SUPPLEMENTAL MATERIAL

EXPANDED MATERIALS AND METHODS

Reagents

K201,4-[3{1-4-benzyl)piperidinyl}propionyl]-7-methoxy-2,3,4,5-tetrahydra-1,4-benzothi azepine (JTV519) was provided by Aetas Co, Ltd. (Tokyo Japan) and dantrolene was from Sigma. K201 is a small molecule (461Da), and it was developed by a pharmaceutical company but never released clinically yet. K201 was first found to have a protective effect against Ca^{2+} overload–induced myocardial injury, presumably by preventing the Ca^{2+} overload that results from increased release from the intracellular Ca^{2+} store.[1] Recent work including ours suggests that K201 has a channel-stabilizing effect on the defective channel gating of RyR2 in heart failure and lethal arrhythmia.[2,3]

Generation of knock-in mice with the RyR2 S2246L mutation (Supplemental Figure 1).

We isolated the template clone containing exons 39-55 from C57BL/6J mouse genomic library. The 5'-arm (7.6Kb fragment containing exons 39 to 42), the exon 43 fragment with the S2246L mutation and the 3'-arm (2.3Kb fragment containing the exon 44) were amplified by PCR using the template. These three fragments were inserted into the pGEM-11zf(-) vector with the MC1-neo cassette between the mutant exon 43 and the 3'-arm.

The targeting vector constructed as above was linearized with *ClaI* and transfected into the CMT (CMTI-2 EmbryoMax ; Millipore Corp.) embryonic stem (ES) cells by electroporation. The cells were plated in 100-mm dishes and were incubated for 24 hours. Positive and negative

selections were performed using Geneticin and diphtheria toxin A (DT-A) fragment, respectively. About 100 clones were selected and analyzed by PCR using the primer F (5'-AGGATCTCGTCGTGACCCATG -3') and primer R (5'-ACCAAGGACTAAGCCATTGCAG -3') and then confirmed by Southern blot analysis. The selected ES cells were injected into the blastosysts of BALB/c mice and chimeric animals were obtained. The chimeric mice gave offspring with germ-line transmission, and mice heterozygous for the targeted $RyR2^{+}/RyR2^{S2246L\text{-}neo}$ were established. Sperms from the heterozygous mice were external fertilized with eggs from C57BL/6 female mice. The neo cassette was removed by injection of the Cre-expression circular plasmid (pCAGGS-Cre) [4, 5] into the oocytes. The genotypes of the F1 generations were determined by PCR and Southern blot analysis of genomic tail DNA. Primers F1 (5'-AGATCTCACTGTGACTGAGCATC-3'), F2(5'-TAGATAGCATCTTTGCGATGCAG-3') and R1(5'-TGCCTTACACTTGTAAACAGTGC-3') were used in the PCR analysis.

Histology.

Hearts from S2246L/+ KI and WT mice, aged 16–20 weeks, were fixed with 10% formalin. A complete, full-circumferential section, at the level of the two left ventricular papillary muscles, was selected for morphometric analysis. Hematoxylin-Eosin and Trichromic Masson stains were performed for each section of ventricle.

Echocardiography.

Cardiac function was analyzed by a HDI-5000 ultrasound machine (Philips, Netherlands) equipped with a 15-MHz probe, as described previously.[5] S2246L/+ KI and WT mice were initially anesthetized with 4–5% isoflurane (mixed with oxygen) and maintained with 1–2%

isoflurane during echocardiography.

ECG Telemetry

ECG was monitored in a conscious state for S2246L/+ KI (n=4) and WT (n=4) mice by using ECG telemetry, as described previously.[5] Briefly, transmitters (Data Sciences International, St. Paul, MN) were implanted in the back space with s.c. electrodes in a lead II configuration. Telemetry was recorded 48 h after surgery in a conscious state for 30-min intervals at baseline and after exercise with treadmill (Panlab, Barcelona, Spain). After recording the ECG before the treatment with dantrolene, these KI mice were treated with either dantrolene (20mg/kg/day, i.p) [6] or K201 (12 mg/kg/day, i.p.) [7] for 7-10 days before being exposed to the exercise with treadmill. Arrhythmias were defined as follows: non-sustained ventricular tachycardia was defined as a series of 4 to 10 consecutive repetitive ventricular ectopic beats (VEBs), sustained VT (SVT) was defined as a run of >10 consecutive VEBs.[8]

Preparation of SR vesicles

We prepared SR vesicles from mice LV, as described previously.[5] Left ventricles were homogenized in a solution containing 30 mmol/L Tris-malate, 0.3 mol/L sucrose, protease inhibitor cocktail (complete, EDTA-free, Roche), at pH 7.0. The homogenate was centrifuged at 4,000 g for 15 minutes and the resultant supernatant was filtered through four layers of cheesecloth before centrifugation at 10,000 g for 15 minutes. The supernatant was again filtered through cheesecloth and centrifuged at 60,000 g for 30 minutes. The pellet was resuspended in a solution containing 0.1 mol/L KCl, 20 mmol/L Tris-malate, 0.3 mol/L sucrose, protease inhibitor cocktail (complete, EDTA-free), at pH 7.0 to give a final concentration of about 10-20 mg protein/mL. This fraction was rapidly frozen in liquid nitrogen and stored at -80°C.

