Reduced junctional Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger activity contributes to sarcoplasmic reticulum Ca\textsuperscript{2+} leak in junctophilin-2-deficient mice

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Wang W, Landstrom AP, Wang Q, Munro ML, Beavers D, Ackerman MJ, Soeller C, Wehrens XH. Reduced junctional Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger activity contributes to sarcoplasmic reticulum Ca\textsuperscript{2+} leak in junctophilin-2-deficient mice. Am J Physiol Heart Circ Physiol 307: H1317–H1326, 2014. First published September 5, 2014; doi:10.1152/ajpheart.00413.2014.—Expression silencing of junctophilin-2 (JPH2) in mouse heart leads to ryanodine receptor type 2 (RyR2)-mediated sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} leak and rapid development of heart failure. The mechanism and physiological significance of JPH2 in regulating RyR2-mediated SR Ca\textsuperscript{2+} leak remains elusive. We sought to elucidate the role of JPH2 in regulating RyR2-mediated SR Ca\textsuperscript{2+} release in the setting of cardiac failure. Cardiac myocytes isolated from tamoxifen-inducible conditional knockdown mice of JPH2 (MCM-shJPH2) were subjected to confocal Ca\textsuperscript{2+} imaging. MCM-shJPH2 cardiomyocytes exhibited an increased spark frequency width with altered spark morphology, which caused increased SR Ca\textsuperscript{2+} leakage. Single channel studies identified an increased RyR2 open probability in MCM-shJPH2 mice. The increase in spark frequency and width was observed only in MCM-shJPH2 and not found in mice with increased RyR2 open probability with native JPH2 expression. Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger (NCX) activity was reduced by 50% in MCM-shJPH2 with no detectable change in NCX expression. Additionally, 50% inhibition of NCX through Ca\textsuperscript{2+}-administration alone was sufficient to increase spark width in myocytes obtained from wild-type mice. Additionally, superresolution analysis of RyR2 and NCX colocalization showed a reduced overlap between RyR2 and NCX in MCM-shJPH2 mice. In conclusion, decreased JPH2 expression causes increased SR Ca\textsuperscript{2+} leakage by directly increasing open probability of RyR2 and by indirectly reducing junctional NCX activity through increased dyadic cleft Ca\textsuperscript{2+}. This demonstrates two novel and independent cellular mechanisms by which JPH2 regulates RyR2-mediated SR Ca\textsuperscript{2+} leak and heart failure development.

calcium; heart failure; junctophilin; ryanodine receptor; sodium/calcium exchanger

INTRACELLULAR CALCIUM cycling within Ca\textsuperscript{2+}-release units (CRUs) plays a critical role in normal cardiomyocyte contraction. Plasma membrane (PM) depolarization activates voltage-gated L-type Ca\textsuperscript{2+} channels, allowing for Ca\textsuperscript{2+} influx across the cardiac dyad. This Ca\textsuperscript{2+} influx triggers Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR) via ryanodine receptors type 2 (RyR2), a process known as Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}-release (CICR). RyR2-mediated Ca\textsuperscript{2+}-release triggers mechanical myocyte contraction through binding of Ca\textsuperscript{2+}-sensitive myofilaments. In this way, membrane excitation results in mechanical contraction in a process known as excitation-contraction coupling (ECC) gain (7). While this process is critical for physiological beating of the heart, aberrant SR Ca\textsuperscript{2+} release during diastole can trigger arrhythmia by activation of the Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger (NCX) located on the PM (8, 17). Moreover, persistent SR Ca\textsuperscript{2+} leak via RyR2 can result in impaired contractility because of SR Ca\textsuperscript{2+} depletion and loss of ECC gain as seen in heart failure (HF) (30). The activity of the RyR2 Ca\textsuperscript{2+}-release channel is modulated by various accessory proteins and posttranslational modifications (1, 26).

