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Journal

Heart Rhythm, 19(10)

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Publication Date

2022-10-01

DOI

10.1016/j.hrthm.2022.05.034

Peer reviewed

HHS Public Access

Author manuscript

Heart Rhythm. Author manuscript; available in PMC 2023 December 22.

Published in final edited form as:

Heart Rhythm. 2022 October ; 19(10): 1725–1735. doi:10.1016/j.hrthm.2022.05.034.

Stimulation of the mitochondrial calcium uniporter mitigates chronic heart failure–associated ventricular arrhythmia in mice

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Abstract

BACKGROUND—An aberrant increase in the diastolic calcium concentration ($[Ca^{2+}]_i$) level is a hallmark of heart failure (HF) and the cause of delayed afterdepolarization and ventricular arrhythmia (VA). Although mitochondria play a role in regulating $[Ca^{2+}]_i$, whether they can compensate for the $[Ca^{2+}]_i$ abnormality in ventricular myocytes is unknown.

OBJECTIVE—The purpose of this study was to investigate whether enhanced Ca^{2+} uptake of mitochondria may compensate for an abnormal increase in the $[Ca^{2+}]_i$ of ventricular myocytes in HF to effectively mitigate VA.

METHODS—We used a HF mouse model in which myocardial infarction was induced by permanent left anterior descending coronary artery ligation. The mitochondrial Ca^{2+} uniporter was stimulated by kaempferol. Ca^{2+} dynamics and membrane potential were measured using an epifluorescence microscope, a confocal microscope, and the perforated patch-clamp technique. VA

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Disclosures: The authors have no conflicts of interest to disclose.

Appendix

Supplementary data

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.hrthm.2022.05.034.

was induced in Langendorff-perfused hearts, and hemodynamic parameters were measured using a microtip transducer catheter.

RESULTS—Protein expression of the mitochondrial Ca^{2+} uniporter, as assessed by its subunit expression, did not change between HF and sham mice. Treatment of cardiomyocytes with kaempferol, isolated from HF mice 28 days after coronary ligation, reduced the appearance of aberrant diastolic $[Ca^{2+}]_i$ waves and sparks and spontaneous action potentials. Kaempferol effectively reduced VA occurring in Langendorff-perfused hearts. Intravenous administration of kaempferol did not markedly affect left ventricular hemodynamic parameters.

CONCLUSION—The effects of kaempferol in HF of mice implied that mitochondria may have the potential to compensate for abnormal $[Ca^{2+}]_i$. Mechanisms involved in mitochondrial Ca^{2+} uptake may provide novel targets for treatment of HF-associated VA.

Keywords

Calcium waves; Delayed afterdepolarization; Heart failure; Mitochondrial calcium uniporter; Mitochondrial calcium uptake; Ventricular arrhythmia

Introduction

The number of patients with heart failure (HF) is increasing worldwide.[1] Sudden cardiac death due to ventricular arrhythmia (VA) is a major cause of death in patients with HF, so VA management is crucial. Structural and functional changes in ryanodine receptor 2 (RyR2) on the sarcoplasmic reticulum (SR) lead to calcium (Ca^{2+}) leakage from the SR via RyR2.[2,3] This Ca^{2+} binds to other RyR2s, leading to the propagation of calcium-induced calcium release, resulting in increased Ca^{2+} concentration throughout the cell cytoplasm. Moreover, the compensatory process for the increase in cytoplasmic Ca^{2+} concentration leads to delayed afterdepolarization (DAD) as a result of an inward sodium current via the sodium-calcium exchanger and the generation of an action potential, thereby inducing VA development. Therefore, compensating for abnormalities in Ca^{2+} handling, that is, the physiological control of intracellular Ca^{2+}, is a potential therapeutic strategy for treatment of VA.[4] However, because most currently available antiarrhythmic drugs do not alter Ca^{2+} dysregulation, it is crucial to develop effective therapeutic options for HF-associated VA.

Complex Ca^{2+} uptake systems and efflux mechanisms are in place to modulate mitochondrial Ca^{2+} homeostasis.[5,6] Mitochondrial Ca^{2+} release and uptake control local Ca^{2+} levels between the SR and mitochondria, thus playing an important role in the regulation of intracellular Ca^{2+} handling.[7]

Enhanced Ca^{2+} uptake into mitochondria suppresses isoproterenol (Iso)-induced Ca^{2+} waves (CaWs) in cardiomyocytes isolated from catecholamine-induced polymorphic ventricular tachycardia model mice.[8] However, the effects of increased mitochondrial Ca^{2+} uptake on Ca^{2+} dysregulation and arrhythmogenesis in HF remain unclear.

In the present study, we investigated whether increased Ca^{2+} uptake into mitochondria mitigates the increase in diastolic Ca^{2+} and VA in HF. As the mitochondrial Ca^{2+} uniporter (MCU), which consists of 4 core components, has been identified as the main Ca^{2+} importer

across the inner mitochondrial membrane, we used kaempferol, a well-defined activator of MCU activity, to enhance Ca^{2+} uptake into the mitochondria[9,10]

Methods

Extended methods are provided in the Supplementary Methods.

Ethics approval

All procedures and animal care were performed in accordance with the Animal Care Guidelines for the Care and Use of Laboratory Animals of the Hokkaido University Graduate School of Medicine as well as the relevant national and international guidelines, including the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. This study was approved by our Institutional Animal Research Committee (Study Approval No. 18–0077).

Monitoring of Ca^{2+} uptake into cardiac mitochondria, the mitochondrial membrane potential, and intracellular Ca^{2+} dynamics

To measure Ca^{2+} uptake in mitochondria, we monitored the reduction in extramitochondrial Ca^{2+} using Fluo-5N and a spectrofluorometer (RF-6000, Shimadzu, Kyoto, Japan). Of the 2–4 mg mitochondrial protein isolated from a single sham and HF mouse, 1 mg mitochondrial protein was used to measure extramitochondrial Ca^{2+} fluorescence with kaempferol, and another 1 mg protein was used to measure the fluorescence without kaempferol. Ventricular myocytes were loaded with tetramethylrhodamine methylester (TMRM) or Fluo-4 AM. TMRM fluorescence was monitored using a laser scanning confocal microscope (LSM-710, Carl Zeiss, Oberkochen, Germany). Local cytosolic Ca^{2+} signals were imaged using an IX-83 microscope (Olympus, Tokyo, Japan) with a Rolera EM-C^2 electron multiplying cooled charge-coupled device camera (QImaging, Surrey, BC, Canada). Depolarization-induced calcium transients in Fluo-4 AM-loaded cardiomyocytes were elicited by electric field stimulation at 0.5 Hz. Ca^{2+} sparks were measured using a laser scanning confocal microscope (LSM-710, Carl Zeiss).