Peptides used and peptide synthesis

We used the 3 domain peptides, as described previously [5];

DPc10: DP2460-2495

2460GFCPDHKAAMVLFLDRVYGIEVQDFLLHLLEVGFLP2495

DP2246:DP2232-2266

2232PAMRGSTPLDVAAASVMDNNELALALREPDLEKVV2266

DP2246mut:DP2232-2266mut

2232PAMRGSTPLDVAAA***L***VMDNNELALALREPDLEKVV2266

Peptides was synthesized on an Applied Biosystems model 431A synthesizer employing Fmoc (N-(9-fluorenyl)methoxycarbonyl) as the alpha-amino protecting group, or purchased from Sigma. The peptides were cleaved and de-protected with 95% trifluoroacetic acid and purified by reversed-phase high-pressure liquid chromatography.

Construction and expression of RyR2 fragments.

The peptides corresponding to various regions of RyR2 (1–610, 494–1000, 741–1260, 985–1503, 1245–1768, 1741–2270, 1981–2520 and 2234–2750) were PCR amplified with oligonucleotide primers designated to contain two restriction enzyme sites, as described previously.[9]

Purification of the full length RyR2 using SDS gel elution technique

SR vesicles were applied in the 4% SDS gel and SR proteins are separated in the 4% gel, as described previously.[9] One side of the SDS gel was cut and stained with Coomassie blue to know the position of the RyR2 band. Then, RyR2 band of the unstained gel was cut. Using

Biorad electro-eluter (model 422), RyR2 was eluted from the gel as follows. Sliced RyR2 bands were put into the glass tubes of the eluter. The MLCO of 12kDa-15kDa membrane caps were used. RyR2 protein was eluted from the gel with the voltage of 100V for 4 hrs in the eluting buffer containing 25 mM Tris base, 192 mM Glycine and 0.1% SDS. The buffer of eluted RyR2 was exchanged and RyR2 was concentrated using a solvent absorption concentrator (Vivapore10/20, MWCO 30000). Purified RyR2 was resuspended in the sample solution to a final protein concentration of 1 mg/mL and stored at -80 °C.

Site-directed fluorescent labeling of the RyR2

Specific fluorescent labeling of RyR2 in SR vesicles was performed using the cleavable hetero-bifunctional cross-linking reagent sulfosuccinimidyl 3-((2-(7-azido-4-methylcoumarin-3-acetamido) ethyl) dithio)propionate (SAED) from PIERCE, with DPc10 or DP2246 as a site-specific carrier. The method for the site-directed fluorescent labeling of the RyR2 is as described previously.[5, 10, 11] First, peptide-SAED conjugate was formed by incubating 0.5 mmol/L peptide with 0.5 mmol/L SAED in a 20 mmol/L HEPES (pH 7.5) solution for 60 minutes at 22 °C in the dark. The reaction was quenched by 20 mmol/L lysine. Free SAED was removed using ion exchange column (GE HiTrap Q XL). The peptide-SAED conjugate (5 μmol/L in a final concentration) was mixed with 2 mg/mL SR protein in the sample solution containing a 1 mmol/L EGTA/calcium buffer (1.0 μmol/L free Ca^{2+}) in the dark and photolyzed with UV light in a Pyrex tube at 4 °C for 2 minutes. Beta-mercaptoethanol was added (100 mmol/L in a final concentration) to cleave the disulfide bond of SAED. After incubation on ice for 1 h, the mixture was centrifuged at 100,000g for 15 minutes, and the sedimented vesicles were resuspended in the sample solution to a final protein concentration of 10 mg/mL.

The methylcoumarin-acetamido (MCA) is a small fluorescent molecule, and can be quenched by a quenching agent. The specific methyl-coumarin-acetamido (MCA) labeling of the RyR in cardiac SR vesicles was confirmed by a fluorometric scan (excitation at 360 nm, emission at 440 nm) after electrophoresis had been carried out on 4% SDS-polyacrylamide gel.

