A novel RyR2-selective stabilizer prevents and ameliorates severe arrhythmias in stress-induced ventricular tachycardia

Nagomi Kurebayashi, PhD[1*]; Masami Kodama, PhD[1,2]; Takashi Murayama, PhD[1]; Masato Konishi, MD, PhD[1]; Masami Sugihara, MD, PhD[3]; Hana Inoue, PhD[4]; Ryosuke Ishida, PhD[5]; Koichiro Ishii, MSc[1]; Shuichi Mori, PhD[5]; Yukari Endo, MD, PhD[1]; Xi Zeng, MSc[5]; Yukiko U. Inoue, PhD[6]; Takayoshi Inoue, PhD[6]; Satoru Noguchi, PhD[6]; Hajime Nishio, MD, PhD[7]; Utako Yokoyama, MD, PhD[4]; Junko Kurokawa, PhD[2]; Hiroyuki Kagechika, PhD[5*]; Takashi Sakurai, MD, PhD[1]

[1]Department of Cellular and Molecular Pharmacology, Juntendo University Graduate School of Medicine, Tokyo, Japan

[2]Bio-informational Pharmacology, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka, Japan

[3]Department of Clinical Laboratory Medicine, Juntendo University Graduate School of Medicine, Tokyo, Japan

[4]Department of Physiology, Tokyo Medical University, Tokyo, Japan

[5]Laboratory for Biomaterials and Bioengineering, Institute of Integrated Research, Institute of Science Tokyo, Tokyo, Japan

[6]National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan

[7]Department of Legal Medicine, Hyogo Medical University, Nishinomiya, Japan

Short title: A novel RyR2-targeted antiarrhythmic agent

***Corresponding authors**:

Nagomi Kurebayashi, Ph.D., Department of Cellular and Molecular Pharmacology, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

Tel.: +81-3-5802-1035; Fax: +81-3-5802-0419; E-mail: nagomik@juntendo.ac.jp

Hiroyuki Kagechika, Ph.D., Laboratory for Biomaterials and Bioengineering, Institute of Integrated Research, Institute of Science Tokyo, 2-3-10 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan

Tel.: +81-3-5280-8032; Fax: +81-3-5280-8127; E-mail: kage.chem@tmd.ac.jp

Abstract

Background: Excessive activation of the type 2 ryanodine receptor (RyR2) causes lethal arrhythmias such as catecholaminergic polymorphic ventricular tachycardia (CPVT). Stabilization of RyR2 in the closed state is expected to prevent the occurrence of these arrhythmias, but there are no clinically available antiarrhythmic drugs that are exclusively RyR2-specific. In this study, we investigated the antiarrhythmic effect of a novel high-affinity and selective RyR2 modulator, Ryanozole, which we have recently developed, using CPVT mouse models harboring mutant RyR2s.

Methods: *In vitro* effects of Ryanozole were assessed by ER Ca^{2+}-based assay using RyR2-expressing HEK293 cells and [^{3}H]Ryanodine binding assay. To evaluate *in situ* and *in vivo* effects of Ryanozole, we used two lines of mice, RyR2-R420W and -K4750Q, with different arrhythmia severity. Intracellular Ca^{2+} signals were monitored with Cal520 in isolated ventricular cardiomyocytes. Antiarrhythmic effects of drugs (Ryanozole, flecainide, verapamil and atenolol) were evaluated by electrocardiography (ECG) in anesthetized R420W and K4750Q mice challenged with catecholamine and in conscious K4750Q mice that showed activity-dependent arrhythmias. ECG and echocardiographic parameters were analyzed before and after drug administration.

Results: Ryanozole suppressed wild type and mutant RyR2s with 15−40 nM IC_{50}, and the inhibition was more potent at low $[Ca^{2+}]_{cyt}$ than at high $[Ca^{2+}]_{cyt}$. In isolated cardiomyocytes, Ryanozole suppressed isoproterenol-induced Ca^{2+} waves and sparks without affecting action potential-evoked Ca^{2+} transients. Ryanozole effectively suppressed adrenaline-induced arrhythmias and terminated spontaneous arrhythmias during daily activity. Unlike conventional antiarrhythmic drugs, Ryanozole did neither affect ECG parameters nor impair cardiac contractility.

Conclusions: This study suggests that selective suppression of RyR2, especially at low $[Ca^{2+}]_{cyt}$, is highly effective in preventing arrhythmias in CPVT without impairing cardiac function. Ryanozole is a promising therapeutic candidate for the prevention and treatment of arrhythmias caused by RyR2 hyperactivation.

Non-standard Abbreviations and Acronyms.

Ad/Caf: adrenaline–caffeine

$[Ca^{2+}]_{cyt}$: cytosolic Ca^{2+} concentrations

CPVT: catecholaminergic polymorphic ventricular tachycardia

DMSO: dimethyl sulfoxide

ECG: electrocardiography

ICD: Implantable cardioverter defibrillator

NCX: Na^{+}-Ca^{2+} exchanger

RyR2: type 2 ryanodine receptor

RyR2-S3A: phospho-null triple mutant S2807A/S2813A/S2030A RyR2

RyR2-S3D: phospho-mimetic triple mutant S2807D/S2813D/S2030D RyR2

VT: ventricular tachycardia

WT: wild-type

Introduction

Type 2 ryanodine receptor (RyR2) is a Ca^{2+} release channel in the sarcoplasmic reticulum (SR) and plays a pivotal role in cardiac muscle excitation–contraction coupling. Genetic variants in the *RYR2* gene, both gain-of-function and loss-of-function ones, are associated with arrhythmogenic heart diseases, including catecholaminergic polymorphic ventricular tachycardia (CPVT) and idiopathic ventricular fibrillation (IVF) [1-6], and more than 300 arrhythmogenic variants have been reported so far. Of these, the *RYR2* variants associated with CPVT are of the gain-of-function type and account for approximately 90% of diseases associated with *RYR2*. The severity of arrhythmias in these variant carriers varies widely: some develop ventricular arrhythmias for the first time in adulthood in response to severe stress, while others suffer frequent arrhythmias from infancy in response to mild exercise or emotional stress [7]. The main cellular mechanism underlying CPVT is as follows: when gain-of-function mutant RyR2 is further activated during strong sympathetic stimulation, spontaneous Ca^{2+} release occurs without Ca^{2+} entry from L-type Ca^{2+} channel (LTCC). This in turn activates inward sodium-calcium exchanger (NCX) currents to cause delayed afterdepolarization and triggered activity, leading to arrhythmias [8-10]. The severity of the arrhythmia is thought to depend mainly on the degree of enhancement of RyR2 activity caused by each mutation, which determines the feasibility of spontaneous Ca^{2+} release [11].

CPVT is a disease for which current treatments have limited effectiveness. The major conventional antiarrhythmic drugs for CPVT include β-blockers, the Na^{+} channel blocker flecainide, and the Ca^{2+} channel blocker verapamil [12,13]. However, these drugs sometimes fail to prevent arrhythmias [14]. In addition, these drugs have inevitable adverse effects on cardiac function: Na^{+} channel blockers decrease cardiac conduction velocity by slowing upstroke of action potentials in atrial and ventricular cells, whereas β-blockers and Ca^{2+} channel blockers can suppress atrioventricular conduction and weaken cardiac contractility. Implantable cardioverter defibrillators (ICDs) are recommended in severe cases, but patients with ICDs are at risk of receiving inappropriate shocks,

which may worsen the arrhythmias [15,16]. There is thus an urgent need to develop drugs with greater efficacy and fewer side effects for treating CPVT caused by various *RYR2* variants.

On the basis of the pathogenic mechanisms of CPVT described above, it is highly anticipated that compounds that stabilize RyR2 in the closed state can suppress arrhythmias. However, no RyR2-specific drugs are currently available. To date, several drugs and compounds have been proposed to prevent the hyperactivity of mutant RyR2, including flecainide, dantrolene, carvedilol, EL20, and S107 [17-20]. However, many of these drugs also interact with molecules other than RyR2 and may have multiple effects on cardiac function. Thus, there is no proof of concept for the efficacy of specific stabilization of RyR2 in CPVT.

