SUPPLEMENTAL MATERIAL

In situ confocal imaging in intact heart reveals stress-induced Ca^{2+} release variability in a mouse CPVT model of $RyR2^{R4496C+/-}$ mutation

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Extended Methods

Animal model

Animal experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication No.85 to 23, revised 1985) and were approved by the Institutional Animal Care and Use Committee at the University of Iowa. The generation and characterization of $RyR2^{R4496C+/-}$ heterozygous mice has been described recently (1) and is identical to the mice made by Priori group.(2) Both male and female $RyR2^{R4496C+/-}$ and wildtype littermates at ages of 2-4 months were used for studies.

In situ confocal Ca^{2+} imaging in intact hearts with / without ex vivo electrocardiogram

Methods were adapted from published reports.(3, 4) Mice were heparinized (100 IU i.p.) and euthanized by pentobarbital (120 mg/kg, i.p.). Excised hearts were perfused with Rhod-2 AM (0.3 mM) containing Kreb- Henseleit's solution (in mM: 120 NaCl, 24 $NaHCO_3$, 11.1 Glucose, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 0.42 KH_2PO4, oxygenated with 95% O_2 and 5% CO_2) at room temperature for 30 min via retrograde Langendorff perfusion system. Hearts were later transferred to another Langendorff apparatus (37 ºC) attached to the confocal microscope system after Rhod-2 loading was completed. The heart was placed onto a recording chamber for *in situ* confocal imaging (linescan) of Ca^{2+} signals from epicardial myocytes under sinus rhythm. To avoid motion artifacts in Ca^{2+} imaging, blebbistatin (10 μM, Sigma) and BDM (2,3-butanedione monoxime, 10 mM, Sigma) were added to the perfusion solution. Two ECG probes (silver wires) were placed on the apical subepicardium and right atrium, respectively. The pseudo ECG signal was continuously acquired by a Differential AC Amplifier (Model 1700, A&M Systems), digitized at 1K Hz online with Digidata 1440A A-D converter, stored with pClamp 10 software, and analyzed offline with Clampfit 10 (Molecular Devices, CA). The confocal linescan images were acquired at a rate of 3.07 ms or 1.93 ms per line. Unless otherwise specified, Ca^{2+} transients were autonomously elicited by electrical signals from sinoatrial node.

In situ confocal AP imaging in intact heart

These studies were adapted from a published report.(5) Similar to *in situ* Ca^{2+} imaging, a fast voltage-sensitive dye, ANNINE-6plus (3 μg/ml) was used to load the intact hearts through Langendorff-perfusion to track the dynamic changes of transmembrane potential. ANNINE-6plus fluorescence was recently shown to report changes in transmembrane voltage in cardiomyocytes with high fidelity (verified with direct microelectrode recordings of APs).(5) We recorded ANNINE-6plus fluorescence using linescan confocal imaging at a scan rate of 1.54 ms per line from epicardial myocytes of intact hearts under sinus rhythm. Since changes in transmembrane potential are linearly correlated with ANNINE-6 plus fluorescence changes,(5) we used the absolute fluorescence ratio ($\Delta F/F_0$) as an index of AP amplitude, and defined the time from peak to 50% decay as APD_{50}.

Ca^{2+} imaging in adult single isolated ventricular myocytes and in primary cultured neonatal myocytes

Adult ventricular myocyte dissociation and confocal Ca^{2+} imaging were performed as previously described.(6) Briefly, isolated myocytes loaded with Rhod-2 AM were subjected to external field stimulation (3 Hz / 5 Hz), and Ca^{2+} transients were recorded using linescan confocal imaging during steady state conditions at baseline and at least 3 minutes after epinephrine (Epi)+ caffeine (Caff) perfusion. This avoids the transient effects of caffeine on myocytes.