Junctophilin-2 (JPH2) was first identified as a structural component of the CRU, necessary for proper spacing of the PM and SR membrane, which comprise the cardiac dyad (19, 23). Reduced levels of JPH2 expression are associated with HF and pressure-induced cardiac hypertrophy (16). The loss of JPH2 was shown to increase variability in the distance between the PM and SR, whereas the mean distance remained unaltered. Moreover, JPH2 has also been shown to directly bind to and inhibit RyR2 function, suggesting that JPH2 might have nonstructural roles in cardiomyocytes (11, 21). Cardiac-specific conditional knockdown of JPH2 resulted in increased RyR2 sparking and channel opening with resultant decrease in CICR and ECC gain (27). Reduced CICR corresponded to decreased ejection fraction, and the mice died of HF. In addition to RyR2-mediated Ca\textsuperscript{2+} leakage, delayed Ca\textsuperscript{2+} extrusion from the cytosol was observed, suggesting impaired NCX function despite unaltered global NCX1 protein expression levels (11, 25). However, it has remained unknown whether JPH2 is required for subcellular targeting of NCX and/or its functional activity near RyR2 Ca\textsuperscript{2+}-release channel clusters.

Here, we provide novel evidence that reduced JPH2 expression increases RyR2 open probability (P\textsubscript{o}) and simultaneously impairs NCX function, resulting in increased junctional Ca\textsuperscript{2+} levels. This is evidence since an increased Ca\textsuperscript{2+} spark frequency (CaSpF), spark mass, and spark width can promote SR Ca\textsuperscript{2+} leakage via RyR2. We conclude that JPH2 serves as a structural stabilizer of the dyad, as well as an inhibitor of RyR2 through regulation of junctional NCX activity, which may promote the development of HF when expression levels of JPH2 are decreased.
MATERIALS AND METHODS

Mouse models. All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine, conforming to the Guide for the Care and Use of Laboratory Animals. Mice (4–6 mo of age) were anesthetized using inhaled 1.5% isoflurane in 100% oxygen, and mice were euthanized using cervical dislocation under general anesthesia. Control cells were obtained from B6129-Tg (Myh6-cre/ESR1) mice. Jmk/J mice (Mer-Cre-Mer, or MCM) were obtained from Jackson Laboratory (No. 005650). Tamoxifen-induced JPH2 knockdown cells were obtained from MCM-shJPH2 mice, a model for low-cardiac output HF (25). To compare spark parameters with other murine models of RyR2 dysfunction with wild-type JPH2 protein expression, FKBPI2.6 knockout mice (29), RyR2-S2814D mice (26), and MDX (C57BL/10ScSN-Dmdmdx/J, No. 001801 from Jackson Laboratory) (2) mice were compared.

Western blot analysis. Heart lysates were prepared from flash-frozen hearts and s1-fractionated on 7.5% (for JPH2) and 12% (for GAPDH) SDS-polyacrylamide gels (25). Gels were transferred onto polyvinylidene difluoride membranes and probed using custom-made anti-JPH2 antibody (1:5,000) (25) or anti-GAPDH (1:5,000; Millipore, Temecula, CA) monoclonal antibody. Blots were developed using Alexa Fluor 680-conjugated anti-mouse (Invitrogen Molecular Probes, Carlsbad, CA) and/or IR800Dye-conjugated anti-rabbit fluorescent secondary antibodies (Rockland Immunocchemicals, Gilbertsville, PA) and scanned on an Odyssey infrared scanner (Li-Cor, Lincoln, NE). Protein signal densities were normalized to the corresponding GAPDH signal densities, whereas phosphorylation signal densities were normalized to the corresponding total protein signal densities.

Ventricular myocyte isolation. Mouse ventricular myocytes were isolated as previously described (26). Following Langendorff perfusion and enzymatic digestion, myocytes were incubated with Fluo-4-isolated as previously described (26). Following Langendorff perfusion, myocytes were incubated with Fluo-4 and enzymatic digestion, myocytes were incubated with Fluo-4-iso.

Measurement of NCX current. NCX current (I_{NCX}) was recorded as described (10). Rabbit-anti-RyR2 primary antibody (Sigma, HPA016607) and mouse-anti-NCX1 antibody (Swant, R3F1) were used followed by incubation with highly cross-absorbed Alexa 680-labeled goat anti-mouse IgG and Alexa 750-labeled goat anti-rabbit IgG antibodies (1:200, Invitrogen). Cells were transfected into a mounting medium containing 90% (vol/vol) glycerol, 10% (vol/vol) PBS, 10% (wt/vol) glucose, 5 mM cytostatic Ca2+ (pH 7.2) with KOH. Once transfected, experiments were conducted in the same internal solution without saponin.