Recently, we established an effective platform for screening RyR2 modulators using HEK293 cells expressing RyR2 and ER Ca^{2+} sensor protein R-CEPIA1er [21-23]. We identified several compounds that selectively modulate RyR2 activity without affecting RyR1 or RyR3 [23,24]. In addition, we developed a series of tetrazole compounds (Ryanozoles) that selectively stabilize RyR2 in the closed state [24]. Among these Ryanozoles, TMDJ-035, which we will refer to here as Ryanozole, had the highest affinity for RyR2 with IC_{50} of 15 nM [24]. In this study, we found that Ryanozole suppresses the RyR2 activity more strongly at low cytoplasmic Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$) than that at high $[Ca^{2+}]_{cyt}$. We investigated the in vitro and in vivo effects of Ryanozole on two lines of mice with CPVT-associated RyR2 mutations. Ryanozole effectively suppressed abnormal Ca^{2+} signals without affecting action potential-evoked Ca^{2+} transients in the isolated myocytes and prevented induced or spontaneous arrhythmias in these mice. Our results provide the first clear evidence that specific stabilization of hyperactive RyR2 in the closed state is effective for preventing and treating CPVT. Notably, Ryanozole did not affect parameters of ECG and echocardiogram. Ryanozole should be a promising therapeutic candidate for the treatment of CPVT without severe side effects.

Methods

Methodological details are presented in the Supplemental Material. All animal-handling procedures were in accordance with the guidelines and approved by the ethics committees of Juntendo University School of Medicine and the National Center of Neurology and Psychiatry (NCNP).

Statistics

Data are presented as mean±SD. Unpaired Student's t test was used for comparisons between two groups. One-way or two-way analysis of variance (ANOVA), followed by Tukey's test, was performed to compare three or more groups. Two-tailed tests were used for all analyses. Statistical analysis was performed using Prism v10 (GraphPad Software, Inc., La Jolla, CA, USA). A P-value < 0.05 was considered significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; ****$P < 0.001$; ns: not significant. Symbols always indicate comparisons between the control (WT or vehicle-treated) and the other tested conditions.

Results

Ryanozole stabilizes various mutant RyR2s in the closed state

Figure 1a shows the chemical structure of Ryanozole TMDJ-035 (denoted here as "Ryanozole") (Fig. 1a), which selectively modulates RyR2 among the three RyR subtypes [24]. Prior to evaluation in mouse models, we further characterized the effect of Ryanozole on RyR2. First, we tested its effects on the wild type (WT) and various RyR2 mutants using an ER Ca^{2+}-based assay [22,23]. In this assay, we measured ER Ca^{2+} signals in HEK293 cells expressing RyR2 and R-CEPIA1er, a fluorescent ER Ca^{2+} sensor protein, and obtained ratios (F/F_0) of fluorescence intensity before (F_0) and after addition of the compounds (F) as an indicator of RyR2 inhibition (Fig. S1a, b). Ryanozole dose-dependently increased the F/F_0 ratio with IC_{50} of 16.5 nM in WT cells (Fig. 1b) and in CPVT-linked mutant cells R420W, K4750Q, R2474S, and R4497C with IC_{50}s of 20.0−42.5 nM (Fig. S1c−f). We also examined the effects of Ryanozole on RyR2s carrying a phospho-null triple mutation (S2807A/S2813A/S2030A) (RyR2 S3A) or a phospho-mimetic triple mutation (S2807D/S2813D/S2030D) (RyR2 S3D) at the three major phosphorylation sites [23,25-27], as RyR2 has been reported to undergo post-translational modifications such as phosphorylation. The effect of Ryanozole was indistinguishable between the two mutant RyR2s, suggesting its phosphorylation-independent effect (Fig. S1g, h). These results suggest that Ryanozole stabilizes RyR2 in the closed state regardless of the presence of disease-associated mutations or phosphorylation at major phosphorylation sites.

Ryanozole strongly suppresses RyR2 activity at low but not high $[Ca^{2+}]_{cyt}$

The inhibitory effect of Ryanozole on RyR2 was further confirmed by another method, [^{3}H]ryanodine binding assay, which provides a useful measure of the RyR activity based on the specific binding of ryanodine to open channels [28,29]. Ryanozole reduced the peak of Ca^{2+}-dependent [^{3}H]ryanodine binding of RyR2 and shifted the Ca^{2+} dependence to the right (Fig. 1c). Dose

dependence of Ryanozole at pCa 5.0 indicates that the IC_{50} is 10 nM (Fig. 1d), which is close to that obtained with cell-based assay (Fig. 1b). Interestingly, IC_{50} at pCa 3.9 is 10 times greater (115 nM) than that at pCa 5.0, suggesting that the suppressive effect of Ryanozole was much less marked at higher Ca^{2+} concentrations. Taken together, these results suggest that Ryanozole suppresses the RyR2 activity more strongly at low cytoplasmic Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$) near basal/resting levels than at high $[Ca^{2+}]_{cyt}$.

Two RyR2 mutations for CPVT mouse models

We previously demonstrated that the severity of arrhythmia in CPVT patients carrying *RYR2* variants can be predicted from the RyR2 channel activity expressed in HEK293 cells [11]. CPVT patients with mild to moderate gain-of-function *RYR2* may develop infrequent ventricular arrhythmias upon intense exercise or emotional stress, while patients with strong gain-of-function *RYR2* develop frequent arrhythmias from a young age upon moderate daily exercise [11]. Because it is desirable for a drug to have two functions in treating arrhythmias (i.e., preventing the generation of catecholamine-induced arrhythmias and stopping arrhythmias that are already occurring during daily exercise), two RyR2 mutant mouse lines with different arrhythmia severity, RyR2-R420W and -K4750Q, were selected to investigate the drug effects. As shown in Fig. S2a, RyR2 activity determined with [^{3}H]ryanodine binding assay was moderately increased in R420W, whereas it was more enhanced in K4750Q in microsomes obtained from HEK293 cells expressing homozygous RyR2.

Ryanozole suppresses abnormal Ca^{2+} release events in cardiomyocytes isolated from RyR2-R420W mice

An RyR2-R420W mouse model was previously created based on cases of sudden unexplained deaths in 13- and 33-year-old victims [30,31]. Arrhythmias have been reported to be induced by the injection of a mixture of adrenaline (2 mg/kg) and caffeine (120 mg/kg), and the frequency of arrhythmias was

found to be higher in homozygous than in heterozygous mice [32]. This difference in arrhythmia susceptibility can be well explained by differences in RyR2 activity as determined from [^{3}H]ryanodine binding in microsomes prepared from mouse ventricles (Fig. S2b, left). We used homozygous R420W mice (hereafter referred to as "R420W mice") in this study.

We first tested the in vitro effect of Ryanozole on R420W mice using isolated cardiomyocytes. It is well known that abnormal Ca^{2+} release events such as Ca^{2+} waves lead to arrhythmias in CPVT. In R420W cells regularly stimulated at 0.5 Hz, many abnormal Ca^{2+} signals were induced by 100 nM isoproterenol. The addition of Ryanozole at 30 and 100 nM effectively reduced these abnormal signals (Fig. 2a). In a total of 74–132 cells (pooled data from the previous study and the present study), the mean rate of occurrence of abnormal Ca^{2+} signals was 0.11±0.39 events·s^{-1}·$cell^{-1}$ with isoproterenol, which was significantly reduced to 0.02±0.13 events·s^{-1}·$cell^{-1}$ and to 0.003±0.018 events·s^{-1}·$cell^{-1}$ by 30 nM and 100 nM Ryanozole, respectively (Fig. 2b). We also performed time-resolved line-scan imaging analysis to investigate whether Ryanozole can suppress different types of Ca^{2+} release events, especially Ca^{2+} sparks, which are hardly detected by whole-cell fluorescence intensity. In the cells in which frequent Ca^{2+} sparks were induced by isoproterenol (Fig 2c, d) (Video S1), Ryanozole (100 nM) almost completely abolished Ca^{2+} sparks as well as Ca^{2+} waves and triggered activities (Fig. 2c, d) (Video S2).