Neonatal myocytes were prepared as follows: primary cultures of neonatal ventricular myocytes were prepared from 1–2 day old WT or $RyR2^{R4496C+/-}$ neonates. Neonatal mice were sacrificed by cervical dislocation. The ventricles were washed with cold PBS and minced finely in 1.5 ml of 0.05% trypsin, 0.2 mM Na-EDTA, and 0.5 mg/ml collagenase II, and then digested for 15 minutes. Ventricles were then resuspended several times in 1-ml Eppendorf tubes and incubated for another 15-30 minutes until the dissociation of cells was complete. Complete media (10% fetal bovine serum (FBS) in Dulbecco's modified eagle medium (DMEM)) was added to terminate digestion. After centrifugation, cells were resuspended in complete media and pre-plated on a cell culture dish for 2 hours, thereby eliminating non-myocyte cells that adhere to the dish. Un-attached myocytes were plated onto laminin-coated glass surfaces and cultured under 5% CO_2 in complete media for 12 hours. Next, the plating media was replaced with fresh complete media to remove dead cells. Myocytes were used after 48 hours in culture. Confocal Ca^{2+} imaging was performed as for adult ventricular myocytes, except that these neonatal cells exhibit spontaneous beating, thus no external field stimulation was required.

Action potential (AP) recordings in single isolated myocytes

Action potentials of single ventricular myocytes were evoked with 3 / 5 Hz stimulation and were recorded by perforated patch at 36±1°C. The bath solution contained (in mM) 134 NaCl, 10 HEPES, 11 glucose, 4 KCl, 1.8 $CaCl_2$, and 1.2 $MgCl_2$, with pH adjusted to 7.4 with NaOH. The pipette was filled with (in mM) 130 potassium aspartate, 10 NaCl, 10 HEPES, 0.04 $CaCl_2$, 2.0 MgATP, 7.0 phosphocreatine, 0.1 NaGTP, and 240 μg/ml amphotericin B, with pH adjusted to 7.2 with KOH. Action

potential duration (APD) was assessed as the time from the AP upstroke to 50% repolarization to baseline (APD_{50}).

Data analysis and statistics

Confocal Ca^{2+} / AP images were analyzed offline with custom routines composed with IDL image analysis software (ITT VIS Inc., Boulder, CO).(7) Pseudo ECG data were processed offline with Clampfit 10. Data were expressed as mean ± SE (♦, mean plus/minus error bars) and median with interquartile range in boxplots. Multiple regression analysis was performed to determine the correlation coefficient and significance. Student's t-tests were applied for pair-wise comparisons. Bonferroni procedure following a global test based on linear mixed effects model was performed for multiple group comparisons (NCSS, LLC, Kaysville, Utah). The hearts were treated as random effects and the conditions (e.g., WT control, WT Epi + Caff, R4496C+/- Control and R4496C+/- Epi + Caff) as a fixed effect. A compound symmetry correlation structure was assumed for linear mixed effects model tests. A p value of <0.05 was considered statistically significant.

Supplemental Figure legends

Figure S1. **Variance of Ca^{2+} transient amplitude in single isolated myocytes**. Isolated myocytes loaded with Rhod-2 AM were subjected to external field stimulation at 3Hz, and Ca^{2+} transients were acquired at steady state. **A-B**, representative examples of confocal images from WT and $RyR2^{R4496C+/-}$ myocytes under control and at least 3 minutes after Epi+Caff treatment. Note the frequent spontaneous Ca^{2+} waves during the diastolic phase in $RyR2^{R4496C+/-}$ myocyte under Epi+Caff stimulation. **C.** Variance analysis of Ca^{2+} transient amplitude. Action potential-triggered Ca^{2+} transients, but not spontaneous propagating Ca^{2+} waves, were included in this analysis. n= 911, 758, 500 and 306 events, respectively. **D.** a boxplot of means of variance, n= 51, 58, 47 and 30 cells from 4 animals, respectively. No difference in variance in Ca^{2+} transient amplitude was detected between WT and $RyR2^{R4496C+/-}$ myocytes perfused with Epi+Caff, suggesting CRV observed in intact $RyR2^{R4496C+/-}$ hearts was not a Ca^{2+} release defect due to the mutation in ventricular myocytes themselves. **E-F**, Variance analysis of Ca^{2+} transient amplitude under 5-Hz field stimulation in myocytes treated with Epi+Caff. No difference in variance in Ca^{2+} transient amplitude was detected between WT and $RyR2^{R4496C+/-}$ myocytes perfused with Epi+Caff at 5 Hz stimulation too.