Calcium imaging. Once steady-state Ca2+ transient was observed, pacing was stopped for 20 s and Ca2+ sparks were counted. Steady-state SR Ca2+ content was estimated by rapid application of 10 mM caffeine after pacing. NCX activity (1/τ) was calculated during caffeine application as the inverse of the rate of decrease of cytosolic Ca2+ (τ). Spark parameters were measured including spark width [full width at half-maximum amplitude (FWHM)], spark amplitude [peak fluorescent intensity/baseline fluorescent intensity (F/F0)], spark duration [full duration at half-maximum (FDHM)], and CaSpF. All spark recordings were conducted with identical conditions for all mouse lines, and alignments were normalized to respective controls. All statistics were expressed as means ± SE. SR Ca2+ leak was calculated using the following equation: FWHM × FDHM × F/F0 × CaSpF (15).

Measurement of NCX current. NCX current (I_{NCX}) was recorded as described previously (28). Briefly, the whole cell patch-clamp method was employed to observe cell membrane current. Patch-pipette resistances were 1–3 MΩ before sealing. The pipette solution contained (in mM) 50 aspartic acid, 30 CsOH, 20 TEACI, 5 MgSO4, 5 HEPES, 10 glucose, 5 Na2-ATP, and 11 EGTA. Intracellular [Ca2+] was set to the desired value by including the appropriate concentration of CaCl2. The pH was adjusted to 7.2 with CsOH, increasing the final CsOH concentration to about 80 mM. The external NCX-Tyrode solution contained (in mM) 137.7 NaCl, 2.3 NaOH, 1 MgCl2, 1.8 CaCl2, 10 glucose, 5 HEPES, 2 BaCl2, and 2 CsCl. The solution also contained 20 μM verapamil (pH 7.4). Different values of extracellular [Ca2+] were obtained by changing CaCl2 without substitution. The 2 mM Ca2+-sensitive current was measured with a ramp protocol with a holding potential of −4 mV (calculated NCX equilibrium potential) with 1-s duration to construct the I_{NCX}-membrane potential relationships. The pipette [Ca2+] was 300 nM.

SR store calcium measurement and inhibition of NCX. SR Ca2+ store was measured using transient amplitude (F/F0) after 10 mM caffeine application. Spark measurements with partial pharmacological NCX inhibition were done with 10 μM caffeine to promote recurrent sparks from a same location as caffeine concentration up to 100 μM has no substantial effect on the spark amplitude, width, and time course. Averaged spark size of greater than five sparks was compared before and within 1 min after adding 1 mM cadmium. Only cells without detectable changes in baseline cytosolic Ca2+ after Ca2+ were included for analysis.

Single channel RyR2 recording. Single channel recordings of RyR2 are acquired under voltage-clamp conditions at 0 mV. The transchannel (1.0 ml of 250 mM HEPES, 53 mM CaOH2, and 50 mM KCl, pH 7.35), representing the intra-SR compartment, was connected to the head stage input of a bilayer voltage-clamp amplifier (26). The cis channel (1.0 ml of 250 mM HEPES, 125 mM Tris, 50 mM KCl, 1.0 mM EGTA, and 0.5 mM CaCl2, pH 7.35), representing the cytoplasmic compartment, was held at virtual ground. Free [Ca2+] was calculated by CHELATOR software. At the conclusion of each experiment, trypan blue (5 μM) or ruthenium red (20 μM) was applied to confirm RyR2 channel identity. Each recording lasts 3–5 min. Recordings were filtered at 1,000 Hz. Fifty percent threshold was used to calculate P0, mean open time (in ms), and mean close time (in ms).