Electrophysiological recording

Myocytes were patch-clamped in the current clamp mode (perforated patch-clamp technique) using an Axopatch 200B amplifier and pCLAMP-6.10 software (Molecular Devices, Sunnyvale, CA), as described previously.[8]

Induction of arrhythmia and *in vivo* hemodynamic analyses

Arrhythmia inducibility was evaluated in Langendorff-perfused hearts from HF and sham mice. VA events were induced by electrical stimulation or continuous infusion of 1 μM Iso. Hemodynamic parameters were monitored using a 1.4F micromanometer-tipped catheter (Millar Instruments, Houston, TX).

Statistical analysis

Data are given as mean ± SEM. We used the Student *t* test or 1-way analysis of variance followed by the Tukey multiple-comparison test for normally distributed data to detect

differences between groups. Poisson-distributed count data were fitted using a mixed-effect Poisson regression model. Paired *t* tests were used to compare the 2 related sample means. Categorical data were compared using the Fisher exact test. $P < .05$ was considered to indicate a significant difference between groups. Statistical analyses were performed using Graph Pad Prism Version 8 (GraphPad Software, San Diego, CA) and SAS Version 9.4 (SAS Institute Inc., Cary, NC).

Results

Characteristics of sham and HF mice

Echocardiography was performed on all mice in the sham (n = 61) and HF (n = 123) groups. HF mice showed significant dilation of the left ventricle (LV) and reduced percent fractional shortening (%FS) compared with sham mice (Supplemental Figures 1 and 2). LV weight/body weight (BW) and lung weight/BW were significantly higher in HF group mice compared to sham mice on day 28 postsurgery (Table 1). LV diameter and posterior wall thickness were significantly increased, whereas LV %FS was significantly decreased in HF mice than in sham mice. No significant differences were observed in the heart rates between groups.

To investigate the key Ca^{2+} handling proteins, we investigated the expression of sarcoplasmic/endoplasmic reticulum Ca^{2+}ase 2a (SERCA2a), Ser-2808 phosphorylated RyR, and Ser-2814 phosphorylated RyR in the LV myocardium using western blot analysis. Although SERCA expression and phosphorylation of RyR at the protein kinase A site (Ser-2808) did not change significantly, phosphorylation at the calmodulin kinase II site of the RyR (Ser-2814) increased (Supplemental Figures 3 and 4).

Kaempferol increases mitochondrial Ca^{2+} uptake without altering the mitochondrial membrane potential

The protein expression level of the mitochondrial Ca^{2+} uniporter was similar in the cardiomyocytes of sham and HF mice (Figures 1A–C and Supplemental Figure 4). We evaluated extramitochondrial Ca^{2+} clearance using isolated cardiac mitochondria from sham and HF mice and found that kaempferol substantially increased mitochondrial Ca^{2+} uptake in both HF (Figures 1D and 1E, and Supplemental Figure 5) and sham mice (Supplemental Figures 6A, 6B, and 7). Enhanced mitochondrial Ca^{2+} uptake by kaempferol was suppressed by the MCU blocker ruthenium red in wildtype mice (Supplemental Figure 6C). Mitochondrial Ca^{2+} overload induces mitochondrial permeability transition pore (mPTP) opening and depolarization of the mitochondrial membrane, leading to mitochondrial dysfunction and eventually cell death.[11] We confirmed that carbonyl cyanide-ptrifluoromethoxyphenylhydrazone (FCCP) strongly depolarized the mitochondrial membrane; however, kaempferol did not alter the mitochondrial membrane potential ($\Delta\psi_m$) (Figure 1F and Supplemental Figure 8).

Kaempferol abolishes arrhythmogenic Ca^{2+} events (CaWs and Ca^{2+} sparks)

Under control conditions, CaWs were not observed in HF cardiomyocytes during electrical stimulation at 0.5 Hz; however, treatment with Iso resulted in the detection of frequent

CaWs (Supplemental Figures 9 and 10). To solely analyze diastolic CaWs, we recorded CaWs for 30 seconds after electrical stimulation. The number of cells exhibiting spontaneous diastolic CaW signals and the average number of CaWs in each cardiomyocyte were analyzed. In sham mice, CaWs were not increased upon administration of either Iso or Iso with kaempferol (Supplemental Figure 11). In contrast, Iso treatment resulted in an increased number of cells exhibiting CaW signals, and the average number of CaWs in HF mice also increased. Although treatment with 10 μM kaempferol did not reduce the number of cells displaying CaW signals, it significantly reduced the average number of CaWs. Treatment with ruthenium red in this background reversed the kaempferol-induced reduction in the average number of CaWs (Figures 2A–2C).

We observed an increase in Ca^{2+} spark frequency after treatment with Iso, which was reduced upon treatment with kaempferol and efsevin (Figures 2D and 2E, and Supplemental Figure 12).

Kaempferol reduces spontaneous action potentials in HF cardiomyocytes

In the presence of 0.5 μM Iso, the frequency of spontaneous action potentials (SAPs) and DADs were increased compared with that of the vehicle. These increase was normalized upon treatment with 10 μM kaempferol. Although kaempferol marginally decreased the resting potentials, it did not affect the maximum amplitude of SAPs or the action potential duration at 90% repolarization (Figure 3). We evaluated the resting membrane potential (RMP) of cells with and without SAPs regardless of Iso and kaempferol administration. We found no significant difference in RMP between cells with and those without action potentials (APs) (Supplemental Figure 13A). Similarly, the RMP of cells treated with Iso was comparable between those with and those without APs (Supplemental Figure 13B).

Kaempferol mitigates Iso-induced VA in Langendorff-perfused hearts

We analyzed the inducibility of sustained ventricular arrhythmias (SVAs) by burst pacing under β-adrenoreceptor stimulation with Iso (100 nM). In sham mice, SVA was rarely induced in response to burst pacing under conditions of stimulation with 100 nM Iso (Supplemental Figure 14). In HF mice, SVAs were frequently induced during perfusion with 100 nM Iso, whereas 10 μM kaempferol significantly inhibited VAs without changing the heart rate significantly (Figures 4A and 4B, and Supplemental Figure 15).

Because we could not exclude the re-entrant mechanism in the evaluation of SVAs by burst pacing, we performed an induction test at an increased concentration of Iso (1 μM) without electrical stimulation. In the sham group, spontaneous events of short VA were not observed. In contrast, short VAs were observed in approximately 50% of the HF group mice, and kaempferol significantly inhibited the generation of spontaneous short VAs (Figures 4C and 4D).