Ryanozole prevents induced arrhythmias in R420W mice

We next tested the in vivo effect of Ryanozole on the induced arrhythmias using R420W mice. Isoflurane-anesthetized R420W mice aged between 8 and 12 weeks were subjected to an adrenaline and caffeine stress test based on a previous report [32]. In individual animals, a baseline ECG was recorded for 10 min, followed by drug or vehicle injection; 10 min later an adrenaline (2 mg/kg)–caffeine (120 mg/kg) (Ad/Caf) mixture was injected, and the ECG was recorded for another 30 min. Typical ECG records are shown in Fig. 3a. In the vehicle-pretreated mouse, sustained ventricular

arrhythmias were frequently observed (Fig. 3a, top). In the mouse pretreated with 30 mg/kg Ryanozole, such ventricular arrhythmias were largely suppressed (Fig. 3a, bottom). We classified ECG waveforms into four types: normal rhythm, T-wave alternans, bigeminy, and ventricular tachycardia (VT) (Fig. 3b). The proportion of time for each rhythm/arrhythmia during 30 min after injection was determined for individual mice and the mean values are plotted in Fig. 3c. Severe arrhythmias such as VT were frequently observed in the vehicle-pretreated mice. Ryanozole dose-dependently suppressed the proportion of VT at 3 mg/kg or higher (Fig. 3c). The proportion of normal rhythm was significantly higher at 30 mg/kg Ryanozole.

Creation of K4750Q mice

We newly created a knock-in mouse carrying the K4750Q mutation as a severe CPVT model for the evaluation of Ryanozole (Fig. S3a). The heterozygous *RYR2*-K4750Q variant has been reported to cause frequent polymorphic ventricular tachycardia in a young, 6-year-old carrier[7] and to greatly promote RyR2 activity in an *in vitro* expression system[11,33]. Heterozygous RyR2-K4750Q mice were born and grew normally. In contrast, homozygous RyR2-K4750Q mice were born very rarely (3% of the total litters in heterozygous mating) (Fig. S3b) and, even when they were born, they were small and mostly died before the age of 3 months. [^{3}H]Ryanodine binding in microsomes from heterozygous K4750Q mouse ventricles was substantially higher than that from homozygous R420W mice (Fig. 4a, see also Fig. S2b), although that from the homozygous K4750Q ventricle was the highest (Fig. S2b, right). The body weight, heart to body weight ratio (HW/BW), and lung to body weight ratio (LW/BW) of heterozygous K4750Q mice were not significantly different from those of WT and R420W (Fig. S3c) and basal ECG parameters (PR interval, QRS interval, and heart rate) were normal (Fig. S3d), consistent with the clinical consensus that typical CPVT is not associated with cardiac hypertrophy or heart failure[13,34]. We used heterozygous K4750Q mice (hereafter referred to as “K4750Q mice”) as another CPVT mouse model to evaluate the antiarrhythmic effects of drugs.

Ryanozole suppresses abnormal Ca^{2+} release events in cardiomyocytes isolated from K4750Q mice

We determined the in vitro effect of Ryanozole on the K4750Q mice using isolated cardiomyocytes. In the presence of 100 nM isoproterenol, more than 90% of RyR2-K4750Q cells were spontaneously active with abnormal Ca^{2+} signals, which was a higher rate than for R420W cells (Fig. 4b). The mean rate of occurrence of abnormal Ca^{2+} signals was 1.23±1.51 events·s^{-1}·$cell^{-1}$ with 100 nM isoproterenol (Fig. 4c). Ryanozole effectively suppressed the abnormal Ca^{2+} signals in a dose-dependent manner: the mean rate of occurrence was significantly reduced to 0.21±0.43 events·s^{-1}·$cell^{-1}$ and to 0.016±0.060 events·s^{-1}·$cell^{-1}$ by 30 nM and 100 nM Ryanozole, respectively (Fig. 4b, c). Time-resolved line-scan imaging revealed that, in the K4750Q cardiomyocytes showing isoproterenol-induced Ca^{2+} sparks (Fig. 4d, e) (Video S3), Ryanozole (100 nM) strongly suppressed the occurrence of Ca^{2+} sparks as well as Ca^{2+} waves and triggered activities (Fig. 4d, e) (Video S4).

Ryanozole suppresses induced arrhythmias in K4750Q mice

We next tested the in vivo effect of Ryanozole on the K4750Q mice. In contrast to the R420W mice, ventricular arrhythmias could be induced in the K4750Q mice by adrenaline alone without caffeine. The experimental protocol was basically the same as that for R420W except that adrenaline alone was used instead of the Ad/Caf mixture. Typical ECG records are shown in Fig. 5a. In the vehicle-pretreated mice, arrhythmias were frequently observed, in which T-wave alternans and bigeminy were included (Fig. 5a, top). Such arrhythmias were largely suppressed in the Ryanozole-pretreated mice (Fig. 5a, bottom). Ryanozole dose-dependently suppressed the rate of arrhythmias at 3 mg/kg or higher (Fig. 5b). At 30 mg/kg Ryanozole, almost no arrhythmias were induced by adrenaline administration.

Ryanozole suppresses spontaneous arrhythmias in K4750Q mice

The *RYR2*-K4750Q variant has been reported to cause frequent polymorphic ventricular tachycardia in daily life [7]. We therefore examined whether K4750Q mice also show spontaneous arrhythmias during daily activities. ECG transmitters were implanted subcutaneously in mice at 2–3 months of age and continuous ECG recordings were obtained up to 5 months of age. WT and R420W mice showed no arrhythmias, but all K4750Q mice showed intermittent ventricular arrhythmias, especially at night (Fig. 5c).

We tested the effect of Ryanozole on spontaneous arrhythmias using RyR2-K4750Q mice. Because mice were active at night and showed more frequent arrhythmias, vehicle or Ryanozole was injected in the evening, between 7 and 8 p.m., and ECG records were analyzed for 3 h thereafter. Individual mice were administered various doses of Ryanozole or vehicle in a random order within 3 weeks. While numerous ventricular arrhythmias were observed after the injection of vehicle (Fig. 5d top), they were substantially suppressed after injection of 1 mg/kg (Fig. 5d middle) and almost completely disappeared with 30 mg/kg Ryanozole (Fig. 5d, bottom). On average, the rate of ventricular arrhythmia was significantly reduced with 1 mg/kg Ryanozole or more for at least 2 h (Fig. 5e). Although the suppressive effect of Ryanozole diminished over time, a significant effect still remained after 3 h at 30 mg/kg. Importantly, Ryanozole was also effective upon oral administration, for which higher doses were required to obtain effective suppression (Fig. 5f, g).

Ryanozole does not impair cardiac function

Conventional antiarrhythmic drugs for CPVT have adverse effects such as conduction disturbances, bradycardia, and decreased cardiac contractility. To determine whether Ryanozole affects cardiac function, we examined its effects on normal Ca^{2+} transients in cardiomyocytes and ECG parameters and echocardiographic parameters in vivo in WT and K4750Q mice.

Action potential-evoked Ca^{2+} transients were recorded in ventricular myocytes stimulated at

0.33 Hz (Fig. 6a), and peak amplitude and 50% decay time were compared at various concentrations of Ryanozole (Fig. 6b). In WT cells, peak F/F_0 was significantly decreased, whereas 50% decay time was significantly increased by Ryanozole at 100 and 300 nM, but not at 30 nM (Fig. 6b, top). The effects of Ryanozole were fully reversed by washout of the drug. In RyR2-K4750Q cells, meanwhile, no significant change in either peak F/F_0 or 50% decay time was observed (Fig. 6b, bottom). In WT cells, it is likely that inhibition of RyR2 by Ryanozole leads to a decrease in peak amplitude, which then causes prolonged decay of Ca^{2+} transients via the attenuation of Ca^{2+}-dependent inactivation of the channel. In RyR2-K4750Q cells, inhibition of leaky RyR2 could result in an increase in the SR Ca^{2+} content, which could mask the inhibitory action of the drug on RyR2.