Figure S2. **No apparent CRV was observed in 2-day cultured neonatal myocytes with the R4496C mutation**. Ca^{2+} transients were recorded in multi-connected neonatal myocytes under conditions of spontaneous beating. **A**, Representative images of Ca^{2+} transients from WT and $RyR2^{R4496C+/-}$ neonatal myocytes treated with Epi+Caff. Green bars indicate the boundary of neighboring cardiomyocytes. **B.** Variance analysis of the amplitude of Ca^{2+} transients, n=3711, 4450, 1752, 7521 events for WT, WT with

Epi+Caff, R4496C$^{+/-}$ and R4496C$^{+/-}$ with Epi+Caff, respectively. **C.** A boxplox of means of variance, n=22, 11, 11, 14, respectively. No difference was found between WT and RyR2$^{R4496C+/-}$ myocytes under either control or Epi+Caff treatment.

Figure S3. AP variation was synchronized between neighboring cells in RyR2$^{R4496C+/-}$ hearts. **A**, Raw and normalized images from a RyR2$^{R4496C+/-}$ heart under Epi+Caff perfusion. **B**, Spatial average of ANNINE-6plus fluorescence from Cell 1 and Cell 2 as indicated in Panel A, suggesting a synchronized pattern in AP alteration in physiologically-coupled RyR2$^{R4496C+/-}$ myocytes. Of note, the synchronized AP alterations were not observed in WT hearts under the same condition.

Figure S4. **AP studies in single isolated myocytes using conventional current clamp recordings. A-B**, Superimposed AP traces (100) from WT and RyR2$^{R4496C+/-}$ myocytes under control conditions and with adrenergic stress (Epi+Caff) at 3Hz stimulation. **C-D**, Boxplots of average data on APA and APD_{50} (3 Hz). **E-F**, Boxplots of variance of APA and APD_{50} under different conditions (3 Hz). **G-J**, Boxplots of average data at 5Hz timulation. No differences were detected in AP amplitude (APA), duration (APD_{50}), or in variance of these parameters between WT and RyR2$^{R4496C+/-}$ hearts under both control and adrenergic stress.

Figure S5. The influences of 2,3-butanedione monoxime (BDM) and blebbistatin (BB) on field stimuation Ca^{2+} transient amplitude and kinetics of single myocytes. A. Representative examples of steady state Ca^{2+} transients from WT and RYR2$^{R4496C+/-}$ myocytes under 3-Hz field stimulation, in control and BDM and BB perfusion. **B**. Average data on Ca^{2+} transient parameters (amplitude, time to peak and decay time), n=30, 20, 30, 20 cells from 3-4 hearts.

References

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2. Cerrone M*, et al.* (2005) Bidirectional ventricular tachycardia and fibrillation elicited in a knock-in mouse model carrier of a mutation in the cardiac ryanodine receptor. *Circ Res* 96(10):e77-82.
3. Aistrup GL*, et al.* (2006) Pacing-induced heterogeneities in intracellular Ca^{2+} signaling, cardiac alternans, and ventricular arrhythmias in intact rat heart. *Circ Res* 99(7):e65-73.
4. Rubart M, Wang E, Dunn KW, & Field LJ (2003) Two-photon molecular excitation imaging of Ca^{2+} transients in Langendorff-perfused mouse hearts. *Am J Physiol Cell Physiol* 284(6):C1654-C1668.

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6. Song LS*, et al.* (2002) Ca^{2+} signaling in cardiac myocytes overexpressing the $\alpha 1$ subunit of L-type Ca^{2+} channel. *Circ Res* 90(2):174-181.

7. Cheng H*, et al.* (1999) Amplitude distribution of calcium sparks in confocal images: theory and studies with an automatic detection method. *Biophys J* 76(2):606-617.