Kaempferol does not alter the steady-state Ca^{2+} transient amplitude

The maximum fluorescence intensity of 10 μM caffeine-induced Ca^{2+} transients was decreased in the presence of 10 μM kaempferol compared to that in the presence of a vehicle (Figures 5A and 5B, and Supplemental Figure 16). In contrast, the first field-stimulated Ca^{2+}

transient amplitude did not differ in the presence or absence of kaempferol (Figures 5C–5E). However, the time to 50% decay ($t_{1/2}$) and tau decay of the Ca^{2+} transient were extended in the presence of kaempferol (Figures 5F and 5G), and baseline Fluo-4 fluorescence intensity was not different between the 2 groups (Figure 5H).

Kaempferol reduces episodes of epinephrine- and caffeine-induced VA and does not affect hemodynamic *in vivo*

To assess the potency of kaempferol in suppressing arrhythmia, we administered kaempferol to HF mice using implantable osmotic minipumps. All mice recovered well from surgery and showed no signs of abnormal behavior. After 3 days, kaempferol showed no effect on echocardiographic and electrocardiogram parameters (Supplemental Figures 17 and 18). The frequency of TUNEL-positive cells was not significantly different between vehicle-treated HF mice and kaempferol-treated HF mice (Supplemental Figure 19). We then injected mice with a bolus of 2 mg/kg epinephrine and 120 mg/kg caffeine (epi/caff) to activate the adrenergic response. Kaempferol reduced the episodes of SVA under catecholaminergic stimulation (Figures 6A and 6B).

We compared LV hemodynamic parameters before and 30 minutes after kaempferol administration (5 mg/kg BW). LV systolic pressure, LV diastolic pressure, positive change in pressure over time (dp/dt), and negative dp/dt did not differ between the 2 conditions, that is, before and after kaempferol administration in sham and HF mice (Figures 6C–6H, and Supplemental Figure 20). Kaempferol decreased complex I + II–linked respiration in mitochondria isolated from sham and HF cardiomyocytes (Supplemental Figure 21).

Discussion

In this study, we investigated the role of mitochondrial Ca^{2+} uptake in the modulation of Ca^{2+} handling in HF ventricular cardiomyocytes. The major findings were as follows. (1) Mitochondrial Ca^{2+} uptake through kaempferol-stimulated MCU prevented the formation of diastolic CaWs, Ca^{2+} sparks, and action potentials. (2) Mitochondrial Ca^{2+} uptake suppressed Iso with/without pacing-induced VAs. (3) Mitochondrial Ca^{2+} uptake did not alter hemodynamic parameters.

Treatment to improve Ca^{2+} handling abnormalities associated with HF

Abnormal intracellular Ca^{2+} handling due to Ca^{2+} leakage via RyR2 is one of the key hallmarks of HF. Impaired Ca^{2+} homeostasis may lead to defects in excitation–contraction coupling and cardiac dysfunction, and may also trigger VA due to DAD.[12] Most antiarrhythmic drugs target cell membrane receptors and channels, such as beta-1 adrenergic receptor or Na^1, K^1, and Ca^{2+} channels, most of which do not rectify defective intracellular Ca^{2+} handling. Stabilization of Ca^{2+} handling abnormalities may be a promising therapeutic approach to HF. In cardiomyocytes, the governing mechanism of Ca^{2+} handling and its rate-limiting factor are unknown. However, mitochondrial Ca^{2+} uptake and release could affect diastolic cytosolic Ca^{2+} concentration,[13] and mitochondrial Ca^{2+}-uptake proteins ensure that the mitochondrial calcium uniporter acts as a gatekeeper at low $[Ca^{2+}]_i$, and as a positive regulator at high $[Ca^{2+}]_i$.[14,15] Thus, mitochondria play a role in the regulation

of intracellular Ca^{2+}. Our results suggest that enhanced mitochondrial Ca^{2+} uptake in HF has an antiarrhythmic effect. Therapeutic strategies that promote mitochondrial Ca^{2+} uptake may be an alternative to the current treatments of VA in HF. In HF, as the protein expression of the mitochondrial Ca^{2+} uniporter was unchanged and kaempferol enhanced mitochondrial Ca^{2+} uptake, kaempferol may be able to mitigate VA. Thus, it may be necessary to classify HF patients based on the capacity of mitochondrial Ca^{2+} uptake, but further research is needed in this direction.

Adverse effects of enhancing mitochondrial Ca^{2+} uptake

We also evaluated the adverse effects of enhancing mitochondrial Ca^{2+} uptake. Excessive mitochondrial Ca^{2+} uptake often induces permeabilization of the mitochondrial membrane and induction of apoptosis by opening mPTP.[16] However, we found that kaempferol did not alter $\Delta\psi_m$, at least during the short term. In contrast, mPTP opening by FCCP clearly dissipated $\Delta\psi_m$, potentially leading to mitochondrial membrane permeabilization. Furthermore, continuous infusion of kaempferol *in vivo* did not affect cardiac function. Moreover, the currently used antiarrhythmic drugs have proarrhythmic and negative inotropic side effects, which limit their clinical application.[17] However, kaempferol had no effect on hemodynamics in the short term. Ca^{2+} uptake–based therapeutic agents do not affect hemodynamics unlike conventional antiarrhythmic drugs.

Similar to previous studies, we observed that kaempferol inhibited mitochondrial energy production.[18–20] However, this result is inconsistent with the findings of another study, according to which a moderate increase in mitochondrial Ca^{2+} results in enhanced energy production.[21] Many aspects of Ca^{2+} handling and energy production in cardiomyocytes still are unclear, and the role of the mitochondrial Ca^{2+} uniporter complex also is not known. Further studies investigating the potential adverse effects of increased mitochondrial Ca^{2+} uptake in terms of changes in cellular bioenergetics are required.

Study limitations

First, this study did not measure mitochondrial Ca^{2+} directly in isolated cardiomyocytes because this is hindered by the spatial and temporal resolution. Therefore, mitochondrial Ca^{2+} uptake was evaluated in isolated mitochondria (Figures 1D and 1E). Furthermore, our results indicated the antiarrhythmic effect of 3 days of continuous kaempferol administration. Although a previous study reported no adverse effects of long-term kaempferol administration,[9] the long-term effects of increased mitochondrial Ca^{2+} uptake in the heart and other organs and the associated cellular bioenergetics require detailed study, which may lead to clinical applications. Second, although kaempferol enhanced Ca^{2+} uptake in the isolated mitochondria and reduced CaWs in HF cardiomyocytes, which were reversed by ruthenium red, kaempferol might have affected cytosolic Ca^{2+} via mechanisms other than MCU activation, such as ATP production and reactive oxygen species–related mechanisms. We observed that kaempferol prolonged the tau decay in the depolarizationinducedCa^{2+}transient, which could not be explained directly by enhanced mitochondrial Ca^{2+} uptake. SERCA and the sodium–calcium exchanger play major roles in regulating cytosolic Ca^{2+} in each heartbeat. We observed a decrease in complex I + II–linked mitochondrial respiration with kaempferol, suggesting decreased ATP production,

which potentially leads to reduced SERCA activity and prolonged tau decay. However, the influence of mitochondrial Ca^{2+} uptake on beat-to-beat $[Ca^{2+}]i$ oscillation remains largely unknown. Finally, we observed a slightly lower RMP with kaempferol administration, suggesting an increased hyperpolarizing current. However, we did not observe a significant relationship between the presence of SAP and RMP. Furthermore, we could not clarify the mechanism by which RMP was hyperpolarized by kaempferol and whether hyperpolarized RMP affected the suppression of Ca^{2+} handling and arrhythmia inducibility in the present study.