Immunostaining and superresolution imaging. Mouse ventricular cardiomyocytes were immediately fixed in 2% paraformaldehyde for 10 min following isolation. Immunofluorescent staining was carried out as described (10). Rabbit-anti-RyR2 primary antibody (Sigma, HPA016607) and mouse-anti-NCX1 antibody (Swant, R3F1) were used followed by incubation with highly cross-absorbed Alexa 680-labeled goat anti-mouse IgG and Alexa 750-labeled goat anti-rabbit IgG antibodies (1:200, Invitrogen). Cells were transfected into a mounting medium containing 90% (vol/vol) glycerol, 10% (vol/vol) PBS, 10% (wt/vol) glucose, 5 mM cysteamine, 0.5 mg/ml glucose oxidase, and 50 μg/ml catalase. Suspended cells were mounted on a coverslip (thickness No. 1.5) and sealed to a slide using nail varnish. The superresolution imaging system uses a Nikon TE2000 inverted microscope with a custom objective holder, a piezo focuser (P-725, Physik Intrumente, Germany), an EMCCD camera (DV887, Andor Technology, UK), a custom image-splitting device for simultaneous detection of multiple color channels, custom illumination optics, and several laser sources. Samples were imaged either near or ~5 μm from the cell surface. Fluorescence was excited using a single 671-nm diode laser (Viasho, China), which was coupled to produce a highly inclined light sheet within the sample (24). Data were simultaneously recorded from both Alex 680 and Alex 750 fluorescence, and single molecule events were fitted in both detection channels simultaneously with color assignment being performed on the basis of the ratio of the intensities as described (4). A series of ~25,000 raw frames (50 ms/frame) were captured for each image reconstruction and localization events from each frame series were rendered using our visualization algorithms (3).

Rendered dual-color superresolution images were set at a threshold value capturing 0.7 of the total signal to obtain binary masks of RyR2 or NCX distribution, and distance transform-based analysis was performed (9). With the calculation of the amount of labeling at a given distance from the edge of the masks, using the convention that...
distances within the cluster mask were negative whereas distances outside the mask were positive, fractions of total labeling as a function of distance were determined. Fractions were summarized in distance histograms, and cumulative fractions within selected distances were calculated from these histograms. Negative distances up to and including zero were summed to give the total fraction of colocalization between the two labels.

**Statistical analysis.** Data are presented as means ± SE. Student’s t-test was used for comparison between two groups, and the Mann-Whitney U-test was used for colocalization data. A P value < 0.05 was considered statistically significant.

**RESULTS**

**JPH2 knockdown leads to an increase in Ca²⁺ spark amplitude, width, and duration.** To explore the mechanism of increased Ca²⁺ leak from the SR during HF, a detailed analysis of Ca²⁺ sparks from RyR2 was conducted in mice with cardiac-specific knockdown of JPH2, which is associated with impaired cardiac contractility (27). Individual spark properties were analyzed in MCM-shJPH2 mice (70–80% reduction of JPH2) compared with MCM controls (normal JPH2 levels) (27). Images of representative Ca²⁺ sparks and corresponding fluorescence amplitude tracings are shown in Fig. 1A. FWHM of Ca²⁺ sparks in myocytes from MCM-shJPH2 mice (4.25 ± 0.16 µm) was increased by about 70% compared with MCM control myocytes (2.55 ± 0.14 µm; P < 0.001; Fig. 1B). Frequency histograms of spark width demonstrate a nearly equal distribution in frequencies from widths of 2 to 8 µm in MCM-shJPH2 cells, whereas the majority of MCM sparks are <4 µm (Fig. 1B). In addition, MCM-shJPH2 myocytes demonstrated a 10% increase in Ca²⁺ spark amplitude (ΔF/F₀ of 0.27 ± 0.01) compared with MCM controls (0.24 ± 0.01; P < 0.05; Fig. 1C). The FDHM of Ca²⁺ sparks was also increased in MCM-shJPH2 myocytes (32.7 ± 1.0 ms) compared with MCM controls (29.9 ± 1.2 ms; P < 0.05; Fig. 1D). Calculated Ca²⁺ spark mass was greater in MCM-shJPH2 myocytes compared with MCM controls (Fig. 1E). Taken together, these
findings resulted in a 15-fold increase SR Ca\textsuperscript{2+} leakage in MCM-shJPH2 cells (164 ± 46 arbitrary units) compared with MCM controls (11 ± 3 arbitrary units; P < 0.001; Fig. 1F).

**JPH2 knockdown alters RyR2 single channel properties.** Based on the observation that JPH2 knockdown results in increased SR Ca\textsuperscript{2+} leakage and increased Ca\textsuperscript{2+} spark width, we examined whether JPH2 alters RyR2 gating as a mechanism of increased SR Ca\textsuperscript{2+} leak. Prior immunoprecipitation studies have revealed that JPH2 and RyR2 directly interact and that less JPH2 is bound to RyR2 in MCM-shJPH2 mice (6, 25).