We also examined the effects of Ryanozole on action potential characteristics in cardiomyocytes from K4750Q mice (Fig. S4). Abnormal membrane potentials together with abnormal Ca^{2+} signals were frequently observed in K4750Q cardiomyocytes (Fig. S4a, top). In the presence of 100 nM Ryanozole, such abnormal events were significantly suppressed (Fig. S4a, bottom; b). Fig. S4c compares the action potential parameters. Resting potentials (RP), action potential (AP) amplitudes, dV/dt max, and action potential durations (APD) at 90% and 50% repolarization (APD_{90} and APD_{50}, respectively) were rarely affected by Ryanozole at 100 nM (Fig. S4c). Notably, mean APD_{90} was shortened by half by the application of Ryanozole (from 111.0 ± 121.4 ms to 57.7 ± 57.4 ms, $p = 0.195$), although this did not reach significance. This can be explained by the suppression of Ca^{2+} release by Ryanozole, which results in reduction in inward NCX current at the late repolarization phase.

Figure 6c shows typical ECG traces before and after the intraperitoneal injection of Ryanozole. Relative changes in ECG parameters, namely, heart rate, PR interval, and QRS interval, after drug administration are plotted in Fig. 6d. There are no significant differences in the heart rate, PR interval, and QRS interval between vehicle and 30 mg/kg Ryanozole administration.

We also assessed the effects of Ryanozole on cardiac function by echocardiography (Fig. 6e,

f) in WT and K4750Q mice. Intraperitoneal injection of Ryanozole did not change the ejection fraction (EF) in WT mice (Fig. 6e, left; and 6f, left). Interestingly, Ryanozole significantly enhanced EF in K4750Q mice (Fig. 6e, right; and 6f, right), in which the basal EF was marginally lower than in the WT (Fig. 6f). In addition, Ryanozole preserved the diastolic index early (E)/late (A) mitral inflow peak velocity ratio (E/A) in WT and K4750Q mice (Fig. 6g). Taken together, these results indicate that Ryanozole has the advantage of having virtually no adverse effects on cardiac function or electrocardiograms.

Conventional antiarrhythmic drugs affect ECG parameters at their effective doses

To compare the antiarrhythmic effects of Ryanozole with conventional drugs, which suppress arrhythmias by acting on voltage-dependent Na^+ channel ($Na_v1.5$), Ca^{2+} channel ($Ca_v1.2$), or adrenergic β_1-receptor [13], we tested these drugs on our CPVT model mice. The antiarrhythmic drugs examined here included flecainide (20 mg/kg), verapamil (2.5 mg/kg), and atenolol (2 mg/kg). The doses of these drugs were selected based on previous reports [17,35,36]. These drugs actually significantly reduced the frequency of induced ventricular tachycardia in R420W mice (Fig. S5a) and spontaneous arrhythmias in K4750Q mice (Fig. S5b). However, the antiarrhythmic effects were weaker than for Ryanozole (see Figs. 3 and 5), at least under the experimental conditions that we employed.

These drugs affected ECG parameters to different extents (Fig. S5c, d). Flecainide substantially prolonged PR and QRS intervals and reduced heart rate (Fig. S5c, d), verapamil slightly but significantly lengthened PR intervals (Fig. S5d, middle), while atenolol significantly reduced heart rate (Fig. S5d, left). These adverse effects appear to be unavoidable because they emerge as a result of the primary mechanism of action.

Discussion

Here, we explored the therapeutic effects of the RyR2-specific stabilizer Ryanozole on ventricular arrhythmias using two lines of CPVT-associated mutant RyR2 mice. We showed that Ryanozole suppresses Ca^{2+} release more strongly at low $[Ca^{2+}]_{cyt}$ than at high $[Ca^{2+}]_{cyt}$, and that it effectively suppresses arrhythmogenic spontaneous Ca^{2+} sparks and Ca^{2+} waves without significantly affecting physiological Ca^{2+} transients. We demonstrated that Ryanozole is very effective in preventing catecholamine-induced arrhythmias and stopping spontaneous arrhythmias during daily activity. Because Ryanozole does not impair cardiac function at its effective doses, the drug is strongly expected to be a safe and effective option for treating arrhythmia.

It has been postulated that drugs with RyR2-stabilizing action have an antiarrhythmic effect on CPVT and other ectopic arrhythmias. Actually, several compounds/drugs with RyR2-modulating effects have been reported to have antiarrhythmic activity, although most of these compounds still need to be improved in terms of their efficacy and/or specificity towards RyR2. EL20, an analog of tetracaine [37,38], and the unnatural verticilide [39] have been reported to suppress RyR2 and arrhythmias in arrhythmic model mice. A group of compounds such as Rycal drugs that stabilize the association of FK binding protein 12.6 (FKBP12.6/calstabin2) with RyR2 has also been reported to ameliorate abnormal Ca^{2+} release in cardiomyocytes [40,41]. In addition, flecainide is an effective antiarrhythmic drug that mainly targets Na^{+} channels, and its action on RyR2 has been reported to contribute to its antiarrhythmic effect [17,42,43]. Meanwhile, a β-blocker, carvedilol, has also been reported to suppress abnormal Ca^{2+} release via RyR2 [18,44]. Compared with these reagents, Ryanozole is the most potent and specific RyR2 stabilizer. This study provides the first proof of concept that specific stabilization of RyR2 in the closed state is effective in the prevention and treatment of CPVT.

The modulating effects of Ryanozole are not mediated by the associated protein, FKBP12.6, because they inhibited FKBP12.6-unbound RyR2 in HEK293 cells that do not express FKBP12.6. Ryanozole also appears to suppress both calmodulin-unbound and calmodulin-bound RyR2, as the

amount of calmodulin in RyR2-expressing HEK293 cells is insufficient in terms of the molar ratio compared with the amount of RyR2 [45]. It has been reported that RyR2 is subject to post-translational modifications such as phosphorylation [23,25-27]. However, the effect of Ryanozole was indistinguishable between the phospho-null triple mutant (S3A) and phospho-mimetic triple mutant (S3D), suggesting its phosphorylation-independent action (Fig. S1g, h). Thus, Ryanozole may directly target RyR2 to stabilize its activity in the closed state. Importantly, Ryanozole similarly modulates RyR2 carrying four different CPVT mutations, which are localized in the N-terminal, central, and C-terminal regions (Fig. S1c−f). These findings suggest that Ryanozole may be useful for a wide range of CPVT patients with different RyR2 mutations.

Ryanozole strongly suppressed the occurrence of Ca^{2+} waves and Ca^{2+} sparks with relatively minor effects, if any, on action potential-evoked Ca^{2+} transients in isolated cardiomyocytes from CPVT model mice (Fig. 2b, Fig. 4c). This may be related to the virtual absence of effects of Ryanozole on cardiac function (Fig. 6e, f). This important property can be explained by the mechanism of action of the compound: Ryanozole strongly suppresses RyR2 activity at low $[Ca^{2+}]_{cyt}$, while mildly suppressing maximal activity at high $[Ca^{2+}]_{cyt}$ (Fig. 1d). As a result, local Ca^{2+} waves and Ca^{2+} sparks generated at low $[Ca^{2+}]_{cyt}$ may be effectively suppressed, but action potential-evoked Ca^{2+} transients caused by massive Ca^{2+} influx via L-type Ca^{2+} channels may be less suppressed. Similar results were also obtained with other RyR2 inhibitors such as chloroxylenol and riluzole [23]. They also reduced the affinity for activating Ca^{2+} of [^{3}H]ryanodine binding and mildly suppressed peak activity, and also effectively suppressed the occurrence of Ca^{2+} waves and Ca^{2+} sparks with minor effects on action potential-evoked Ca^{2+} transients. Identification of the binding site of Ryanozole and the resulting conformational changes in RyR2 would provide keys to its mechanism of action specific to RyR2.