Conclusion

The stimulation of MCU activity by kaempferol mitigates DAD and VAs in HF and substantially ameliorates cardiac Ca^{2+} dysregulation. Thus, targeting MCU may be a novel strategy for treatment of HF-associated VAs, thereby presenting a basis for further research on alternative approaches to the treatment of VAs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Yuki Kimura and Miwako Yamane for their assistance with the experiments, and Drs Takayuki Yoshida and Mitsuhiro Yoshioka for their assistance in preparing the patch-clamp experiment.

Funding Sources:

This work was partially supported by the Japan Society for the Promotion of Science KAKENHI (Grant Numbers 18K08058 to Dr Watanabe; 18K15874 to Dr Temma; and 19K17584 to Dr Kamada); and the U.S. National Institutes of Health (Grant Number R01GM071779 to Dr Kwon).

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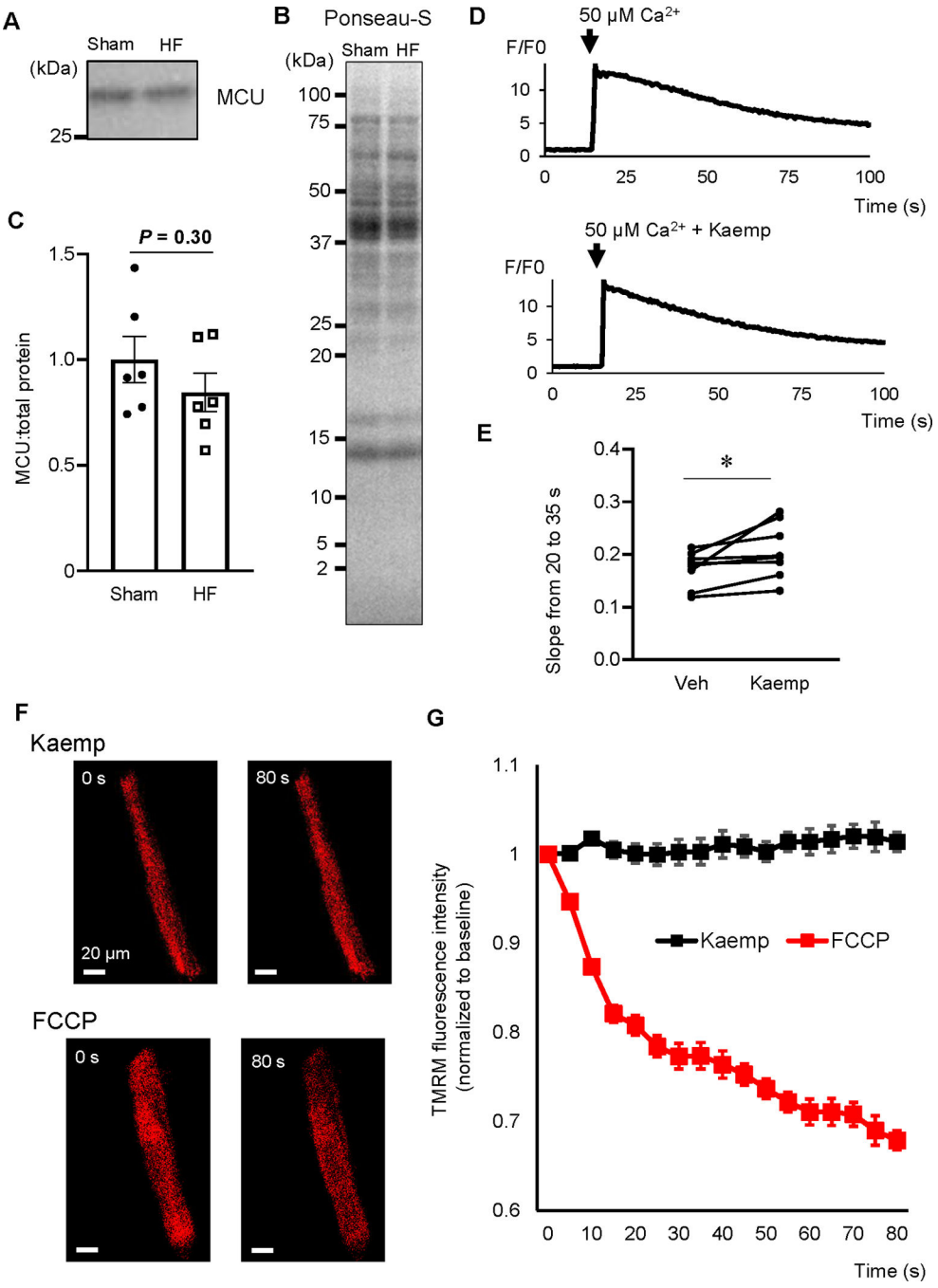

Figure 1.

Kaempferol reduces extramitochondrial Ca^{2+} levels without altering the mitochondrial membrane potential ($\Delta\psi_m$). **A:** Representative western blot depicting MCU expression in sham and HF mice. **B:** Total protein levels determined by Ponceau-S staining were used as internal controls. The size of each molecular weight marker is indicated on the left in kDa. **C:** Ratio of MCU to total protein for the sham (n = 6) and HF (n = 6) groups. The protein level in the sham group was normalized to 1. Statistical analysis was performed using an unpaired *t* test. **D:** Representative traces of fluorescence intensity (F/F0)

in mitochondria from the HF group after the addition of Ca^{2+} (50 μM), with (**bottom**) and without (**top**) kaempferol. Changes in extramitochondrial Ca^{2+} levels were monitored using Fluo-5N fluorescence. **E:** Kaemp increased mitochondrial Ca^{2+} uptake in the HF group (n = 8). Statistical analysis was performed using a paired t test. **F:** TMRM fluorescence was monitored as an indicator of $\Delta\psi_m$. FCCP (10μM) was used as a positive control. Photomicrographs of control cardiomyocytes (0s) and cardiomyocytes 80 s after treatment with kaempferol or FCCP. **G:** Summary data showing changes in TMRM fluorescence in the presence of kaempferol (10 μM) and FCCP (10 μM). FCCP, n = 9; Kaemp, n = 8. Values are given as mean ± SEM. **P* <.05 vs Veh. FCCP = carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone; HF = heart failure; Kaemp = kaempferol; MCU = mitochondrial calcium uniporter; TMRM = tetramethylrhodamine methylester; Veh = vehicle.