To directly test RyR2 gating in the presence of severely reduced JPH2 levels, microsomes containing RyR2 from MCM-shJPH2 and MCM control hearts were reconstituted in lipid bilayers. Single channel recordings were conducted, and representative current tracings are depicted in Fig. 2, A and B. RyR2 channels from MCM-shJPH2 samples (25 channels from 5 mice) demonstrated increased channel \( P_o \) (14.7 ± 3.3%; \( P < 0.05 \); Fig. 2C). In addition, MCM-shJPH2 mean open time was significantly higher in knockdown cells (1.74 ± 0.15 ms) compared with MCM control mice (1.29 ± 0.15 ms; \( P < 0.05 \); Fig. 2D). RyR2 closed time was reduced in a corresponding manner (66.2 ± 24.2 vs. 1117.3 ± 474.9 ms, respectively; \( P < 0.05 \); Fig. 2E). Taken together, these results suggest that reduced expression of JPH2 results in increased RyR2 \( P_o \).

**Increased Ca\textsuperscript{2+} spark width in JPH2 knockdown mice.** To elucidate whether the increase in Ca\textsuperscript{2+} spark width observed with JPH2 knockdown is specific to JPH2 expression silencing versus a secondary effect of increased RyR2 \( P_o \), we next characterized Ca\textsuperscript{2+} sparks in several mouse strains with increased RyR2 \( P_o \) and CaSpF, but with native JPH2 expression levels. Among FKBP12.6 knockout mice, RyR2-S2814D mice, and MDX mice, a significant increase in overall CaSpF was observed in each strain. This increase ranged from a 2.1-fold increase in RyR2-S2814D mice (\( P < 0.01 \) vs. control) to a 3.69-fold increase in MCM-shJPH2 mice (\( P < 0.05 \) vs. control) (see Fig. 3A). In contrast, there was no statistical difference in CaSpF among the wild-type littermates of each respective strain (data not shown). When Ca\textsuperscript{2+} spark width was analyzed, MCM-shJPH2 mice exhibited a significant 1.8-fold increase in FWHM compared control mice (\( P < 0.001 \)). However, FKBP12.6 and RyR2-S2814D mice did not demonstrate an increased FWHM compared with controls. MDX mice demonstrated a significant but modest 1.2-fold increase in spark width compared with control (Fig. 3B). In contrast, Ca\textsuperscript{2+} spark amplitude and spark duration were variably altered among each mutant strain compared with controls (Fig. 3, C and D). Thus these findings suggest that the increase in Ca\textsuperscript{2+} spark width is a result of JPH2-expression silencing, not seen in other mouse strains with an increased RyR2 \( P_o \).

**NCX activity is reduced in myocytes with reduced JPH2 expression levels.** While RyR2 \( P_o \) is increased in JPH2 knockdown, previous investigation has also demonstrated an ~50% reduction in NCX activity and impaired Ca\textsuperscript{2+} extrusion from the cytosol without alterations in NCX1 protein expression in JPH2 knockdown mice (25). As NCX has recently been shown to play a critical role in shaping Ca\textsuperscript{2+} sparks and maintaining spark width, we next explored the possibility that concomitant impairment in NCX may contribute SR Ca\textsuperscript{2+} leakage and Ca\textsuperscript{2+} spark derangement (18). To do this, we used two independent approaches to assess NCX activity. First, cytosolic Ca\textsuperscript{2+} removal kinetics (\( \tau \)) following caffeine-induced Ca\textsuperscript{2+} transient was analyzed, and NCX activity was calculated during caffeine administration (Fig. 4A). These measurements were made with an extracellular [Ca\textsuperscript{2+}] of 1.8 mM and intracellular free [Ca\textsuperscript{2+}] of 314 nM. Quantification revealed a 0.5x-fold decrease in NCX activity in MCM-shJPH2 myocytes compared with control MCM (\( P < 0.05 \), Fig. 4B). The impaired NCX function was not observed in mouse strains with native JPH2 expression.