Fluorescence quenching of the MCA fluorescence attached to the DPc10 binding site

The zipped and unzipped states of RyR2 were evaluated as described previously.[5,10,11] The principle of the fluorescence quench assay of domain unzipping is that a large-size quencher BSA- QSY® 7 is inaccessible to the attached MCA in the zipped state, whereas it becomes accessible to the MCA site in the unzipped state. To form the quencher, QSY® 7 carboxylic acid, was conjugated with BSA by incubating 5 mmol/L QSY® 7 carboxylic acid with 0.5 mmol/L BSA in 20 mmol/L HEPES (pH 7.5) for 60 minutes at 22°C in the dark. Unreacted QSY® 7 carboxylic acid was removed by means of Sephadex G50 gel filtration. Fluorescence quenching by QSY® 7- BSA conjugate (a large-size quencher) was performed by measuring steady-state fluorescence of labeled MCA (excitation at 368 nm, emission at 455 nm) in the presence or absence of chemicals. The data were analyzed using the Stern-Volmer equation.[11]

$F_0/F = 1 + K_Q [Q]$

where F and F_0 are fluorescence intensities in the presence and in the absence of added quencher, respectively; K_Q, quenching constant, which is the measure of the accessibility of the protein-bound probe to the quencher; [Q], the quencher concentration. The principle of the fluorescence quenching assay of domain unzipping is that a large-size quencher QSY® 7 -BSA is not accessible to the attached MCA in the zipped state, whereas it becomes accessible to the MCA in the unzipped state.

Quartz crystal microbalance （QCM） measurements

Binding of DP2246 or DP2246mut to RyR2 fragments was detected by using a 27-MHz quartz-crystal microbalance (QCM) (Initium, Inc., Japan), which is a highly sensitive mass-measuring apparatus, as described previously. [9] The QCM Au electrode was coated with DP2246 and immersed in a solution (500 μl) containing (in mmol/L) 150 NaCl, 20 MOPS (pH 7.4). The amount of drug binding was determined from the frequency changes (ΔF) due to changes in the mass on the electrode (with the sensitivity in the order of sub-nanogram) upon injection of a small volume (2–5 μl) of solution containing RyR2 fragments (30 nmol/L). No significant non-specific binding of RyR2 fragments to Au electrode was detected.

QCM is a very sensitive device for the detection of protein-protein interactions in solution, which are monitored by the linear decrease of the emitted frequency with increasing mass present on the QCM electrode according to Sauerbrey's equation.

The Sauerbrey's equation defined as:

$$\Delta F=-2F_0^2/\{A(\mu q\rho q)^{1/2}\}\Delta m$$

ΔF: Frequency change (Hz)

F_0: Resonant frequency (27MHz)

A: Piezoelectrically active crystal area (Area between electrodes, 4.9mm^2)

μq: Shear modulus of quartz for AT-cut crystal ($\mu q = 2.947x10^{11}$ $g/cm.s^2$)

ρq: Density of quartz ($\rho q = 2.648$ g/cm^3)

Δm: Mass change (g)

Isolation of cardiac cardiomyocytes

The enzymatic isolation of mice cardiomyocytes was performed as described previously.[5, 12] In brief, S2246L/+ KI and WT mice (2 to 3months) 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-containing buffer via the aorta under constant flow. The LV myocardium was minced with scissors in fresh collagenase-containing buffer and the rod-shaped adult mice cardiomyocytes were prepared by retrograde perfusion of the hearts with 95%O_2/5%CO_2-bubbled Minimal Essential Medium (Sigma, St Louis, MO, USA) supplemented with 50 μmol/L [Ca^{2+}], 0.5 mg/mL collagenase B, 0.5 mg/mL, collagenase D, and 0.02 mg/mL protease type XIV. The Ca^{2+} concentration was then gradually increased to a final concentration of 1 mmol/L by changing the incubation medium (50 μmol/L, 100 μmol/L, 300 μmol/L, 600 μmol/L and then 1 mmol/L). The isolated mice cardiomyocytes were transferred to laminin-coated glass culture dishes, and incubated for 12 h at 37°C in a 5%CO_2/95%O_2 atmosphere. To assess SR Ca^{2+} content, caffeine (10 mmol/L) was rapidly perfused. In case of intact cells, caffeine was perfused after field-stimulation at 2 Hz. Experiments were carried out at room temperature.

Analysis of local Ca^{2+} release events with laser scanning confocal microscopy.

The Ca^{2+} events under a fixed intracellular [Ca^{2+}] condition were measured in saponin-permeabilized cardiomyocytes. Ventricular myocytes were superfused with relaxing solution containing EGTA 0.1 mmol/L, ATP 5 mmol/L, HEPES 10 mmol/L, potassium aspartate 150 mmol/L, $MgCl_2$ 0.25 mmol/L, and reduce-glutathione 10 mmol/L, at 23°C. The sarcolemma was permeabilized with saponin (50 μg/mL) for 30s. After permeabilization,

myocytes were placed in a solution containing EGTA 0.5 mmol/L, HEPES 10 mmol/L, K-aspartate 120 mmol/L, ATP 5 mmol/L, free $MgCl_2$ 1 mmol/L, reduced glutathione 10 mmol/L, free $[Ca^{2+}]$ 30 nmol/L (calculated using MaxChelator (http://www.stanford.edu/~cpatton/webmaxcS.htm)), creatine phosphokinase 5 U/ml, phosphocreatine 10 mmol/L, dextran (MW: 40,000) 4%; Rhod-2 0.02 mmol/L, pH 7.2. Rhod-2 were excited by 543 nm laser lines, and fluorescence was acquired at wavelengths of >560 nm. Ca^{2+} spark images were obtained from permeabilized ventricular myocytes before and after the addition of cAMP (0.1-1 μmol/L) in the presence of the CaMKII inhibitor KN-93 (1 μmol/L) and okadaic acid (1 μmol/L) to prevent dephosphorylation by endogenous phosphatases.