In addition to the findings on Ryanozole, this study provides insights into the procedure for testing drug effects using CPVT model mice. We employed two RyR2 mutant lines with different arrhythmia severity. Mice with homozygous RyR2-R420W mutation show a moderate phenotype and require strong stimulation [i.e., catecholamine (adrenaline or isoproterenol) plus caffeine] to exhibit arrhythmias, as in typical CPVT model mice [46-49]. We also generated RyR2-K4750Q mice, which have a more severe phenotype than R420W, exhibiting spontaneous arrhythmias during daily activity. We previously reported that RyR2-I4093V mice, which were created by a random mutation, showed numerous spontaneous arrhythmias and are useful for assessing antiarrhythmic effects [50]. RyR2-K4750Q mice have milder arrhythmia symptoms than I4093V mice, but still exhibit spontaneous intermittent arrhythmias. Thus, mice harboring highly augmented RyR2 mutations may have the propensity to suffer spontaneous arrhythmias and appear to be very useful for evaluating the arrhythmia-blocking effect of drugs and the time course of drug effects.

In this study, Ryanozole was proven to be highly effective in preventing catecholamine-induced arrhythmias and stopping arrhythmias that had already occurred. Moreover, the fact that there were no changes in ECG parameters or cardiac function suggests that Ryanozole is a promising candidate as a new class of antiarrhythmic drug. Because Ryanozole did not suppress cardiac function in the WT and rather improved it in K4750Q mice, Ryanozole may have advantages over conventional drugs in patients with heart failure. In addition, this important property raises the possibility that Ryanozole may also be effective against arrhythmias in heart failure itself, as increased RyR2 leakage has been reported in heart failure [40,41]. In the future, it will be necessary to confirm the drug's effect in large animals, including humans.

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Supplemental Material

Supplemental Methods

Figure S1–S5

Videos S1–S4

Disclosures

The authors declare no competing interests.

Corresponding authors

Correspondence to: Nagomi Kurebayashi (nagomik@juntendo.ac.jp) and Hiroyuki Kagechika (kage.chem@tmd.ac.jp).

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Figure legends

Fig. 1. Effects of Ryanozole on RyR2 activity.

a, Chemical structure of Ryanozole. **b,** Dose-dependent effects of Ryanozole on ER Ca^{2+} signals in HEK293 cells expressing WT RyR2 determined with FlexStation3. Data are mean±SD (n=5). **c, d,** Effects of Ryanozole on [^{3}H]ryanodine binding. **c,** Ca^{2+}-dependent [^{3}H]ryanodine binding in the presence of 0.1 and 1 μM Ryanozole. **d,** Dose-dependent effects of Ryanozole at pCa 5.0 (filled circles) and pCa 3.9 (open circles). Data are mean±SD (n=4).

Fig. 2. Effects of Ryanozole on spontaneous Ca^{2+} release in R420W cardiomyocytes.

a, Typical effects of Ryanozole on isoproterenol (Iso)-induced abnormal Ca^{2+} signals (mean fluorescence intensities in the ROIs in images captured through a 20× objective lens). **b,** Average number of abnormal Ca^{2+} release events before and after the application of Ryanozole (n=74–132, N=8). **c,** Typical time-based line scan images obtained with a 60× objective lens before (top) and after (bottom) Ryanozole application. **d,** Effects of Ryanozole on frequencies of Ca^{2+} waves, Ca^{2+} sparks, and triggered activity (n=11, N=3).

Fig. 3. Effect of Ryanozole on adrenaline-induced arrhythmias in R420W mice.

a, Typical effects of preinjected vehicle (top) and Ryanozole (bottom) on Ad/Caf-induced arrhythmias. Administrations of vehicle/drug and Ad/Caf mixture are indicated by the vertical green and red dotted lines, respectively. Ventricular arrhythmias are indicated with a horizontal thick red line. Note that, when ventricular arrhythmias occur, the HR traces become abnormal, often appearing as thick bands. Bars indicate 5 min. **b,** Classification of cardiac rhythms. ECG records were classified into four types: normal, T-wave alternans, bigeminy, and bidirectional ventricular tachycardia. **c,** Proportion of arrhythmias during 30 min after injection of Ad/Caf (N=10).

Fig. 4. RyR2 activity of K4750Q mice and effects of Ryanozole on spontaneous Ca^{2+} release in K4750Q cardiomyocytes.

a, RyR2 activity determined using [^{3}H]ryanodine binding in ventricular microsomes from heterozygous K4750Q mice (right). [^{3}H]Ryanodine binding in ventricular microsomes from homozygous R420W mice is also shown for comparison (left). **b,** Typical effects of Ryanozole on isoproterenol (Iso)-induced abnormal Ca^{2+} signals detected from the ROIs. **c,** Average number of abnormal Ca^{2+} release events before and after the application of Ryanozole (n=83, N=4). **d,** Typical time-based line scan images before (top) and after (bottom) Ryanozole application. **e,** Effects of

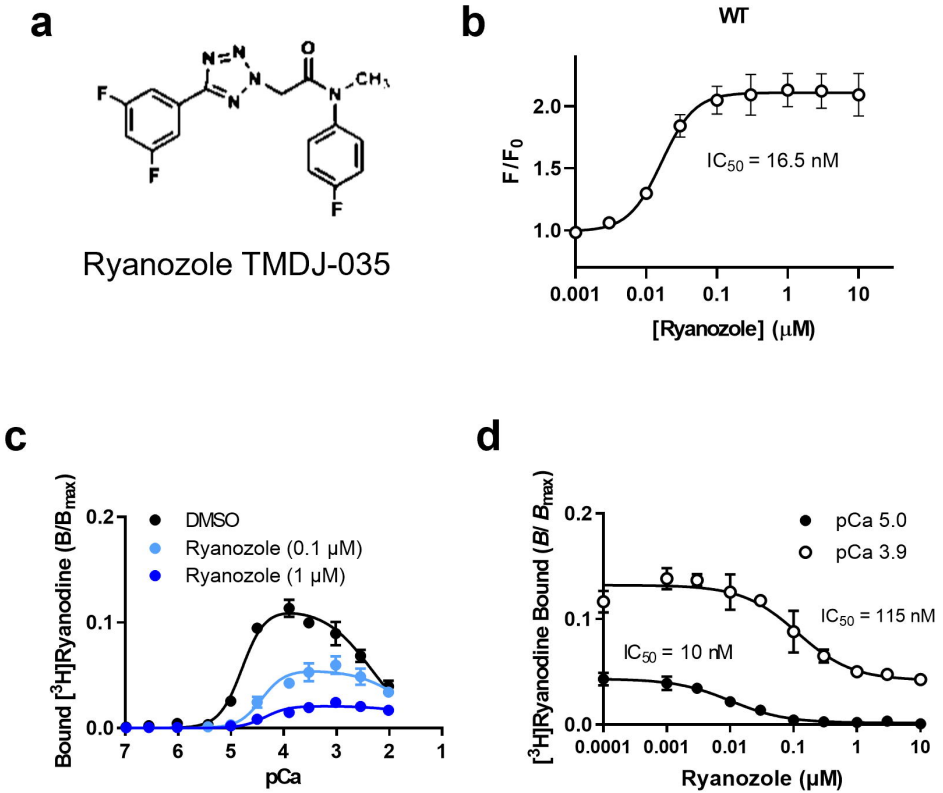
Ryanozole on frequencies of Ca^{2+} waves, sparks, and triggered activities (n=18, N=2).

Fig. 5 Effects of Ryanozole on induced and spontaneous arrhythmias in K4750Q mice.

a, Typical effects of pretreated vehicle (top) and Ryanozole (bottom) on adrenaline (Ad)-induced arrhythmias. Ventricular arrhythmias are indicated with a horizontal thick red line, whereas T-wave alternans is indicated with a thin red line. Black bars indicate 5 min. **b,** Proportion of arrhythmias during 30 min after injection of ad alone after pretreatment with Ryanozole (N=6−7). c, Typical 24-h ECG record. Red arrows indicate sustained ventricular arrhythmias. **d,** Typical ECG records after the intraperitoneal injection of vehicle (top), and Ryanozole at 1 mg/kg (middle) and 30 mg/kg (bottom). **e,** Average proportion of ventricular arrhythmia after the intraperitoneal injection of Ryanozole (n=8–10, N=4). **f,** Typical ECG records after the oral administration of vehicle (top) and Ryanozole (100 mg/kg). **g,** Average proportion of ventricular arrhythmia after the oral administration of Ryanozole (n=7–18, N=4). In **d** and **f**, thin black bar indicates 1 h, and thick red bars indicate ventricular arrhythmias.