![Fig. 2. JPH2 knockdown increases ryanodine receptor type 2 (RyR2) open probability. A and B: representative tracings of RyR2 channel current in reconstituted lipid bilayers obtained from MCM (A) and MCM-shJPH2 (B) mouse hearts. Open probability, opening time (\( T_o \)), and closed time (\( T_c \)) are noted. C–E: histogram demonstrating the mean open probability (C), mean channel opening time (D), and mean channel close time (E) in control MCM (17 channels from 4 mice) vs. MCM-shJPH2 cells (25 channels from 5 mice). *P < 0.05.](http://ajpheart.physiology.org/)

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levels and RyR2 gain of function. To exclude the possibility that altered SR or cytosolic Ca\(^{2+}\) levels could explain this finding, we measured \(I_{\text{NCX}}\) using whole cell patch clamp to maintain identical intracellular and extracellular ionic conditions (Fig. 4, C and D). In MCM-shJPH2 mice, \(I_{\text{NCX}}\) was significantly reduced at −80 mV compared with control cells (−0.26 ± 0.05 vs. −0.39 ± 0.08 pA/pF; \(P < 0.05\); Fig. 4E). This decrease in \(I_{\text{NCX}}\) was in the absence of altered NCX expression based on Western blot analysis (Fig. 4, F and G).

Reduced RyR2-NCX colocalization in JPH2 knockdown mice. Furthermore, superresolution microscopy of immunofluorescence of RyR2 and NCX in cardiac myocytes demonstrated moderate colocalization of RyR2 and NCX in T tubules at cardiac dyads (Fig. 5, A and B). The distance distribution histograms indicate that colocalization of RyR2s and NCX in the MCM-shJPH2 mice is not as tightly organized as in MCM control cells, as seen by broader distributions of the distances of both RyR2 with respect to NCX (Fig. 5C) and NCX with respect to RyR2 (Fig. 5D). In the MCM control myocytes, it was found that 26.5 ± 2.2% of RyR2 was colocalized with NCX, whereas 17.5 ± 1.2% of NCX was colocalized with RyR2 (Fig. 5, E and F). In the JPH2-knockdown mice, colocalization between these two Ca\(^{2+}\)-handling proteins was significantly reduced compared with MCM (16.5 ± 0.9%, \(P < 0.001\); Fig. 5E, and 11.1 ± 0.9%, \(P < 0.01\); Fig. 5F, respectively), indicating a reduction in the proportion of junctional NCX.

NCX pharmacological inhibition increases calcium spark width. Based on the finding of impaired NCX activity and the observed increase in Ca\(^{2+}\) spark width and RyR2-mediated Ca\(^{2+}\)-leakage in JPH2 knockdown cells, we next attempted to determine whether pharmacological inhibition of NCX in wild-type cardiomyocytes increased Ca\(^{2+}\) spark width. Cardiac myocytes were treated with low-dose caffeine (100 μM) to induce recurring sparks followed by 1 mM Cd\(^{2+}\) to inhibit NCX and 10 mM caffeine to ascertain Ca\(^{2+}\) removal kinetics (Fig. 6A). Representative fluorescent intensity tracings and confocal line scans depicting Ca\(^{2+}\) sparks with and without Cd\(^{2+}\) are depicted in Fig. 6B. Addition of Cd\(^{2+}\) resulted in an ∼50% reduction in NCX activity (\(\tau = 3.07 ± 0.77\) s with Cd\(^{2+}\) vs. \(\tau = 8.25 ± 2.14\) s with vehicle; \(P < 0.05\); Fig. 6C). Ca\(^{2+}\) spark width was significantly increased with the addition of 1 mM cadmium (2.17 ± 0.25 μm) compared with vehicle (1.98 ± 0.22 μm, \(P < 0.01\); Fig. 6D). These findings of impaired NCX activity with Cd\(^{2+}\) addition closely mimicked the reduction seen in MCM-shJPH2 cells.