Data were analyzed with SparkMaster, an automated analysis program which allows rapid and reliable spark analysis.[13] The analysis includes general image parameters (number of detected sparks, spark frequency) as well as individual spark parameters (Amplitude, FWHM: full width at half maximum, FDHM: full duration at half maximum).

Determination of the binding of DP2246 to subcellular fractions in saponin-permeabilized cardiomyocytes.

DP2246, fluorescently labeled with Oregon Green 514 (Molecular Probes, OR), was introduced into the saponin-permeabilized WT and KI cardiomyocytes under the same condition as the aforementioned Ca^{2+} spark measurements. Then, the distribution of localized DP2246 was determined by densitometric measurement of Oregon Green-DP2246 fluorescence. Briefly, the fluorescently labeled cardiomyocytes were laser-scanned with the confocal microscope (LSM-510, Carl Zeiss). (numerical aperture, 1.3; excitation at 514 nm; emission 530 nm). The sarcomere-related periodical increase in the Oregon Green 514 fluorescene intensity from baseline was integrated with respect to the longitudinally selected distance (~25 μm) and then

divided the value by the distance.

Evaluation of FKBP12.6 association with the RyR2.

FKBP12.6 bound to the mouse RyR2 was detected by immunoprecipitation and immunoblot analysis, as described previously.[5] Left ventricles were homogenized in a solution containing 30 mmol/L Tris-malate, 0.3 mol/L sucrose, protease inhibitor cocktail (complete, EDTA-free, Roche), at pH 7.0. The homogenate was centrifuged at 4,000 g for 15 minutes and the resultant supernatant was filtered through four layers of cheesecloth before centrifugation at 100,000 g for 30 minutes. The pellet was resuspended in RIPA buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1 mmol/L Na_3VO_4, 1 mmol/L NaF, 0.25% TritonX-100, and protease inhibitors) for immunoprecipitation, in the presence of anti-RyR2 antibody (MA3-925, Affinity BioReagents Inc) for 4 hours at 4°C. After incubating the samples with protein G-Sepharose beads (Amersham; GE Healthcare) at 4°C for 2 hour, proteins were separated in a 4% (for RyR2) or 15% (for FKBP12.6) SDS-PAGE gel, transferred to PVDF membranes, and immunoblotted using the following antibodies: anti-FKBP12.6 (1:1,000) (AF4174, R&D systems), anti-RyR (1:1,000).

Immunoblot analysis

We performed immunoblot analyses for RyR2, P-Ser2808-RyR2, P-Ser2814-RyR2, SERCA2a, PLB, P-Ser16-PLB, P-Thr17-PLB, CASQ, and GAPDH, as described previously.[2] Specific antibodies were against RyR2 (Sigma), P-Ser2808-RyR2 and P-Ser2814-RyR2, (Badrilla), SERCA2a (Affinity Bioregents), PLB (Upstate biotech), P-Thr17-PLB (Badrilla), CASQ (Affinity Bioregents), GAPDH (Chemicon).

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Supplemental Figure legends

Supplemental Figure 1.

A. Schematic representation of the genomic structure of the mouse RyR2; the targeting vector used to generate the knock-in RyR2 S2246L mouse strain and the recombining genomic structure of RyR2 S2246L.

B. The genotypes were determined by PCR on DNA from tail biopsy specimens.

Supplemental Figure 2.

A. Representative images of Hematoxylin-Eosin-stained hearts from WT and S2246L/+ KI mice. **B.** (Top) Western blots of various Ca^{2+} regulatory proteins: RyR2, cardiac ryanodine receptor; P-Ser2808-RyR2, phosphorylated RyR2 at Ser2808; P-Ser2814-RyR2, phosphorylated RyR2 at Ser2814; SERCA2a, SR Ca^{2+}-ATPase; PLB, phospholamban; P-Ser16-PLB, phosphorylated PLB at serine 16; P-Thr17-PLB, phosphorylated PLB at threonine 17; CASQ, calsequestrin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (Bottom) Summarized data of Western blots. Open bar: WT; closed bar: KI. Data represent the mean±SE of 4 hearts.

Supplemental Figure 3.

Summarized data of body weight (BW), heart rate (HR), left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), intra-ventricular septum diastolic thickness (IVSD), left ventricular posterior wall diastolic thickness (LVPWD), left ventricular fractional shortening ((LVEDD-LVESD)/LVEDDx100). N: the number of mice examined.

Supplemental Figure 4.