Figure S1

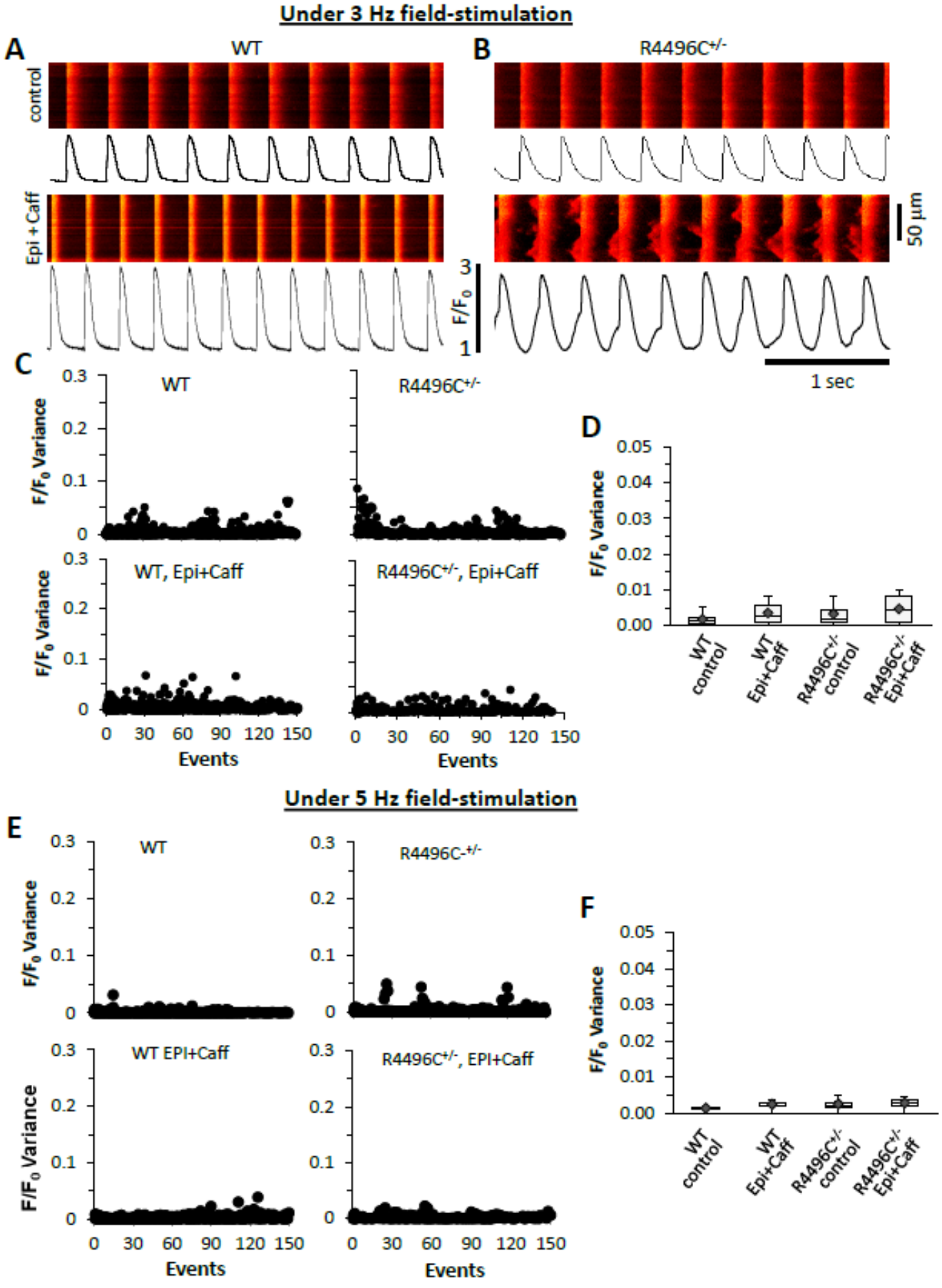

Under 3 Hz field-stimulation
A
WT
control
Epi + Caff
B
R4496C+/-
50 µm
3
F/F0
1
1 sec
C
WT
R4496C+/-
F/F0 Variance
0.3
0.2
0.1
0
WT, Epi+Caff
R4496C+/-, Epi+Caff
0 30 60 90 120 150
Events
D
F/F0 Variance
0.05
0.04
0.03
0.02
0.01
0.00
WT control
WT Epi+Caff
R4496C+/- control
R4496C+/- Epi+Caff
Under 5 Hz field-stimulation
E
WT
R4496C-+/-
WT EPI+Caff
R4496C+/-, EPI+Caff
F
WT control
WT Epi+Caff
R4496C+/- control
R4496C+/- Epi+Caff