Fig. 6 Effects of Ryanozole on action potential-evoked Ca^{2+} transients and cardiac functional properties.

a, Typical Ca^{2+} transients (detected from the ROIs) in the absence and presence of Ryanozole in cardiomyocytes isolated from WT (upper) and K4750Q mice (lower). **b,** Effects of Ryanozole on average peak amplitude and 50% decay time of Ca^{2+} transients in cardiomyocytes from WT and K4750Q mice (n=84−86, N=4). **c,** Typical ECG waveforms before and after the intraperitoneal injection of Ryanozole (30 mg/kg). **d,** Average ECG parameters before and after the intraperitoneal injection of Ryanozole (N=10). **e,** Representative images of M-mode echocardiography from WT mice (left) and K4750Q (right) before and after the intraperitoneal injection of Ryanozole. **f** and **g,** Graphs showing the data from echocardiography regarding left ventricular ejection fraction (EF) (**f**) and E/A ratios (g) before and after the intraperitoneal injection of Ryanozole (N=5).



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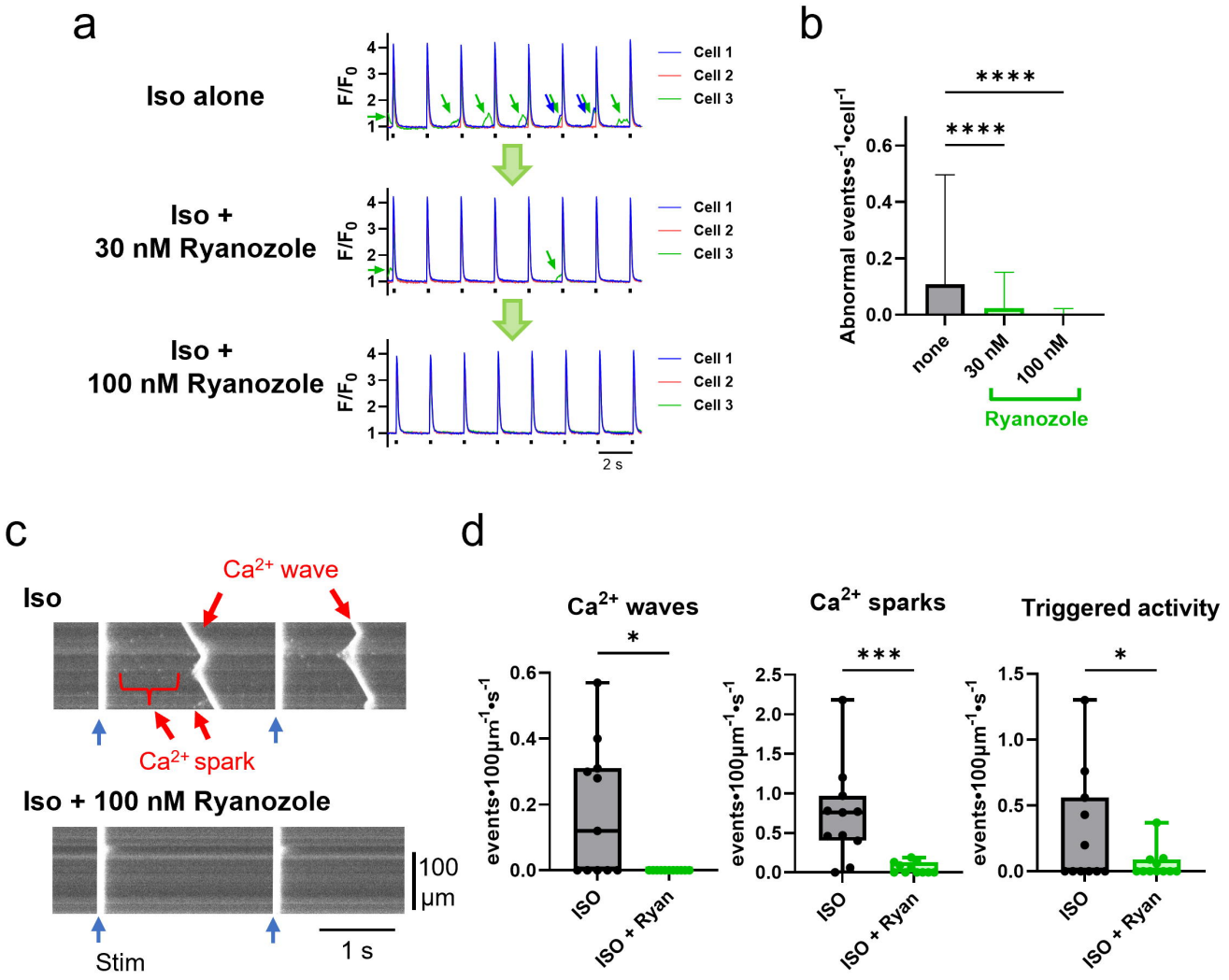

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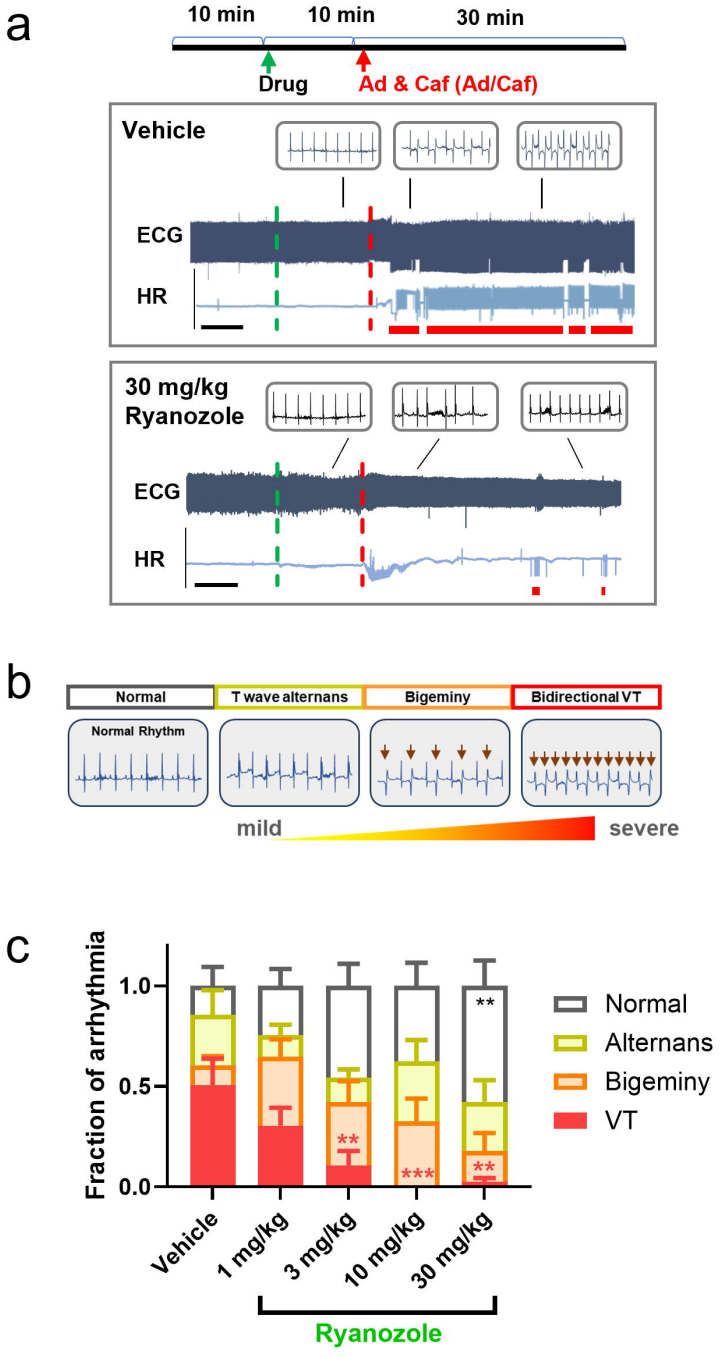