RyR2-FKBP12.6 association in WT and KI cardiac homogenates. (Top) Representative Western blots of the RyR2 and FKBP12.6 after immunoprecipitation (cf. online supplemental methods). (Bottom) Summarized data of the ratio of FKBP12.6 to the RyR2, which was normalized to control condition. cAMP (1 μmol/L) did not dissociate the RyR2-bound FKBP12.6 in WT and KI cardiac homogenates. N: the number of SR preparations; each preparation was obtained from 10-16 hearts.

Supplemental Table 1

ECG characteristics.

	n	HR(beats/min)	QT(ms)	QTc(ms)
WT	6	530±72	24±2	71±8
$RyR2^{S2246L/+}$	6	540±82	24±2	72±7
P value (WT vs KI)		NS	NS	NS

Supplemental Table 2

Ca^{2+} spark characteristics at matched SR Ca^{2+} content in WT and KI cardiomyocytes.

		Ca^{2+} Spark				
	SR Ca^{2+} content (F/F_0)	SpF (Events·100μ m^{-1}·sec^{-1})	Peak (F/F_0)	FWHM (μm)	FDHM (ms)	No. of Sparks
WT+thapsigargin (0.3 μmol/L) (n=4)	1.68± 0.25	0.45± 0.22	1.58± 0.03	1.71± 0.03	22.94± 0.54	**203**
KI (n=4)	1.67± 0.03	10.74± 0.22*	1.58± 0.01	1.77± 0.01*	24.87± 0.14*	**3118**

N: the number of hearts. SpF: Ca^{2+} spark frequency, FWHM: Full width at half maximum, FDHM: Full duration at half maximum. *$p<0.01$ vs. WT+thapsigargin.

Supplemental Table 3

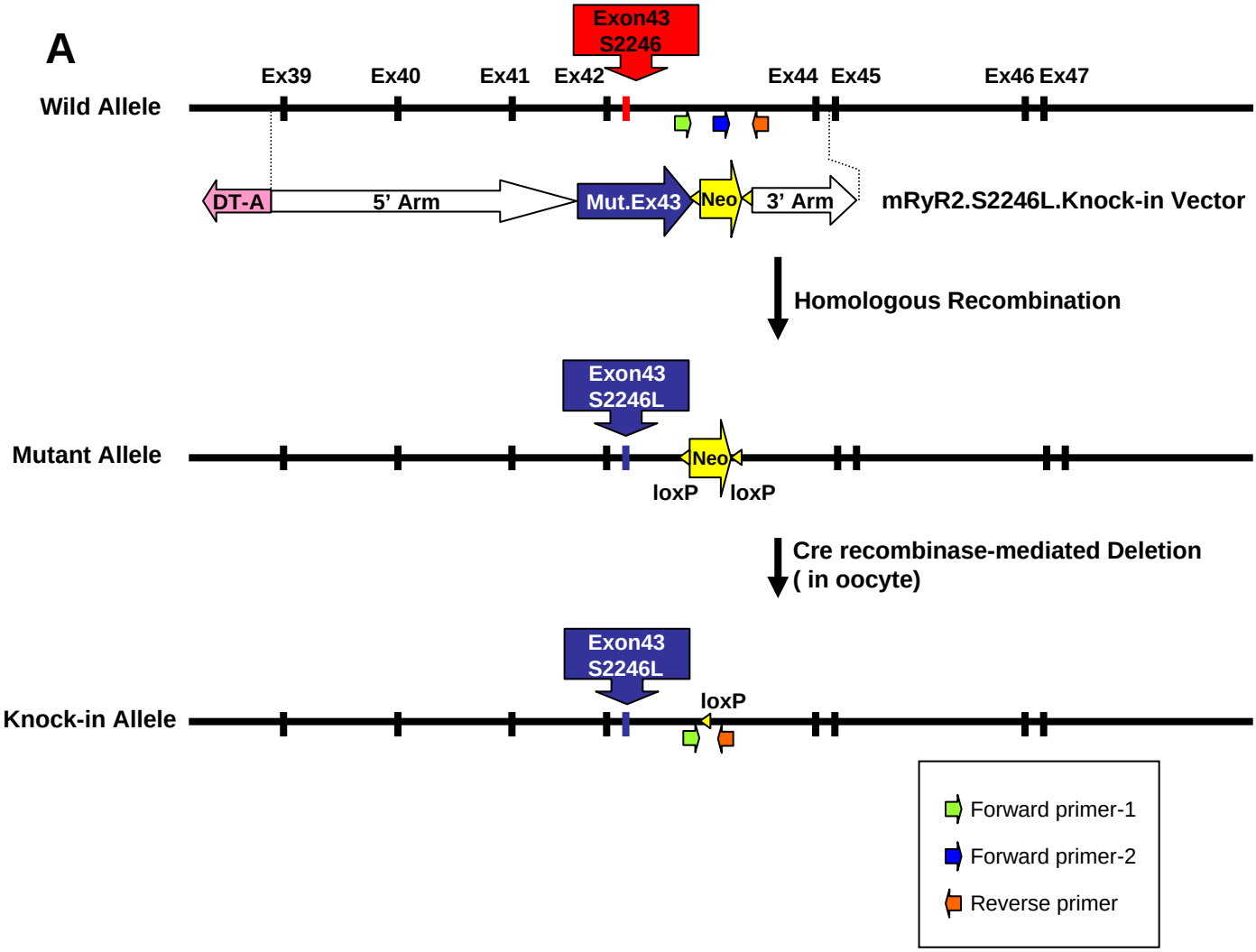
Effect of cAMP on Ca^{2+} spark characteristics in saponin-permeabilized cardiomyocytes