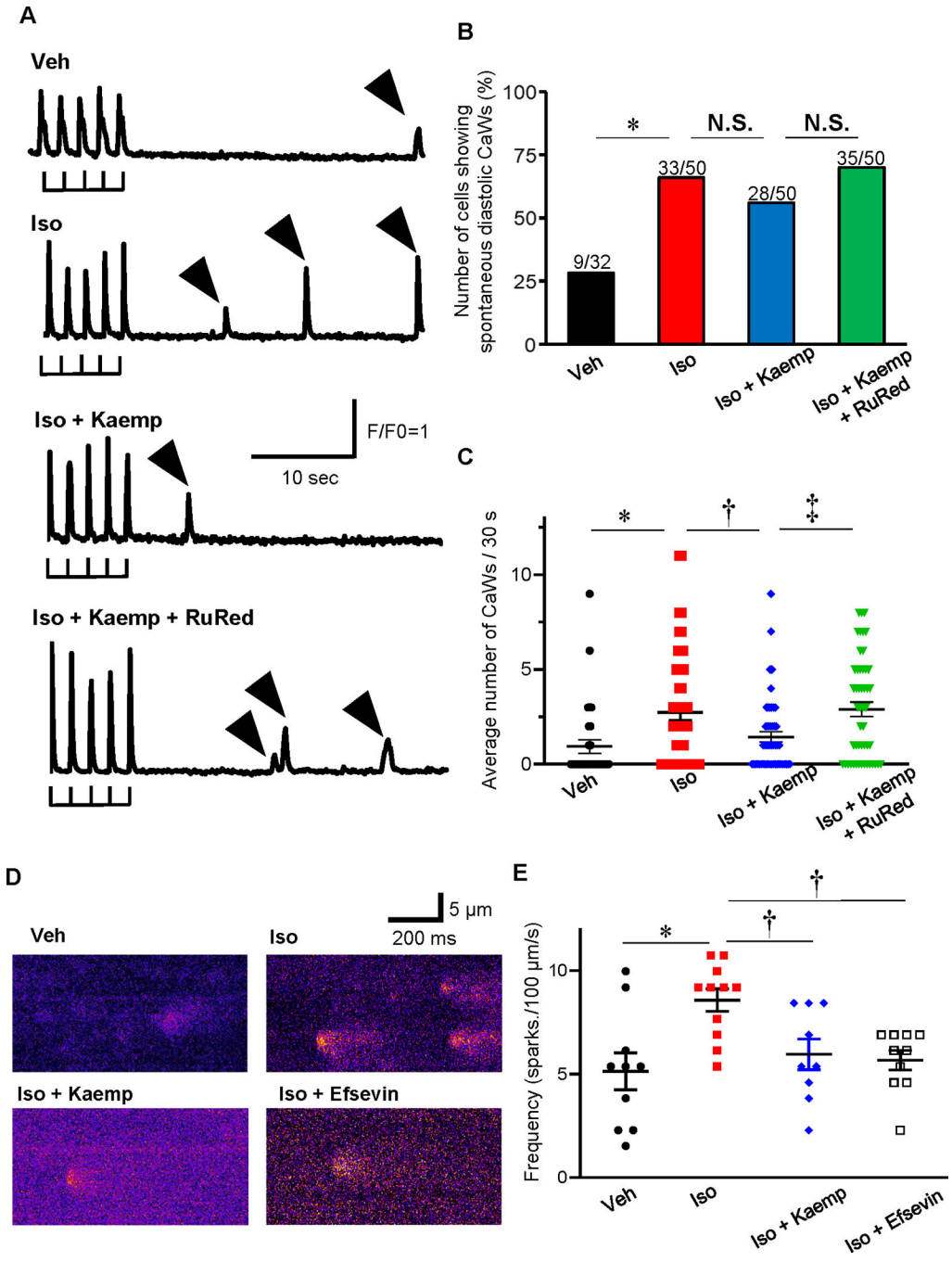

Figure 2.

Kaempferol reduces the diastolic Ca^{2+} waves and Ca^{2+} sparks in HF cardiomyocytes. **A:** Fluorescence intensity plots of line-scans across the long axis of cardiomyocytes from HF mice loaded with Fluo-4 AM, for measurement of intracellular Ca^{2+} levels. Cells were stimulated at 0.5 Hz until they reached steady-state conditions. The last 5 Ca^{2+} transients are shown. After stopping the pulses, the diastolic phase was recorded for 30 seconds to observe spontaneous diastolic CaWs. *Black bars* indicate electrical stimulation. **B:** Summary of the incidences of CaWs in HF mice. Statistical analysis was performed using the Fisher exact

test. **C:** Summary of the average number of CaWs per 30 seconds in HF mice. Statistical analysis was performed using a mixed-effect Poisson regression model. Veh, n =32, N = 4; Iso, n =50, N = 5; Iso plus Kaemp, n =50, N = 4; Iso plus Kaemp and RuRed, n = 50, N = 4. **D:** Representative line-scan confocal images from HF cardiomyocytes revealing spontaneous Ca^{2+} sparks. **E:** Summary of the average frequency of Ca^{2+} sparks in the HF group. Veh, n = 10, N = 6; Iso, n = 11, N = 6; Iso plus Kaemp, n = 9, N = 6, Iso plus Efsevin, n = 10, N = 2; 1-way analysis of variance followed by the *post hoc* Tukey multiple-comparison test. Values are given as mean ± SEM. *P <.05 vs Veh; †P <.05 vs Iso; ‡P <.05 vs Iso 1 kaempferol. CaWs = Ca^{2+} waves; HF = heart failure; Iso = isoproterenol; Kaemp = kaempferol; N.S. = not significant; RuRed = ruthenium red; Veh = vehicle.