Permeabilization reverses increased spark width in JPH2 knockdown cardiomyocytes. To determine whether junctional NCX regulates spark properties by altering dyadic cleft Ca\(^{2+}\) concentration, we next permeabilized MCM and MCM-shJPH2 ventricular myocytes with saponin and perfused with a normalizing Ca\(^{2+}\)-containing solution. Images of representative Ca\(^{2+}\) sparks following permeabilization are demonstrated in Fig. 7A. While intact MCM-shJPH2 myocytes demonstrated increased Ca\(^{2+}\) spark width, following permeabilization, MCM-shJPH2 myocytes demonstrated decreased width (1.84 ± 0.04 μm) compared with controls (2.08 ± 0.04 μm; \(P < 0.01\); Fig. 7B). Despite the decreased Ca\(^{2+}\) spark width following permeabilization, the CaSpF was unaffected as MCM-shJPH2 cells demonstrated a preserved increase in CaSpF after permeabilization (10.5 ± 2.2 sparks/100 μm/s) compared with controls (4.2 ± 0.6 sparks/100 μm/s; \(P < 0.01\); Fig. 7C). While CaSpF remained similar, permeabilization of MCM-shJPH2 cells reversed the previously seen increase in Ca\(^{2+}\) spark amplitude causing a decrease in Ca\(^{2+}\) spark amplitude (0.29 ± 0.01) compared with controls (0.41 ± 0.01; \(P < 0.001\); Fig. 7D). Furthermore, the increase in Ca\(^{2+}\) spark duration was nullified in permeabilized MCM-shJPH2 cells (23.0 ± 0.6 ms) compared with control (22.0 ± 0.4; \(P = \) not significant, Fig. 7E). Taken together, these results demonstrate...
that increased Ca\(^{2+}\) spark width, amplitude, and duration associated with JPH2 expression silencing can be abrogated by cell permeabilization while CaSpF is unaffected.

**DISCUSSION**

**Several roles for JPH2 in cardiac myocytes.** Once viewed as purely structural protein, a more complex role of JPH2 is beginning to emerge. Initially, JPH2 was reported to function as a tether that connects the PM to the SR within the cardiac dyad (19, 23). Recent studies, however, have also demonstrated that JPH2 acts as a critical regulator of cardiomyocyte development by promoting the maturation of transverse tubules (21). In addition, JPH2 was shown to modulate intracellular Ca\(^{2+}\) handling through regulation of RyR2 (6, 25). Missense mutations in the JPH2 gene were identified in patients with hypertrophic cardiomyopathy, and clinical samples of patients with hypertrophic cardiomyopathy were found to have decreased JPH2 expression (11, 12). Furthermore, recent studies have linked mutations in JPH2 and the development of arrhythmias, specifically atrial fibrillation (6). Similarly, conditional, cardiac-specific JPH2 knockdown reduces JPH2-RyR2 binding and triggers severe RyR2-mediated SR Ca\(^{2+}\) leak, which was temporally associated with acute development of HF and increased mortality (25). Loss of JPH2 in ventricular myocytes is associated with a reduced SR Ca\(^{2+}\) transient despite unaltered L-type Ca\(^{2+}\) currents (25). Herein, we demonstrate that increased SR Ca\(^{2+}\)-leakage in JPH2 knockdown mice occurs through two distinct molecular mechanisms: increased RyR2 P\(_{o}\) through decreased negative regulation by JPH2, as well as reduced junctional NCX activity, which increases dyadic Ca\(^{2+}\) levels further.

**JPH2 is a negative regulator of RyR2 gating.** Prior work has demonstrated a direct interaction between RyR2 and JPH2 using immunoprecipitation experiments (9, 25). Here, we provide additional evidence that JPH2 expression silencing results in a fourfold increase in RyR2 P\(_{o}\), further supporting a hypothesis that JPH2 negatively regulates RyR2-gating. This concept is also supported by recent studies that demonstrate a reduced RyR2 P\(_{o}\) following the addition of a small synthetic peptide derived from the JPH2 primary sequence that binds to RyR2 (6). Thus it is conceivable that direct binding of RyR2 by JPH2 is needed for proper RyR2 gating and channel regulation, whereas loss of JPH2 results in increased SR Ca\(^{2+}\) leakage. Our finding that SR leakage is driven primarily by an increased Ca\(^{2+}\) spark width suggests this elementary Ca\(^{2+}\)-release event is altered in a way, which mirrors the molecular changes of the
CRU. Furthermore, this alteration in Ca^{2+} spark characteristics occurs in case of JPH2 expression silencing and is not seen in other models of increased RyR2 $P_o$ with native JPH2 expression levels. Traditionally, Ca^{2+} sparks are believed to be generated by the spontaneous opening of a single RyR2 cluster. Recent evidence using superresolution microscopy suggests that RyR2 clusters may be smaller than previously estimated and may exist in a loosely organized formation of multiple RyR2 clusters (5). Should JPH2 knockdown disrupt this network of RyR2 clusters, it raises the possibility that normal