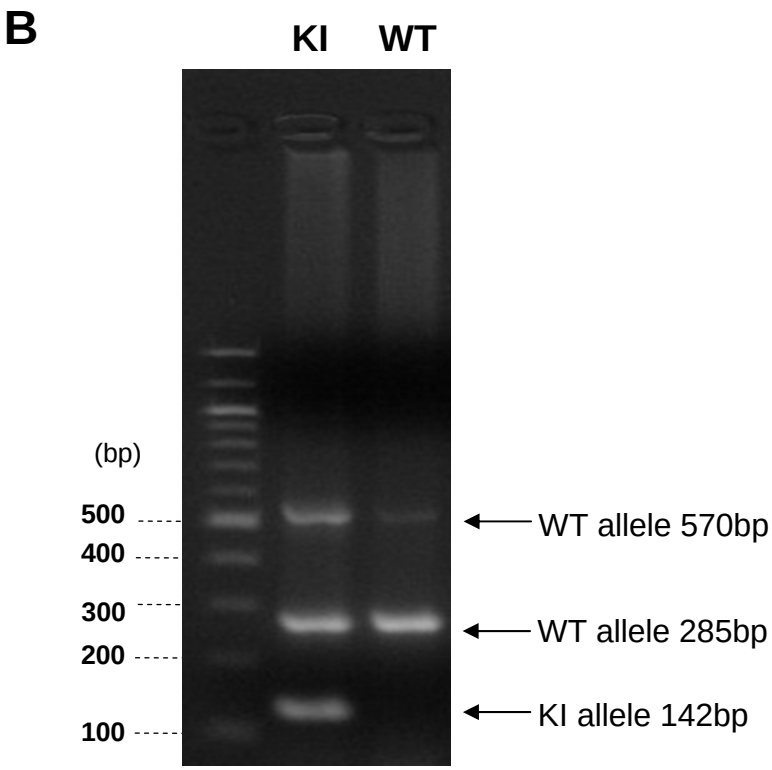
cAMP (μmol/L)		Peak (F/F_0)	FWHM (μm)	FDHM (ms)	No. of Sparks
0	WT (n=4)	1.80±0.01	1.96±0.01	22.12±0.07	**2739**
	KI (n=4)	1.58±0.01*	1.77±0.01*	24.87±0.14*	**3118**
0.1	WT (n=4)	2.01±0.02	2.05±0.01	24.36±0.16	**2721**
	KI (n=4)	1.64±0.00*	1.80±0.01*	25.55±0.13*	**4143**
0.3	WT (n=5)	2.16±0.01	2.16±0.01	27.17±0.18	**4501**
	KI (n=5)	1.66±0.00*	1.92±0.01*	29.41±0.12*	**7630**
1	WT (n=4)	2.18±0.01	2.18±0.01	32.21±0.19	**4156**
	KI (n=4)	1.72±0.01*	1.96±0.01*	35.21±0.13*	**6003**

N: the number of hearts. FWHM: Full width at half maximum, FDHM: Full duration at half maximum

*$p<0.01$ vs. WT; the p values were corrected by a post hoc Scheffe's test.