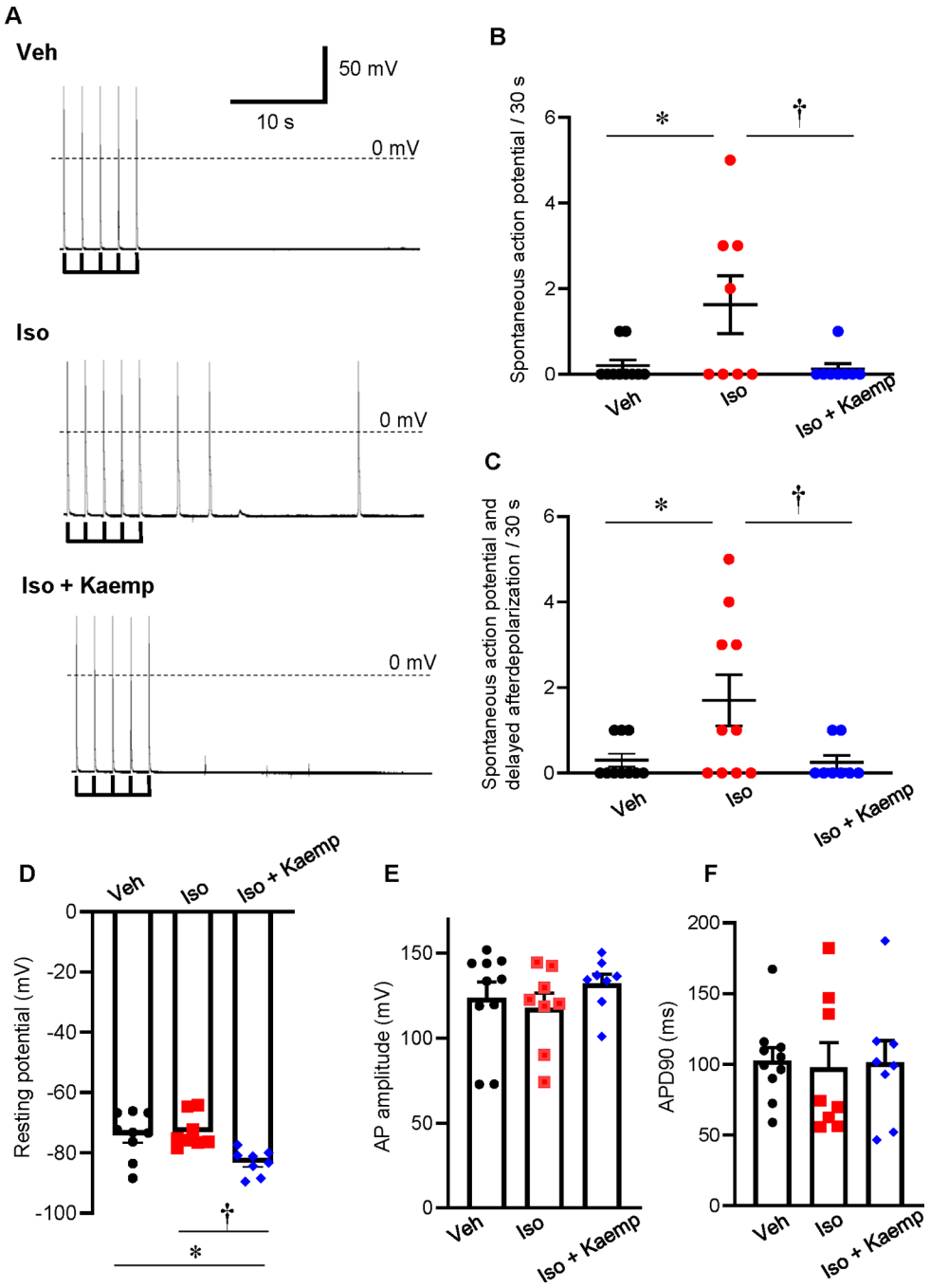

Figure 3.

Kaempferol reduces the spontaneous action potentials of cardiomyocytes from mice with HF. **A:** Representative perforated patch-clamp recordings of cardiomyocytes from HF mice. *Black bars* indicate current stimulation. **B, C:** Summary of the incidences of spontaneous diastolic action potentials (B) and delayed afterdepolarization (**C**) in cardiomyocytes from HF mice. Statistical analysis was performed using a mixed-effect Poisson regression model. **D–F:** Resting potential (**D**), action potential amplitude (**E**), and action potential duration at 90% repolarization (APD90) (**F**) in the indicated cardiomyocytes. Kaemp significantly

decreased the resting potential. Statistical analysis was performed using 1-way analysis of variance (ANOVA) followed by the *post hoc* Tukey multiple-comparison test. Veh, n =10, N = 8; Iso, n = 8, N = 7; Iso plus Kaemp, n = 8, N = 3; 1-way ANOVA followed by the *post hoc* Tukey multiple-comparison test. Values are given as mean ± SEM. *P <.05 vs Veh; †P <.05 vs Iso. AP = action potential; HF = heart failure; Iso = isoproterenol; Kaemp = kaempferol; Veh = vehicle.