Figure S2

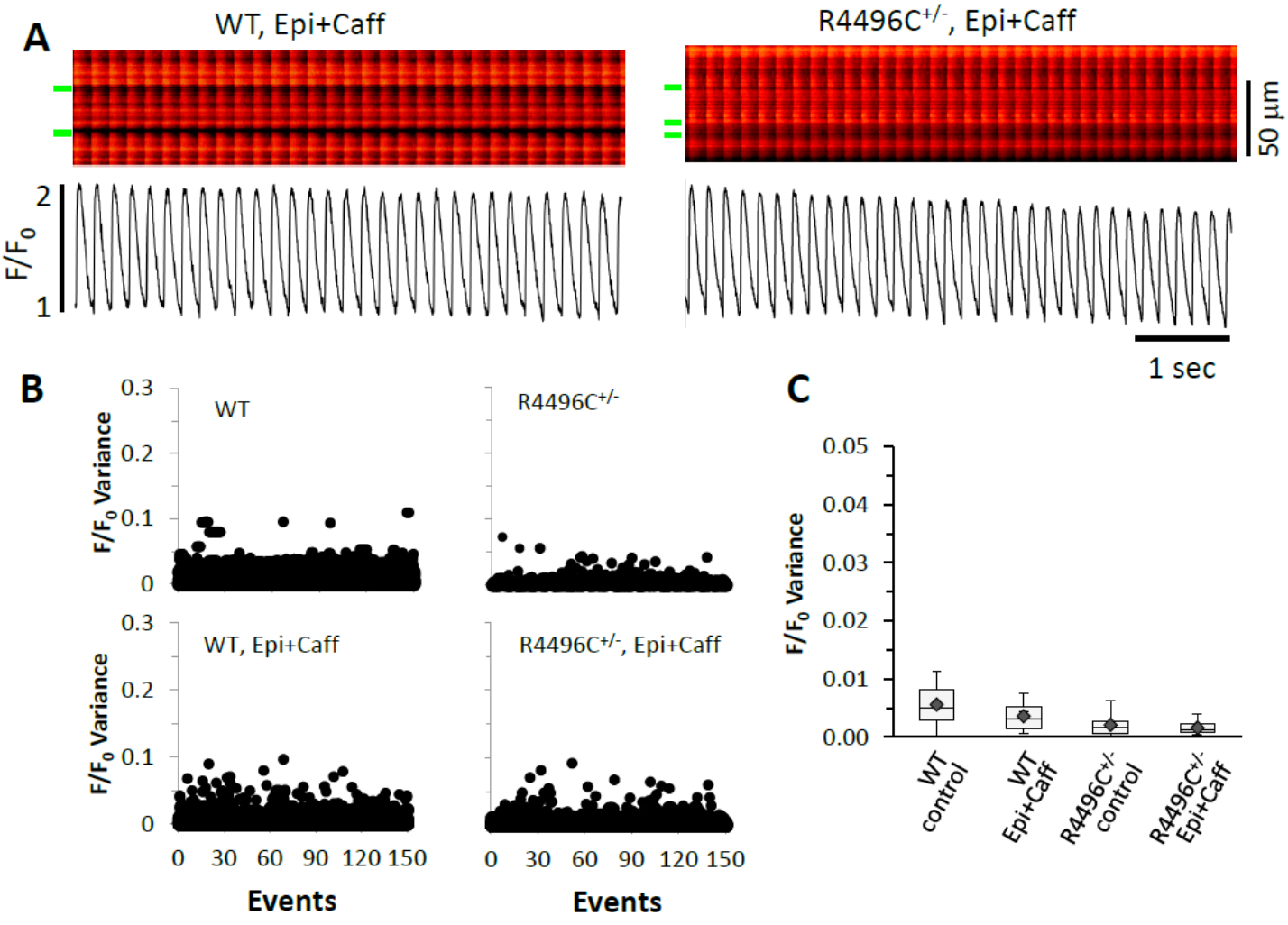

A
WT, Epi+Caff
R4496C+/-, Epi+Caff
50 µm
F/F0
2
1
1 sec
B
WT
R4496C+/-
WT, Epi+Caff
R4496C+/-, Epi+Caff
F/F0 Variance
0.3
0.2
0.1
0
0 30 60 90 120 150
Events
C
F/F0 Variance
0.05
0.04
0.03
0.02
0.01
0.00
WT control
WT Epi+Caff
R4496C+/- control
R4496C+/- Epi+Caff