Supplemental Figure 1

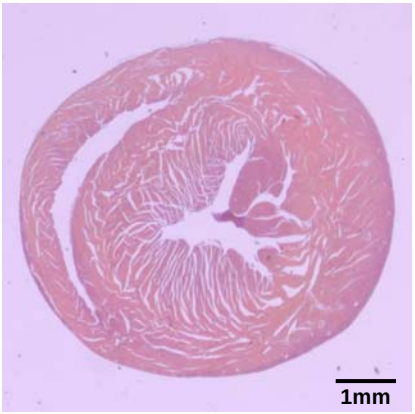




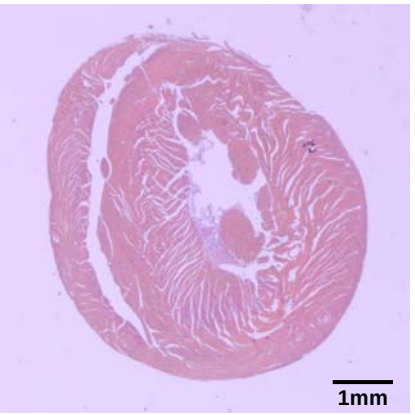
Supplemental Figure 2

A

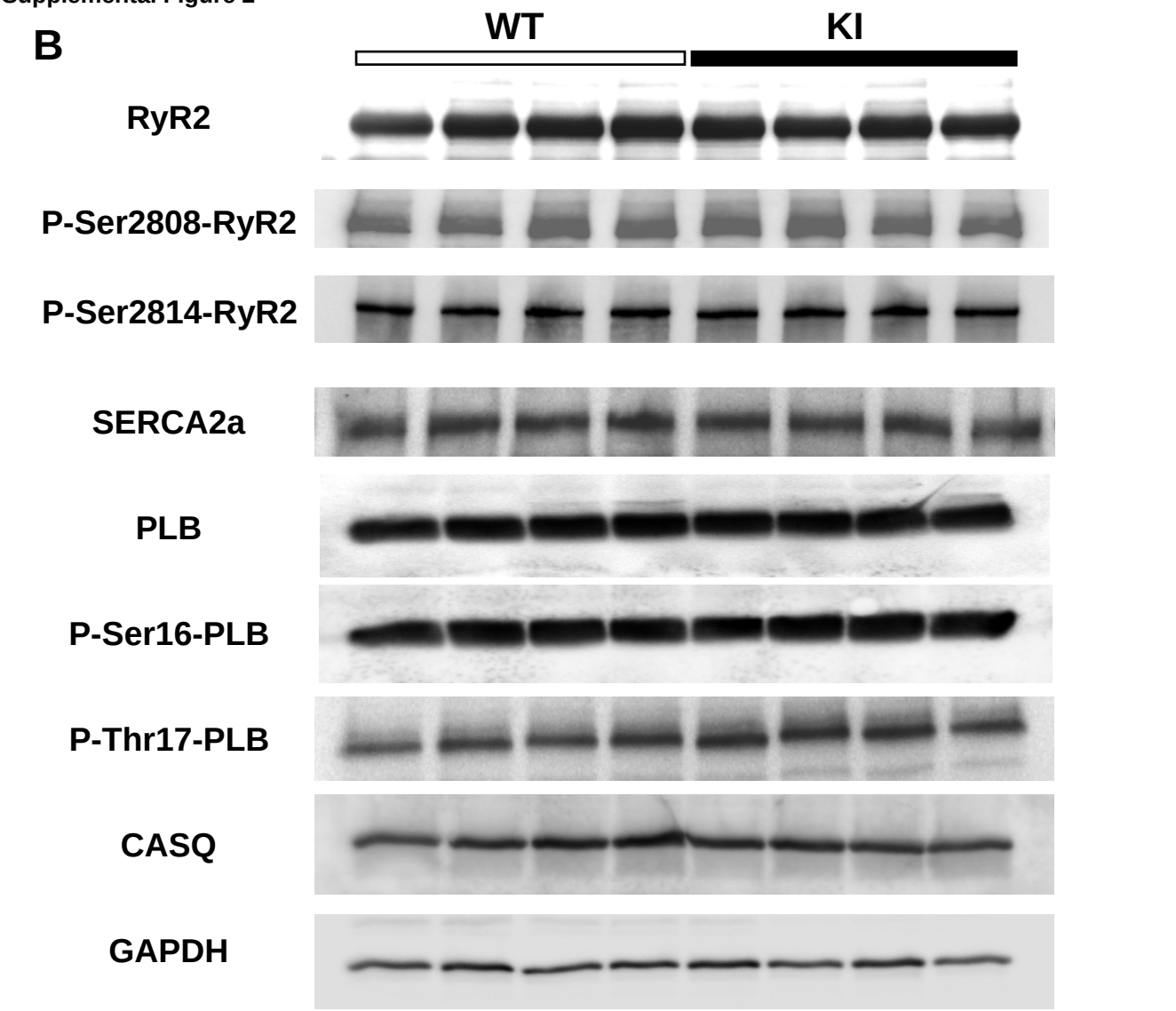
Wild-type (WT) (RyR2 $^{+/+}$)

