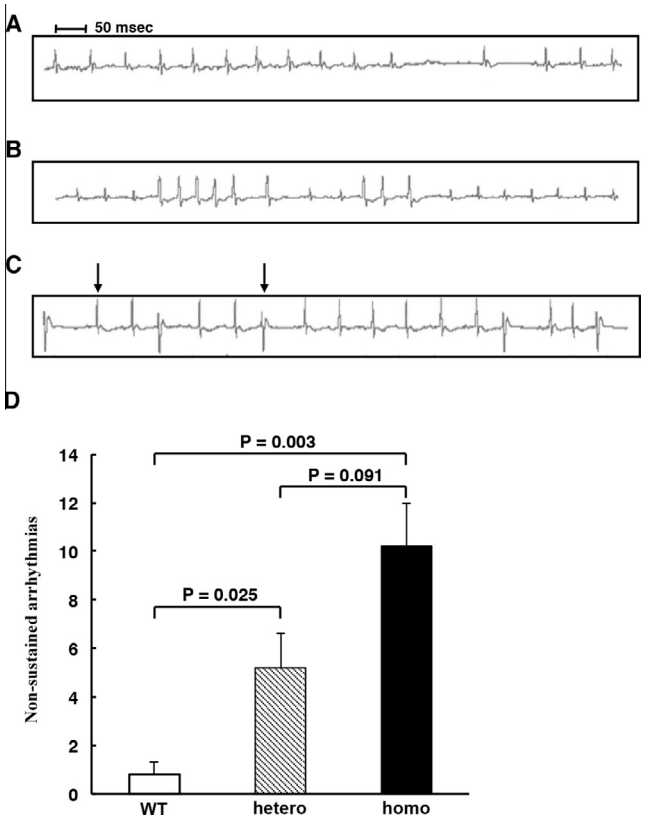

Fig. 2. Various types of arrhythmias were induced in *RyR2*$^{R420W/R420W}$ mice. (A) Isoproterenol (100 μg/head) provokes bradycardia and irregularity of RR distance. (B) VT-like arrhythmia appeared with the administration of adrenalin (2 mg/kg) and caffeine (120 mg/kg). (C) Adrenalin (2 mg/kg) and caffeine (120 mg/kg) revealed trigeminy (every third beat is a premature ventricular beat between the arrows) in a *RyR2*$^{R420W/R420W}$ mouse. (D) Effects of adrenaline and caffeine administration on ECG. Average data obtained from six wild-type (WT), six heterozygote (hetero), and six homozygote (homo) mice over a constantly monitored period of 30 min. Adrenalin (2 mg/kg) and caffeine (120 mg/kg) were administered intraperitoneally at the same time.

Table 2
Occurrence of arrhythmias with drug administration.

		Sinus bradycardia	AV-block	PVC
WT	ISO	0/6	0/6	0/6
	AD + CF	0/6	0/6	1/6
HT	ISO	2/6	2/6	2/6
	AD + CF	1/6	1/6	3/6
HM	ISO	6/6	6/6	6/6
	AD + CF	2/6	0/6	6/6

WT: wild-type, HT: heterozygote, HM: homozygote, ISO: isoproterenol, AD: adrenaline, CF: caffeine.

4. Discussion

In this study, we observed that a knock-in mouse model with R420W mutation exhibited abnormal Ca^{2+} transients in cardiomyocytes and various types of arrhythmias in response to heart stimulants. The prolonged decay time from the peak of the Ca^{2+} transient in cardiomyocytes from the mutant mice in this study coincides with previous studies which report that N-terminal mutation of *RyR2* causes abnormal termination of Ca^{2+} release [15,20]. We also found that the time to the peak of the Ca^{2+} transient was significantly increased in cardiomyocytes from the mutant mice with significantly lower Ca^{2+} transient amplitude in response to depolarization. Thus, the channel with the mutation has the characteristic of a delayed response to depolarization-induced Ca^{2+} transient.

Bidirectional ventricular tachycardia, which is one of typical arrhythmias for CPVT, was not observed in the mutant mice in this study. However, mutant mice did show various types of arrhythmias after administration of heart stimulants. Sinus bradycardia, which was observed in mutant mice in response to heart stimulants, has been reported in CPVT patients [21,22]. In addition, the mouse model with R420W mutation showed high occurrences of various types of arrhythmias in response to heart stimulants. This may be a useful model that aids in the research of CPVT and ARVC and phenotypes of the conditions.

From previous reports, ECG profiles are noted to be different among the model mouse strains with *RyR2* mutations. Mice with the R176Q mutation have been reported to show bidirectional

ventricular arrhythmias in response to adrenergic stress [11]. In contrast, mice with exon-3 deletion of the N-terminal region of *RyR2* showed only bradycardia and no other types of ventricular tachycardia [23].

We have autopsied two cases with the R420W mutation, both who suddenly died during exercise [13,14]. Although R420W mutation is well known to cause juvenile sudden death [5], no sudden death cases have been noted in mutant mice. We have observed just one mouse with the homogenous mutation that suddenly died without any particular autopsy findings after the occurrence of a big earthquake (data not shown). Because the homogenous mutation has not been reported in human patients, the mutation may show a more severe phenotype in humans compared to mice.

Patients with *RyR2* mutations are known to show structurally normal hearts. However, autopsied cadavers with R420W mutation have been reported to show ARVC-like phenotypes of mild fatty infiltration in the heart apex [13]. We examined the heart histology of up to 10-month-old mutant mice and did not detect any differences between the homozygous and wild-type mice. No apparent fatty changes in hearts were observed in the homozygous mutant mice (data not shown). Two other mouse strains carrying R176Q [24] or R4496C [25] mutations of *RyR2* have reported no structural abnormalities in cardiovascular systems.

In this study we investigated cardiac phenotypes of a knock-in mouse model with N-terminal R420W mutation. The mice exhibited abnormal Ca^{2+} release from cardiac sarcoplasmic reticulum as well as a wide variety of arrhythmias on ECG profiles, which may be associated with juvenile sudden death in humans. The mouse model may be useful for examining pathophysiological aspects of *RyR2* mutations, especially those in N-terminal regions.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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