Dantrolene, a Therapeutic Agent for Malignant Hyperthermia, Inhibits Catecholaminergic Polymorphic Ventricular Tachycardia in a RyR2R2474S/+ Knock-In Mouse Model

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Background: Dantrolene, a specific agent for the treatment of malignant hyperthermia, was found to inhibit Ca$^{2+}$ leak through not only the skeletal ryanodine receptor (RyR1), but also the cardiac ryanodine receptor (RyR2) by correcting the defective inter-domain interaction between N-terminal (1–619 amino acid) and central (2,000–2,500 amino acid) domains of RyRs. Here, the in vivo anti-arrhythmic effect of dantrolene in a human catecholaminergic polymorphic ventricular tachycardia (CPVT)-associated RyR2R2474S/+ knock-in (KI) mouse model was investigated.

Methods and Results: ECG was monitored in KI mice (n=6) and wild-type (WT) mice (n=6), before and after an injection of epinephrine (1.0 mg/kg) or on exercise using a treadmill. In all KI (but not WT) mice, bi-directional ventricular tachycardia (VT) was induced after an injection of epinephrine or on exercise. Pre-treatment with dantrolene (for 7–10 days) significantly inhibited the inducible VT (P<0.01). In KI cardiomyocytes, Ca$^{2+}$ spark frequency (SpF; s$^{-1}$·100 μm$^{-1}$: 5.8±0.3, P<0.01) was much more increased after the addition of isoproterenol than in WT cardiomyocytes (SpF: 3.6±0.2). The increase in SpF seen in KI cardiomyocytes was attenuated by 1.0 μmol/L dantrolene (SpF: 3.6±0.5, P<0.01).

Conclusions: Dantrolene prevents CPVT, presumably by inhibiting Ca$^{2+}$ leak through the RyR2. (Circ J 2010; 74: 2579–2584)

Key Words: Calcium; Excitation-contraction (E-C) coupling; Ventricular tachycardia
**Methods**

**ECG Telemetry**
ECG was monitored in R2474S/+ KI mice (n=6) and wild-type (WT) mice (n=6) in a conscious state by using ECG telemetry, as previously described, with slight modification. Briefly, transmitters (Data Sciences International, St. Paul, MN, USA) were implanted in the back space with s.c. electrodes in a lead II configuration. Telemetry was recorded after 96h of recovery from surgery in a conscious state at baseline and after the injection of epinephrine (1 mg/kg of body weight, i.p.) for measurement of the number of VT episodes for 30 min. A subset of telemetered KI (n=6) and WT mice (n=6) were injected with epinephrine (1 mg/kg of body weight, i.p.) and monitored for 30 min. Another set of KI (n=6) and WT mice (n=6) were exercised on a treadmill (Panlab, Barcelona, Spain). After recording the ECG before treatment with dantrolene, these KI mice were treated with dantrolene i.p. (20 mg/kg) for 7–10 days before being exposed to an epinephrine injection or exercise on a treadmill. The QT interval was measured from the beginning of the QRS complex to the end of the T wave based on the 5-min stable ECG segments of sinus rhythm, as previously described. Then, the QT interval was rate-corrected using Bazett’s formula (QT interval divided by the square root of heart rate) and expressed as QTc in the present study. Arrhythmias were defined as follows: non-sustained ventricular tachycardia (VT) was defined as a series of 4–10 consecutive repetitive ventricular ectopic beats (VEBs), and sustained VT (SVT) was defined as a run of >10 consecutive VEBs.

**Isolation of Cardiac Cardiomyocytes**
The enzymatic isolation of mice cardiomyocytes was performed as described previously. In brief, R2474S/+ KI and WT mice (2–3 months old) were anesthetized with pentobarbital sodium (70 mg/kg of body weight, i.p.), intubated and ventilated with ambient air. An incision in the chest was made, and the heart was quickly removed and retrogradely perfused with a collagenase-free buffer via the aorta under constant flow. The LV myocardium was minced with scissors in a fresh collagenase-containing buffer and the rod-shaped adult mouse cardiomyocytes were prepared by retrograde perfusion of the hearts with 95%O2/5%CO2-bubbled Minimal Essential Medium (Sigma, St Louis, MO, USA) supplemented with 50 μmol/L [Ca2+], 0.5 μmol/mL collagenase B, 0.5 mg/mL collagenase D, and 0.02 mg/mL protease type XIV. The Ca2+ concentration was then gradually increased to a final concentration of 1 μmol/L by changing the incubation medium (50 μmol/L, 100 μmol/L, 300 μmol/L, 600 μmol/L and then 1 μmol/L). The isolated mice cardiomyocytes were transferred to laminin-coated glass culture dishes, and incubated for 12h at 37°C in a 5%CO2/95%O2 atmosphere. Experiments were carried out at room temperature.

**Analysis of Local Ca2+ Release Events With Laser Scanning Confocal Microscopy**
Ca2+ sparks in intact isolated cardiomyocytes were measured by confocal microscopy to examine the effect of dantrolene on the local Ca2+ release, as previously described. The Ca2+ sparks were measured using a laser scanning confocal microscope system (LSM-510, Carl Zeiss) equipped with an argon ion laser coupled to an inverted microscope (Axiovert 100, Carl Zeiss) with a Zeiss ×40 oil-immersion Plan-Neofluar objective (numerical aperture, 1.3; excitation at 488 nm; emission >505 nm). Briefly, intact cardiomyocytes were loaded with fluo-4 AM (20 μmol/L; Molecular Probes) for 30 min at room temperature. Line-scan mode was used, where a single cardiomyocyte was scanned repeatedly (520.8 Hz) along a line parallel to the longitudinal axis, avoiding nuclei. To monitor Ca2+ sparks, cardiomyocytes were stimulated until the Ca2+ transient reached a steady state, then stimulation was stopped, and Ca2+ sparks were then recorded during subsequential 10 s rest.