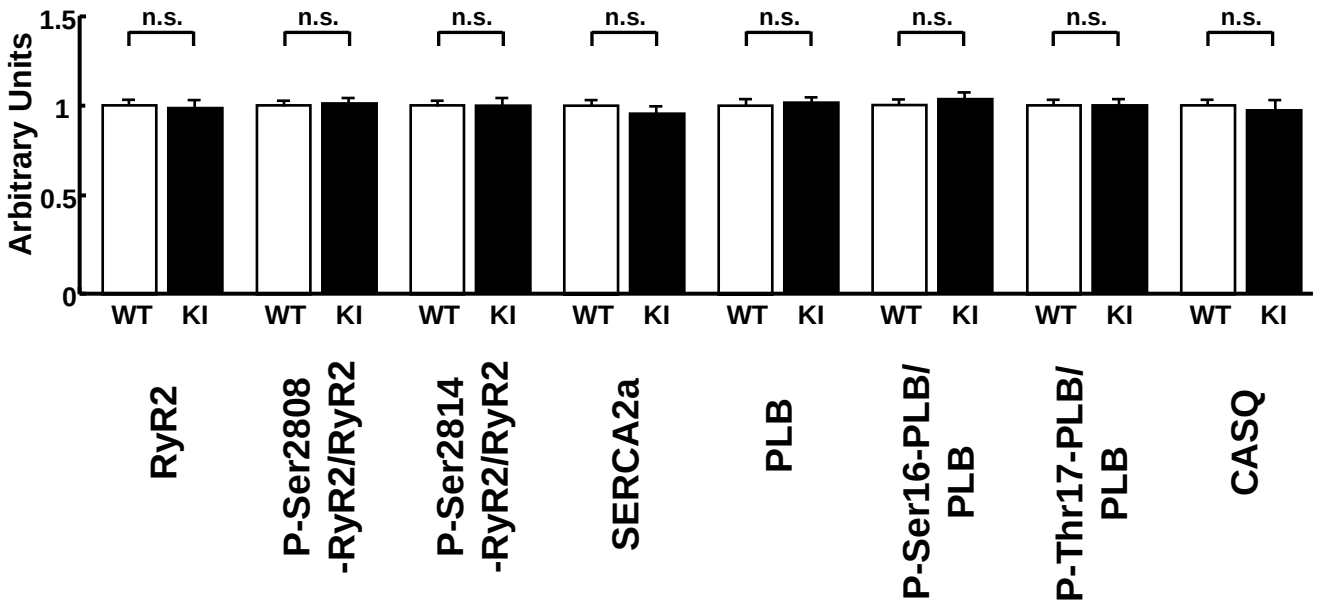


Knock-in (KI) (RyR2 $^{S2246L/+}$)

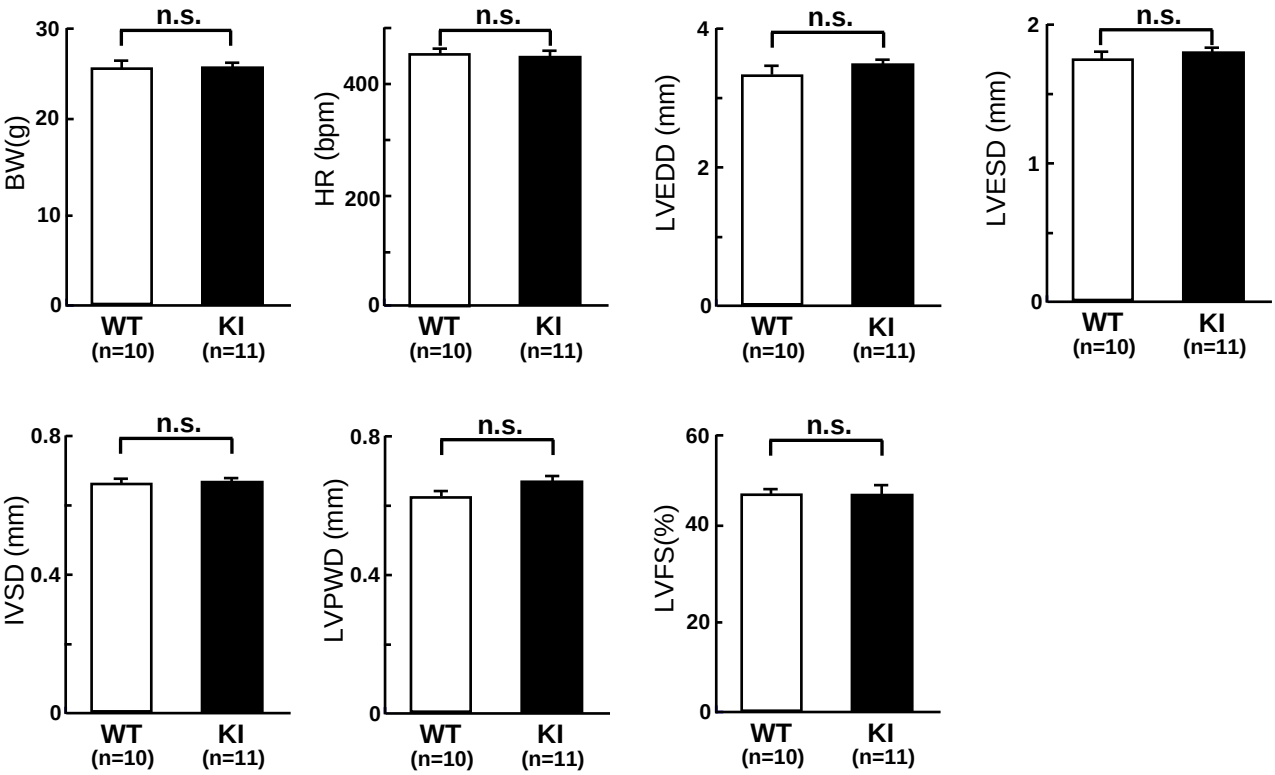


Supplemental Figure 2





Supplemental Figure 3



Supplemental Figure 4