Figure S3

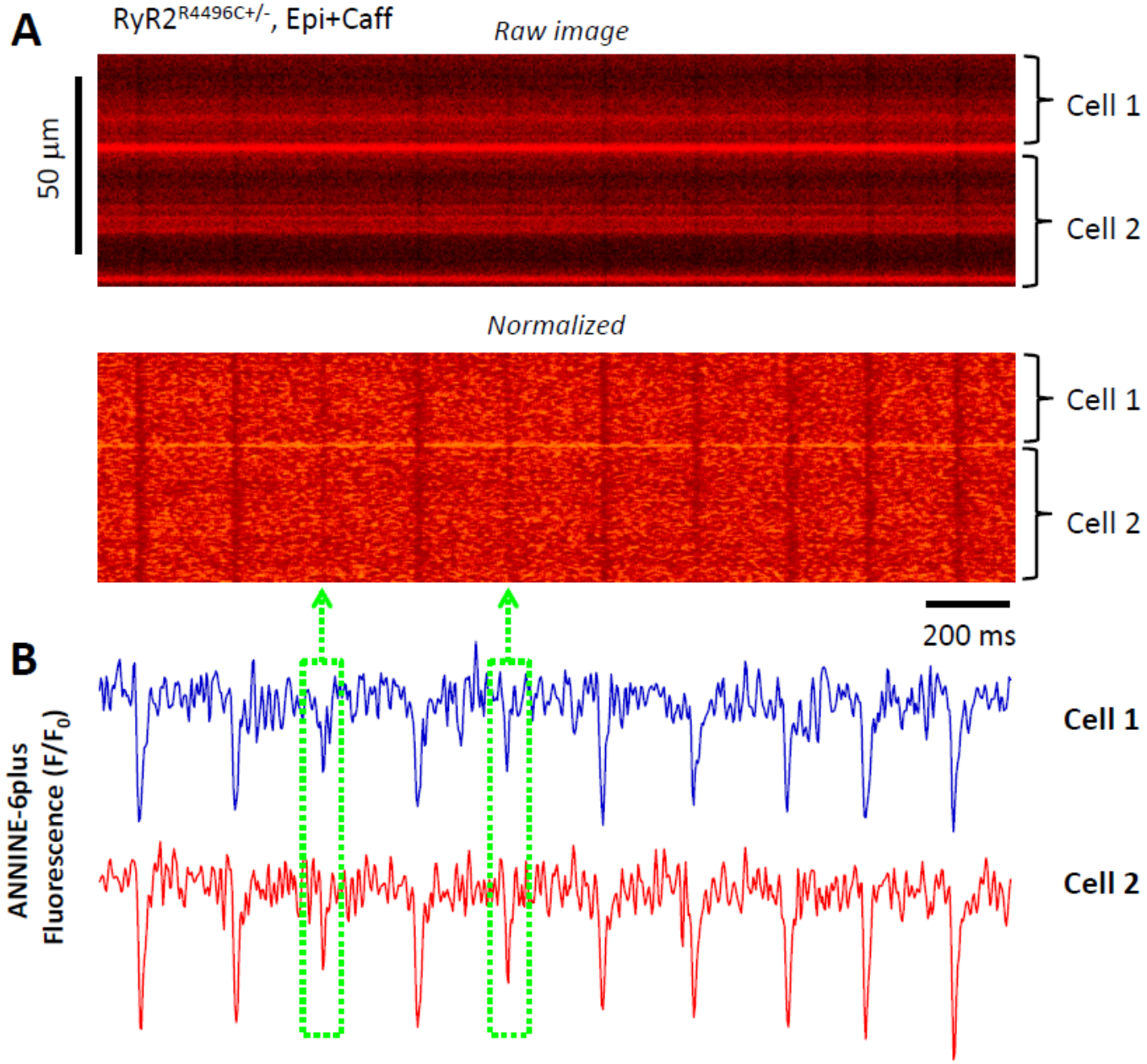

A
RyR2R4496C+/-, Epi+Caff
Raw image
50 µm
Cell 1
Cell 2
Normalized
Cell 1
Cell 2
200 ms
B
ANNINE-6plus
Fluorescence (F/F0)
Cell 1
Cell 2