Fig. 3. Effect of Ryanozole on aderenaline-induced arrhythmias in R420W mice.
a, Typical effects of preinjected vehicle (top) and Ryanozole (bottom) on ad/caf-induced arrhythmias. Administrations of vehicle/drug and Ad/Caf mixture are indicated by the vertical green and red dotted lines, respectively. Ventricular arrhythmias are indicated with horizontal thick red line. Note that when ventricular arrhythmias occur, the HR traces become abnormal, often appearing as thick bands. Bars indicate 5 min. **b,** Classification of cardiac rhythms. ECG records were classified into four types: normal, T wave alternans, bigeminy, and bidirectional ventricular tachycardia (VT). **c,** Dose-dependent effects of preinjected Ryanozole on proportion of arrhythmias during 30 min after injection of Ad/Caf (N=10).

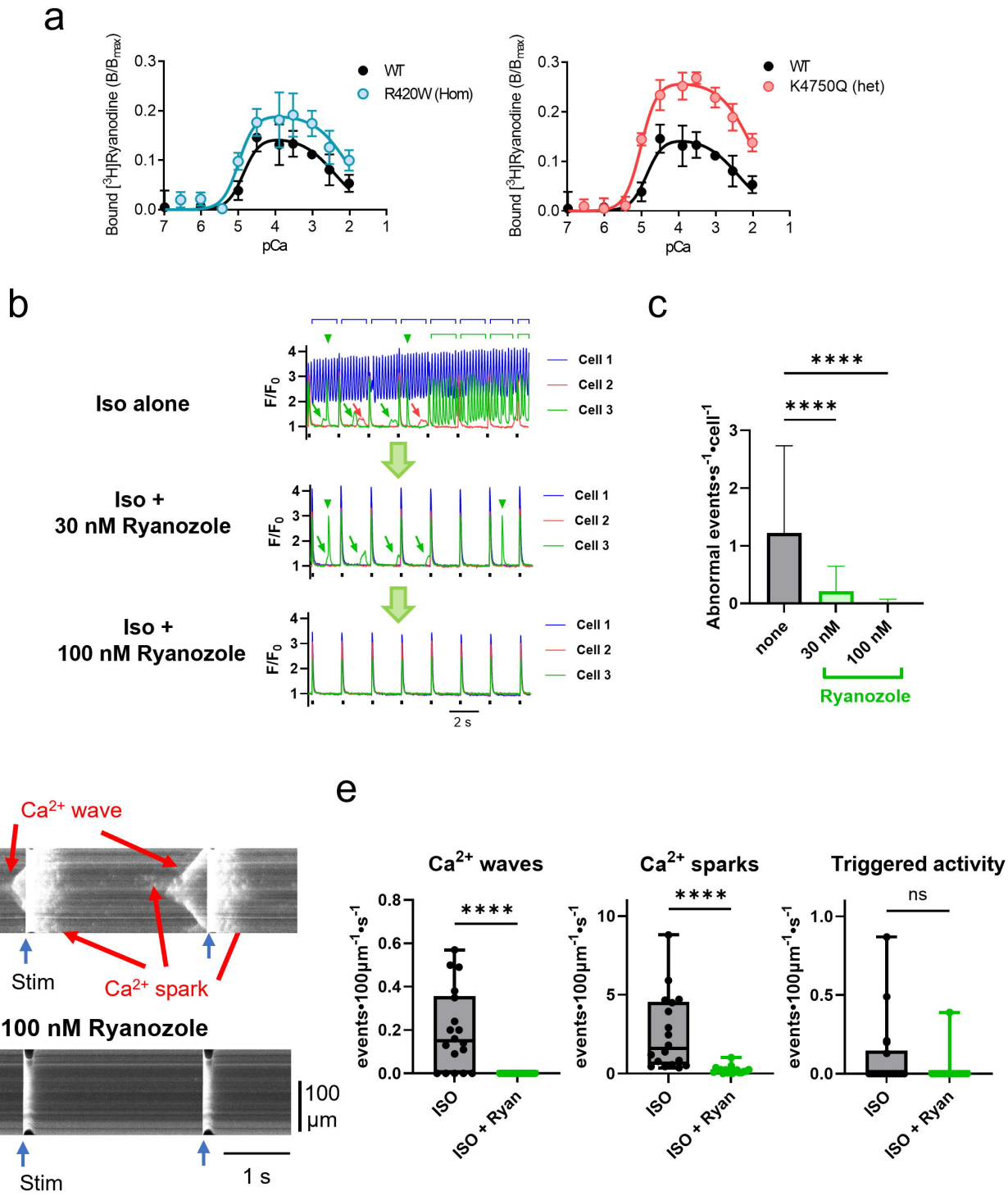

Fig. 4. RyR2 activity of K4750Q mice and effects of Ryanozole on spontaneous Ca^{2+} release in K4750Q cardiomyocytes.

a, RyR2 activity determined with [3H]ryanodine binding in ventricular microsomes from heterozygous K4750Q mice (right). [^{3}H]Ryanodine binding in ventricular microsomes from homozygous R420W mice is also shown for comparison (left). **b,** Typical effects of Ryanozole on isoproterenol (Iso)-induced abnormal Ca^{2+} signals detected from ROIs. **c,** Average number of abnormal Ca^{2+} release events before and after the application of Ryanozole (n=83, N=4). **d,** Typical time-based line scan images before (top) and after (bottom) the Ryanozole application. **e,** Effects of 100 nM Ryanozole on frequencies of Ca^{2+} waves, sparks and triggered activities (n=18, N=2).