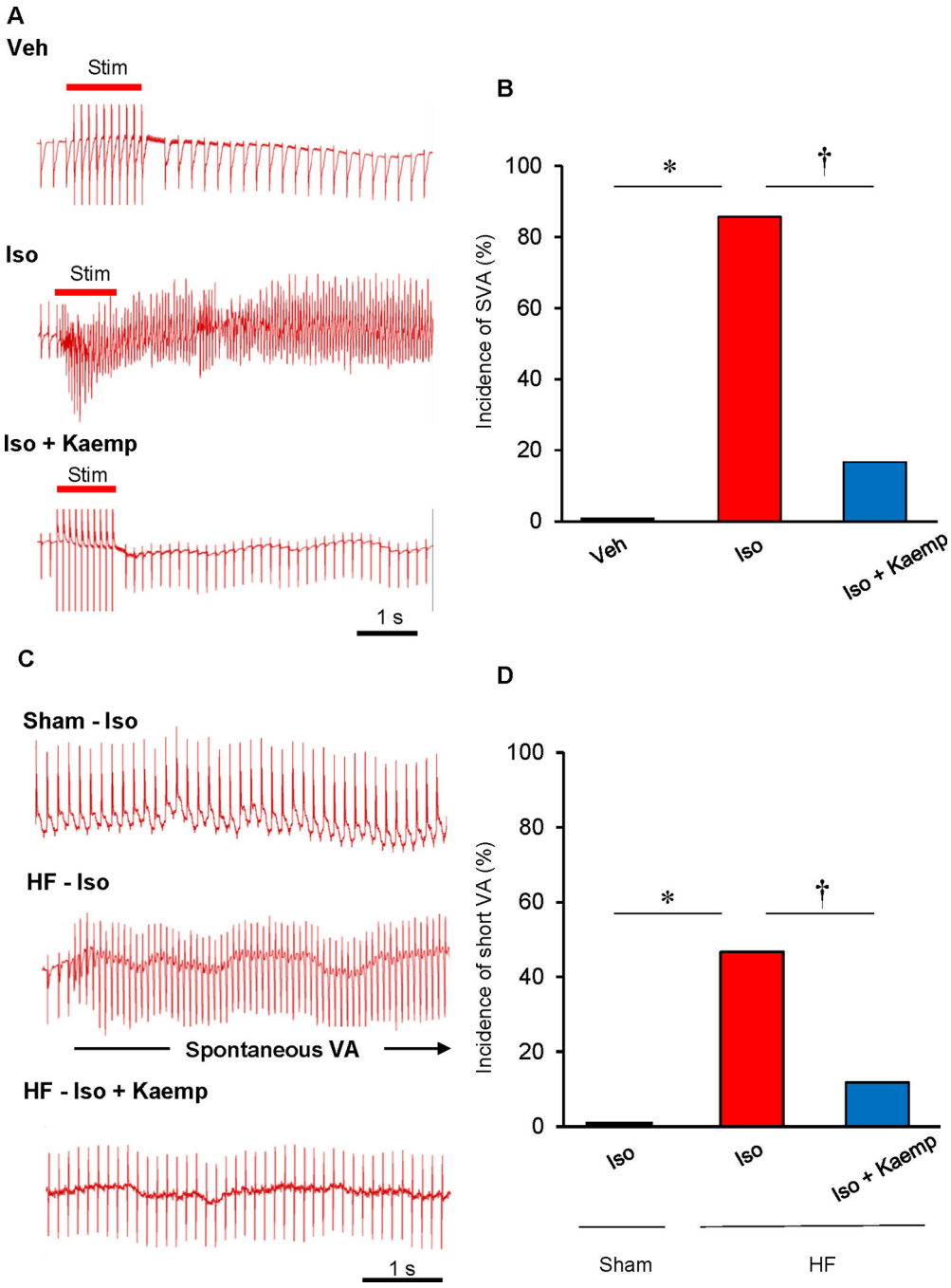

Figure 4.

Kaempferol mitigates VA in Langendorff-perfused hearts. Inducibility of VA was tested by administering 100 nM Iso with burst pacing (**A, B**) and 1 μM Iso without burst pacing (**C, D**). A: Representative electrogram traces obtained from HF mice. **Top:** control; middle: Iso administration; **bottom:** Iso plus Kaemp (10 μM) administration. **B:** Summary of SVA incidences in HF mice. Veh, n = 5; Iso, n = 7; Iso plus Kaemp, n = 6. Statistical analysis was performed using the Fisher exact test. **C:** Representative electrogram traces obtained from sham (**top**) and HF (**middle**) mice in the presence of 1 μM Iso. **Bottom:** Electrogram

trace from HF mice in the presence of Iso plus 10 μM kaempferol. **D:** Sum of short VA incidences in sham and HF mice. The hearts of mice from the sham group (n = 10) were perfused with Iso alone, whereas those from the HF group were perfused with Iso (n = 15) and Iso plus Kaemp (n = 18). *P <.05 vs Veh; †P <.05 vs Iso, Fisher exact test. HF = heart failure; Iso = isoproterenol; Kaemp = kaempferol; SVA = sustained ventricular arrhythmia; VA = ventricular arrhythmia; Veh = vehicle.

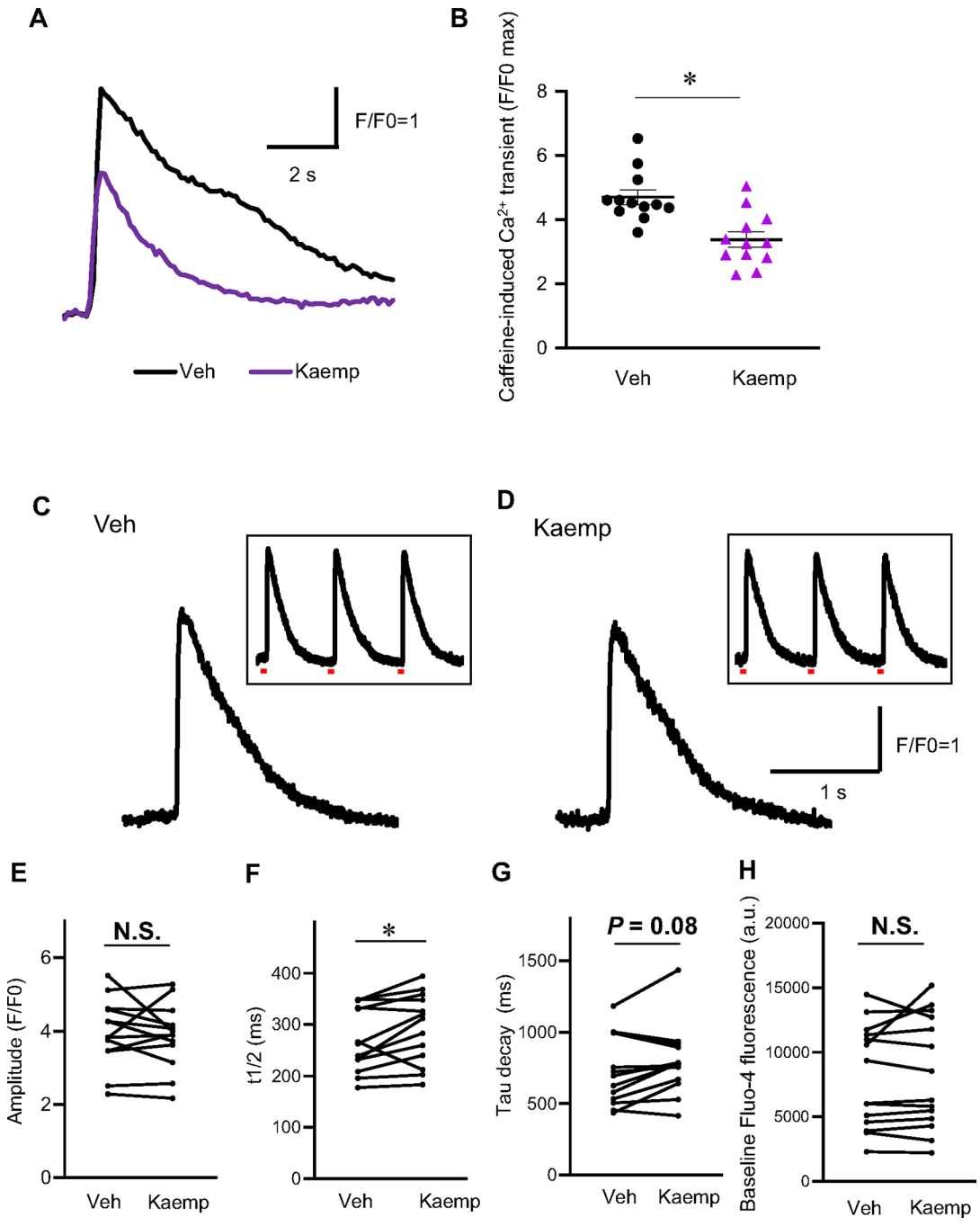

Figure 5.