**Statistical Analysis**
A paired or unpaired t-test was used for statistical comparison of the data between 2 different situations. The chi-square test was used to estimate the effectiveness of dantrolene to prevent occurrence of CPVT. Data are expressed as mean±SD except for the data of Ca2+ spark frequency. The data of Ca2+ spark frequency are expressed as mean±SE. We accepted a P value less than 0.05 as statistically significant.

**Results**
Table shows the ECG characteristics obtained from WT and KI mice by telemetry. There was no significant difference in baseline parameters of HR, QT interval, and QTc between WT and KI mice. In all KI mice, bi-directional VT was induced after injection of epinephrine or on exercise, but not in WT mice (Table). In KI mice, pre-treatment with a daily intraperitoneal injection of 20 mg/kg dantrolene (7–10 days) decreased the frequency of premature ventricular contractions at rest, and prevented the inducible VT (Figures 1–3). In KI (but not WT) mice, pre-treatment with dantrolene significantly increased the total running distance during exercise on a treadmill (Treated: 188.5±77.1 m, vs Untreated: 22.3±9.0 m, P<0.01). In KI cardiomyocytes, Ca2+ spark frequency (SpF; s−1, 100 μm−2) was increased (5.8±0.3, P<0.01) was much more increased after the addition of isoproterenol than in WT cardiomyocytes (SpF: 3.6±0.2) (Figures 4, 5). In KI cardiomyocytes, the SpF was inhibited by 1.0 μmol/L dantrolene (SpF: 3.6±0.5, P<0.01) (Figures 4, 5).
Figure 1. Representative telemetry ECGs in RyR2^{R2474S/+} KI mice on exercise using a treadmill. RyR2, ryanodine receptor; KI, knock-in.

Figure 2. Representative telemetry ECGs in RyR2^{R2474S/+} KI mice before and after an epinephrine injection. RyR2, ryanodine receptor; KI, knock-in.
Figure 3. Summarized data for the effect of dantrolene on the inducible VT in RyR2<sup>R2474S/+</sup> mice. RyR2, ryanodine receptor; VT, ventricular tachycardia.

Figure 4. Effect of dantrolene on the aberrant Ca<sup>2+</sup> sparks in intact RyR2<sup>R2474S/+</sup> KI cardiomyocytes. Ca<sup>2+</sup> sparks were measured at 2 mmol/L extracellular [Ca<sup>2+</sup>]. RyR2, ryanodine receptor.
Catecholaminergic polymorphic VT is regarded as a highly lethal disease and β-blockers can be used as first line treatment of CPVT. Unfortunately, however, the efficacy of β-blockers in CPVT is known to be low. Most of the patients with CPVT require an implantable cardioverter defibrillator, with 50% of implanted patients receiving appropriate shocks during a 2-year follow up.

The most important new aspect of the present study is the finding that pre-treatment with dantrolene, a specific agent for MH, prevented CPVT induced by either epinephrine or exercise, and significantly improved exercise tolerance in KI mice. In addition, in confirmation with our previous report using saponin-permeabilized cardiomyocytes that dantrolene inhibited the PKA-phosphorylation-induced aberrant Ca$^{2+}$ release, it markedly suppressed the isoproterenol-induced spontaneous Ca$^{2+}$ sparks in intact cardiomyocytes as well. The mechanism by which dantrolene prevented CPVT is likely to be attributable to stabilization of the CPVT-associated, mutated RyR2. This notion is based on the following findings, which have been previously noted: (1) in either RyR1 or RyR2, dantrolene specifically binds to the domain with the same amino-acid sequence; Leu$^{590}$-Cys$^{609}$ in RyR1 or Leu$^{600}$-Cys$^{619}$ in RyR2, and (2) dantrolene corrects the defective inter-domain interaction between N-terminal: 1–619 and central: 2,000–2,500 domains (i.e., domain unzipping to zipping), thereby inhibiting Ca$^{2+}$ leak through RyRs.

More recently, we further demonstrated that in the RyR2$^{R2474S/+}$ KI mice, the affinity of calmodulin (CaM) binding to the RyR2 is reduced upon PKA-mediated phosphorylation, which seems to be a critical cause of spontaneous local Ca$^{2+}$ release events. As dantrolene restored a normal level of CaM-binding affinity in the PKA-phosphorylated KI hearts, it is suggested that the defective inter-domain interaction between the N-terminal domain and the central domain of the RyR2 is involved in the reduction of the CaM binding affinity. It is quite advantageous for the clinical use of dantrolene that it showed no appreciable effect on cardiac function in normal hearts, but it substantially improved the contractile function in pacing-induced failing hearts. This beneficial effect is clearly different from other anti-arrhythmic drugs.

The clinical dose of dantrolene for the treatment for MH is less than 7 mg/kg. In the present study, however, we treated KI mice with a relatively high dose of dantrolene (20 mg·kg$^{-1}$·day$^{-1}$). This dosage was determined by a titration study that was aimed to find out the minimum dose to achieve complete prevention of sustained VT on exercise. Before we further move on to clinical studies, we should pay great attention to the effective and safety dosage of dantrolene for the treatment of patients with CPVT or heart failure.

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Disclosures
None.
References