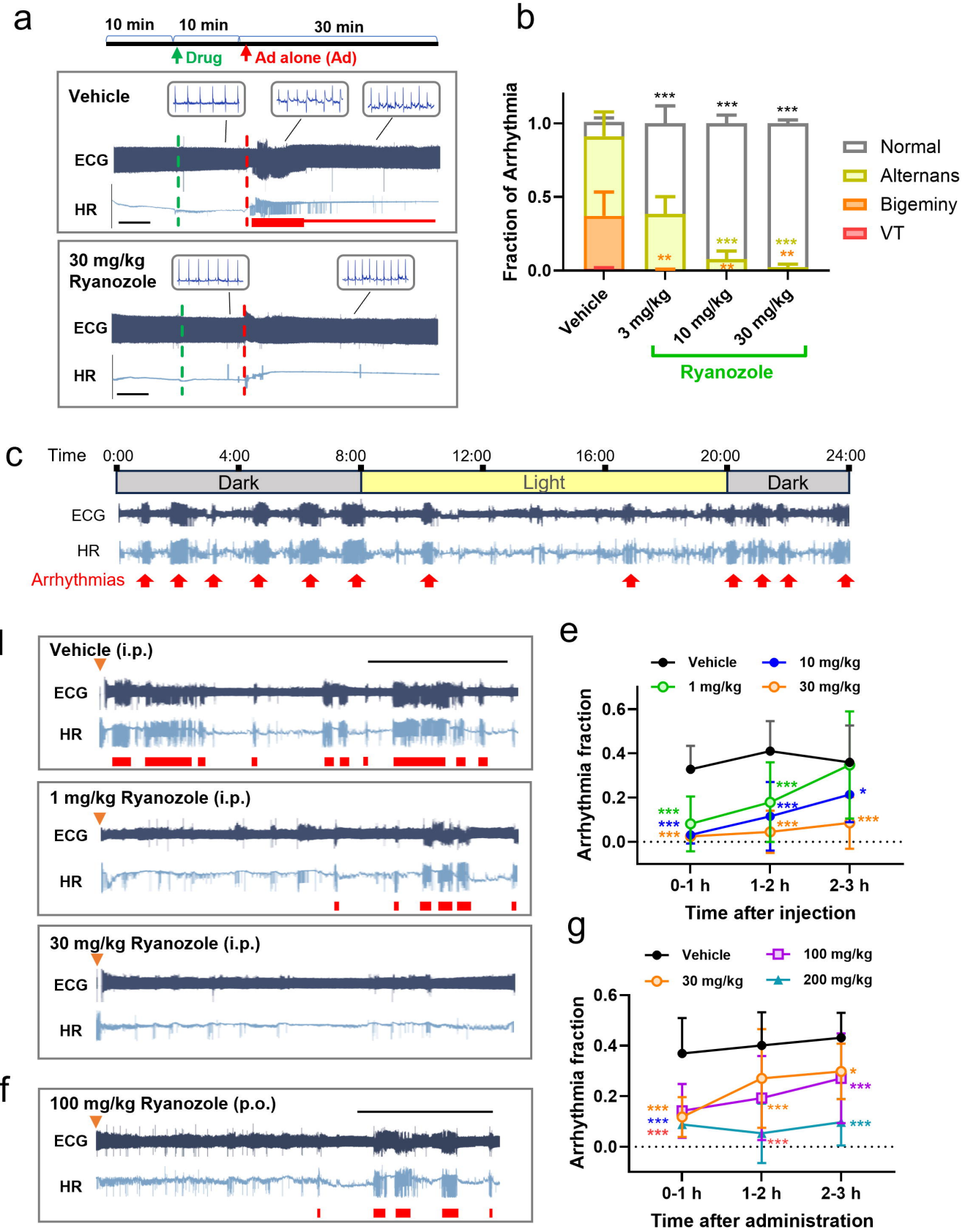

Fig. 5 Effects of Ryanozole on induced and spontaneous arrhythmias in K4750Q mice.
a, Typical effects of pretreated vehicle (top) and Ryanozole (bottom) on adrenaline (Ad)-induced arrhythmias. Ventricular arrhythmias are indicated with a horizontal thick red line, whereas T-wave alternans are indicated with a thin red line. Black bars indicate 5 min. **b,** Proportion of arrhythmias during 30 min after injection of ad alone after pretreatment with Ryanozole (N=6−7). **c,** Typical 24-hour ECG record. Red arrows indicate sustained ventricular arrhythmias. **d,** Typical ECG records after the intraperitoneal injection of vehicle (top), and Ryanozole at 1 mg/kg (middle) and 30 mg/kg (bottom). **e,** Average proportion of ventricular arrhythmia after the intraperitoneal injection of Ryanozole (n=8-10, N=4). **f,** Typical ECG records after the oral administration of vehicle (top) and Ryanozole (100 mg/kg). **g,** Average proportion of ventricular arrhythmia after the oral administration of Ryanozole (n=7-18, N=4). In **d** and **f,** thin black bar indicates 1 h, and thick red bars indicate ventricular arrhythmias.

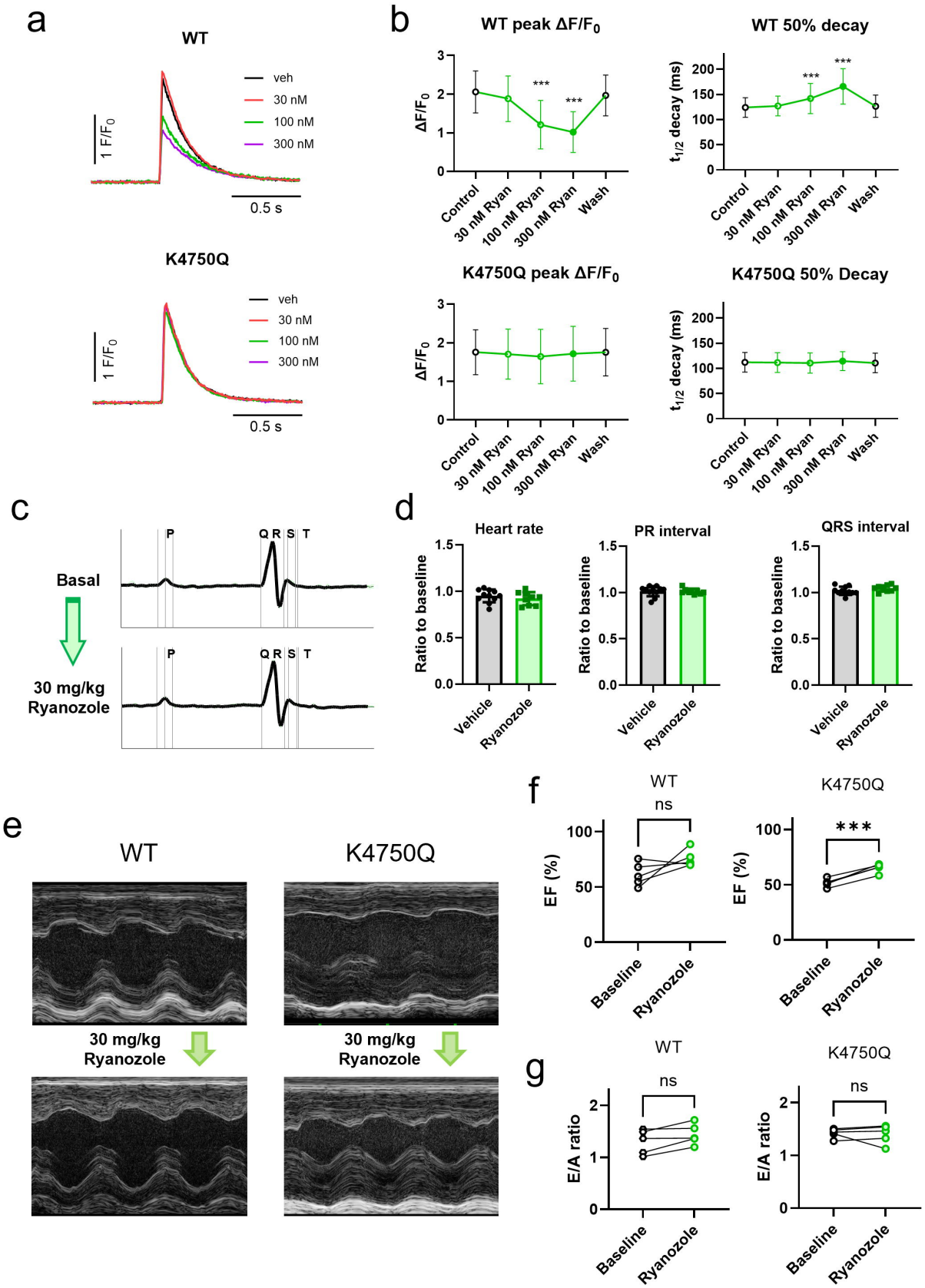

Fig. 6 Effects of Ryanozole on action potential-evoked Ca^{2+} transients and cardiac functional properties. **a,** Typical Ca^{2+} transients (detected from ROIs) in the absence and presence of Ryanozole in an isolated cardiomyocytes from WT (upper) and K4750Q mice (lower). **b,** Effects of Ryanozole on average peak amplitude and 50 % decay time of Ca^{2+} transients in cardiomyocytes from WT and K4750Q mice (n=84–86, N=4). **c,** Typical ECG wave forms before and after the intraperitoneal injection of Ryanozole (30 mg/kg). d, Average ECG parameters before and after the intraperitoneal injection of Ryanozole (N=10, 5 R420W and 5 K4750Q mice). **e,** Representative images of M-mode echocardiography from WT mice (left) and K4750Q (right) before and after the intraperitoneal injection of Ryanozole. **f and g,** Graphs showing the data from echocardiography regarding left ventricular ejection fraction (EF) (**f**) and E/A ratios (**g**) before and after the intraperitoneal injection of Ryanozole (N=5).