Figure S4

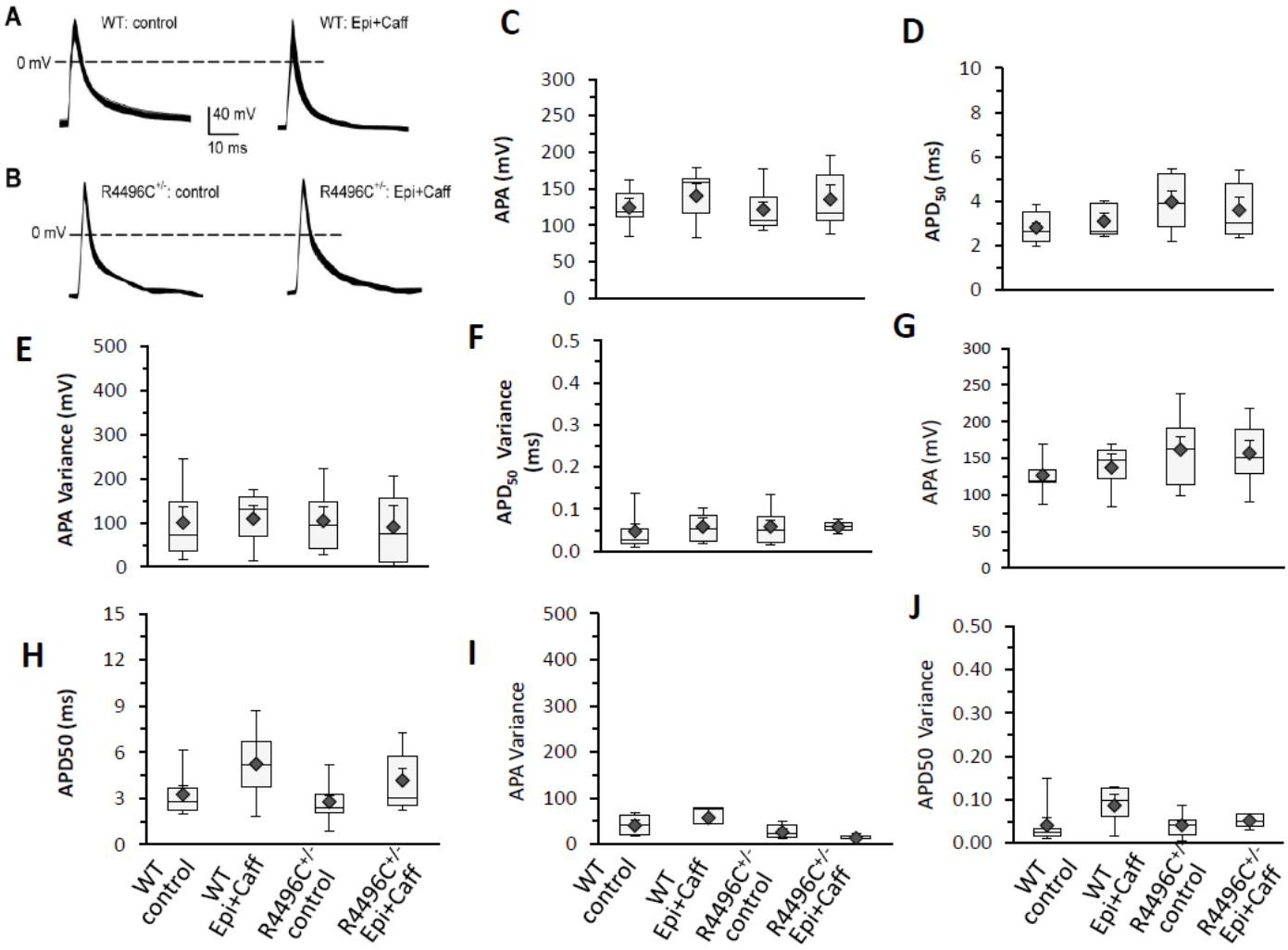
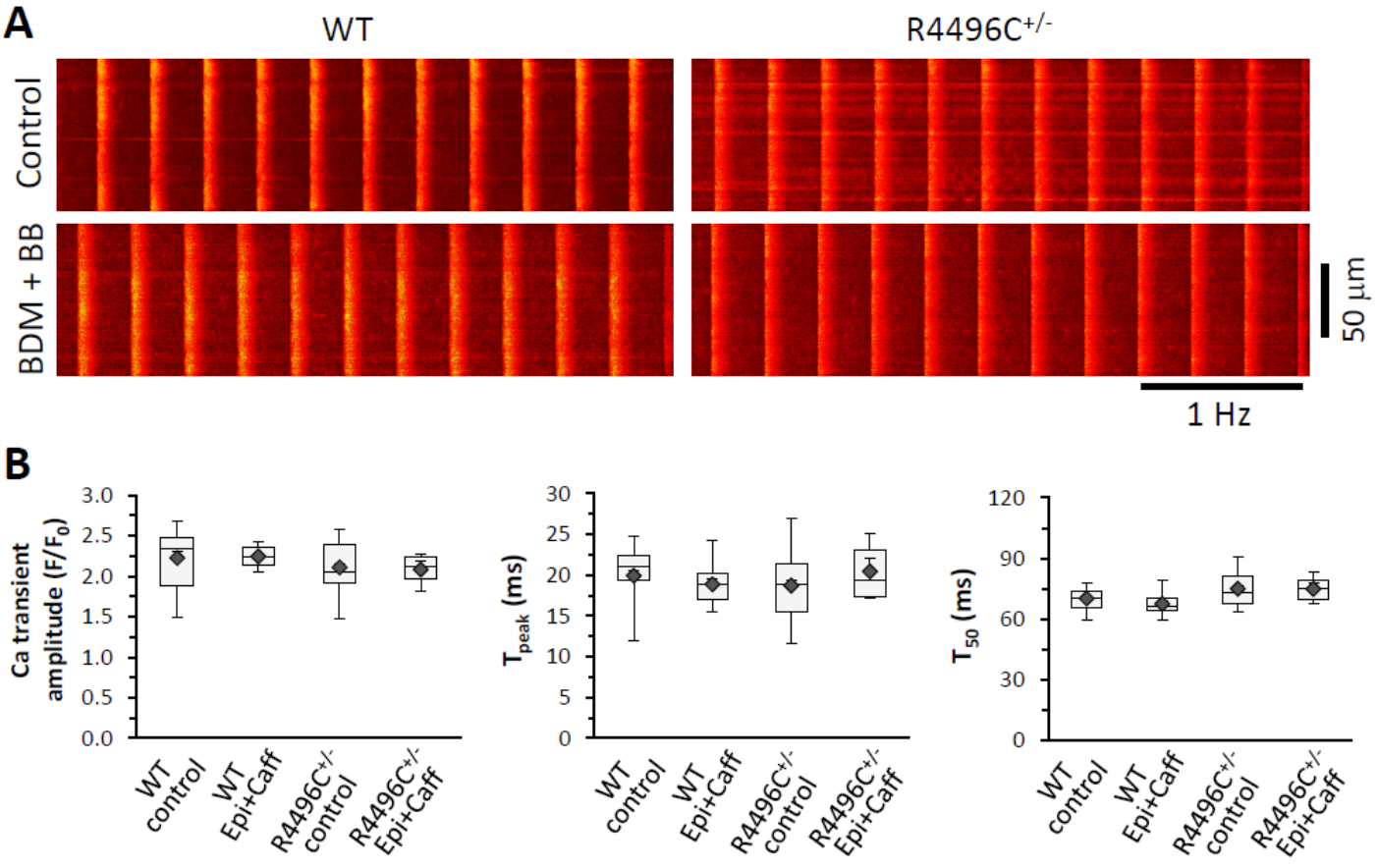

A
WT: control
WT: Epi+Caff
0 mV
40 mV
10 ms
B
R4496C+/-: control
R4496C+/-: Epi+Caff
0 mV
C
APA (mV)
D
APD50 (ms)
E
APA Variance (mV)
F
APD50 Variance (ms)
G
APA (mV)
H
APD50 (ms)
I
APA Variance
J
APD50 Variance
WT control
WT Epi+Caff
R4496C+/- control
R4496C+/- Epi+Caff

Figure S5


A
WT
R4496C+/-
Control
BDM + BB
50 µm
1 Hz
B
Ca transient amplitude (F/F0)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Tpeak (ms)
30
25
20
15
10
5
0
T50 (ms)
120
90
60
30
0
WT control
WT Epi+Caff
R4496C+/- control
R4496C+/- Epi+Caff