Kaempferol decreases caffeine-induced Ca^{2+} transients but does not alter their amplitude. **A:** Ca^{2+} fluorescence traces of the HF group. **B:** Kaemp decreased the maximum amplitude of caffeine-induced Ca^{2+} transients in cardiomyocytes of the HF group. Veh, n = 12, N = 4; Kaemp, n = 12, N = 4; unpaired *t* test. **C, D:** Confocal line-scans across the long axis of isolated cardiomyocytes from HF mice. Representative fluorescence intensity plots are shown. *Red bars* depict the delivery of electrical stimulation. **E:** Kaemp did not change the amplitude of Ca^{2+} transients. **F, G:** The time to 50% decay ($t_{1/2}$) and tau decay of

Ca^{2+} transients were extended by Kaemp treatment. There was no significant difference in baseline Fluo-4 fluorescence intensity between myocytes treated with and without Kaemp. **H:** n = 12, N = 8. **P* <.05 vs Veh, unpaired *t* test. HF = heart failure; Iso = isoproterenol; Kaemp = kaempferol; N.S. = not significant; Veh = vehicle.

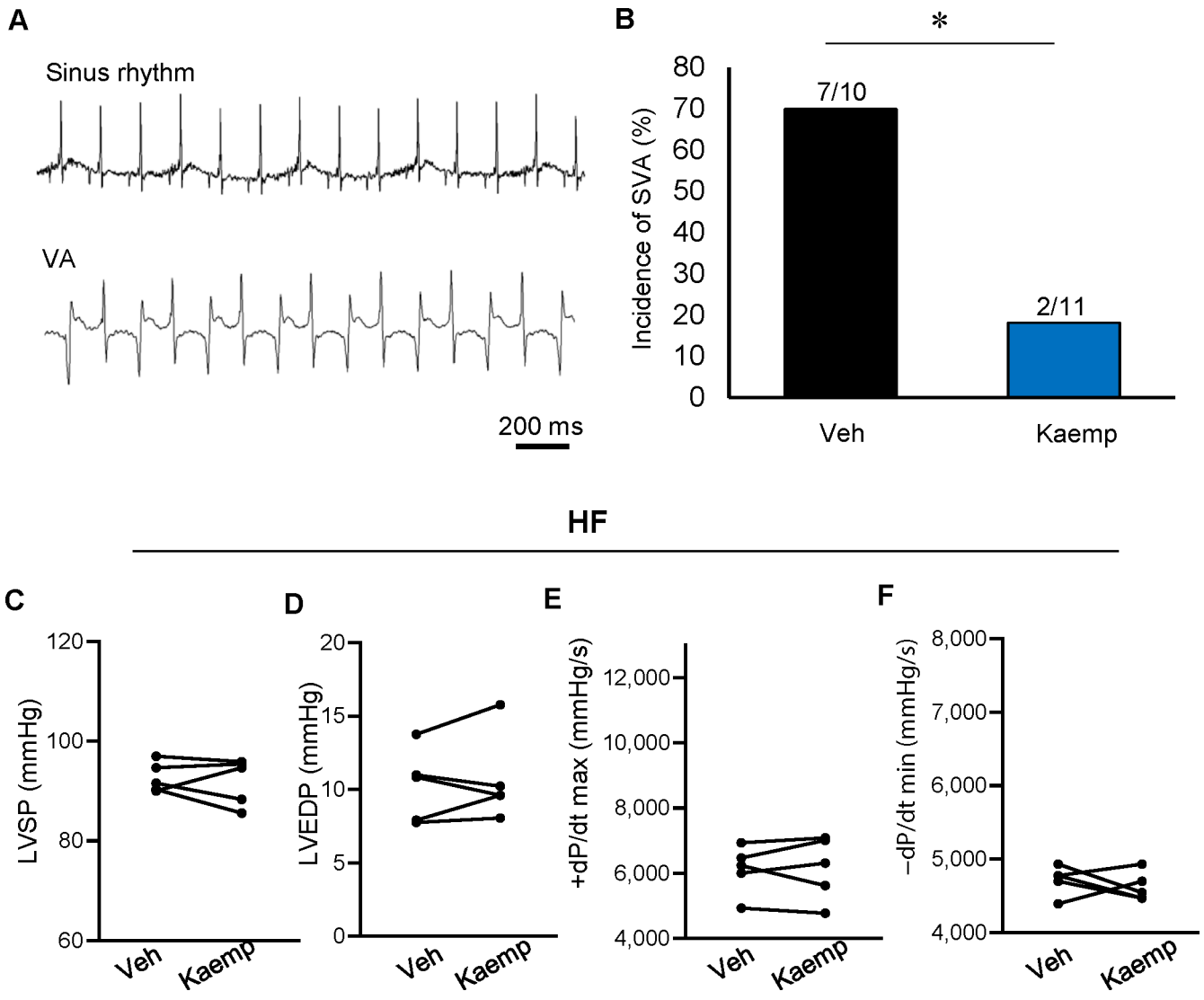

Figure 6.

Kaempferol reduces episodes of VA and does not alter the hemodynamics in HF. **A:** Examples of electrocardiographic recording in sinus rhythm and VA. **B:** Episodes of SVA were recorded in 7 of 10 vehicle-treated mice, but the incidence of VA episodes was significantly reduced to 2 of 11 mice with Kaemp ($P = .030$, Fisher exact test). *$P < .05$ vs Veh, Fisher exact test. **C–F:** Kaemp did not significantly alter LV systolic pressure (**C**), LV diastolic pressure (**D**), positive dp/dt (**E**), or negative dp/dt (**F**) in HF (n = 5) mice. Statistical analysis was performed using a paired *t* test. HF = heart failure; Kaemp = kaempferol; LV = left ventricle; LVEDP = left ventricular end-diastolic pressure; LVSP = left ventricular systolic pressure; SVA = sustained ventricular arrhythmia; VA = ventricular arrhythmia; Veh = vehicle.

Table 1

Characteristics of mice in HF and sham groups at 28 days postsurgery

	Sham (n = 6)	**HF (n = 6)**
Body and organ weights		
BW (g)	25.8 ± 0.6	25.3 ± 0.6
LV weight/BW (mg/g)	3.01 ± 0.06	4.74 ± 0.26 *
Lung weight/BW (mg/g)	5.21 ± 0.08	6.59 ± 0.27 *
	Sham (n = 61)	HF (n = 123)
Echocardiography		
Heart rate (bpm)	720 ± 4	715 ± 2
LV end-diastolic diameter (mm)	3.2 ± 0.02	5.0 ± 0.03 *
LV end-systolic diameter (mm)	1.7 ± 0.01	4.2 ± 0.03 *
Fractional shortening (%)	48.0 ± 0.8	15.1 ± 0.2 *
Anterior wall thickness (mm)	0.8 ± 0.01	0.4 ± 0.01 *
Posterior wall thickness (mm)	0.9 ± 0.02	0.6 ± 0.03 *

Data are given as mean ± SEM.

BW = body weight;HF = heart failure;LV = left ventricle.

* $P < .05$ vs sham.