Fig. 5. Dual-color superresolution fluorescence imaging of RyR2 and NCX. A: overlay of superresolution images of RyR2 (green) and NCX (red) immunofluorescence labeling on interior couplings within the T tubules. B: increased magnification demonstrates colocalization between RyR2 puncta and junctional NCX in some clusters, the proportion of which was reduced in shJPH2 mice compared with MCM. Scale bars: 2 μm in A and 0.25 μm in B. C and D: distribution plots of the fraction of RyR2 labeling as a function of distance to the nearest NCX labeling (C) and NCX labeling as a function of distance to the nearest RyR2 labeling (D) in MCM (left) and MCM-shJPH2 (right) mice. Dark gray bars represent colocalized fraction. E and F: bar graph of mean colocalization of RyR2 with NCX (E) and NCX with RyR2 (F) in MCM (black; $n = 10$ cells) vs. MCM-shJPH2 (white; $n = 11$ cells) cardiac myocytes. **$P < 0.01$; ***$P < 0.001$.
expression levels of JPH2 are necessary for RyR2 cluster formation. Given our findings that Ca\(^{2+}\) spark width and amplitude is increased with JPH2 knockdown, it is possible that an increased number of RyR2 clusters are participating in spark generation perhaps secondary to the increased Ca\(^{2+}\) levels. Indeed, our findings that membrane permeabilization normalized the increased spark width, amplitude, and duration suggest that increased dyadic Ca\(^{2+}\) is playing a role in the increased SR calcium leakage.

**JPH2 regulates functional NCX activity and leads to increased dyadic Ca\(^{2+}\) levels.** In addition to direct regulation of RyR2 gating, JPH2 may also regulate NCX activity, particularly within the cardiac dyad, which may play a role in the genesis of increased dyadic Ca\(^{2+}\) levels. We noted a significant reduction in NCX activity and slowing of cytosolic Ca\(^{2+}\) clearance, which was evidenced by both delayed reuptake/expulsion of cytosolic Ca\(^{2+}\) following caffeine-triggered SR Ca\(^{2+}\) release, as well as by direct measurement of \(i_{\text{NCX}}\) using patch clamping. Furthermore, given that pharmacological impairment of NCX activity results in a qualitatively similar change in spark size as in JPH2 knockdown myocytes, it is possible that functional NCX might regulate spark size and SR Ca\(^{2+}\)-leakage by a novel regulatory mechanism. This possibility is supported by previous studies in NCX knockout mice, which demonstrated increased spark size and spark amplitude (18). Furthermore, it is has been shown that reduced junctional NCX activity elevates baseline dyadic Ca\(^{2+}\) concentration because of reduced extrusion of systolic Ca\(^{2+}\) from the cell (18). It is likely that this increased Ca\(^{2+}\) level would trigger increased activity of the Ca\(^{2+}\)-sensitive RyR2, which in turn could manifest both as increased \(P_o\) and enhanced Ca\(^{2+}\) sparks as seen in our studies. Interestingly, 50% inhibition of NCX function resulted in a moderate 13% increase in spark size, which is significantly less than the 70% increase in our JPH2 knockdown mice. This suggests that there are other variables increasing spark size in JPH2 knockdown mice beyond NCX modulation. However, it is unlikely that other Ca\(^{2+}\) extrusion pathways are important contributors, since the plasmaemmal Ca\(^{2+}\) ATPase only plays a minor role in mouse ventricular myocytes (20). Taken together, these findings support the possibility that reduced JPH2 expression negatively impacts junctional NCX activity with implications for Ca\(^{2+}\) spark formation.

Given the apparent functional impairment in NCX function with JPH2 expression silencing, it is possible that JPH2 regulates NCX function through either a direct or indirect manner. Based on previous findings that NCX expression levels are unaltered in JPH2 knockdown, it is likely that there is functional impairment of the protein. While NCX hosts a large intracellular domain, which could directly bind JPH2, the inability to immunoprecipitate JPH2 with anti-NCX immunoglobulin argues against a direct binding mechanisms. Indeed, it is more likely that the functional NCX impairment is indirect. Further experiments are required to further delineate either of these possibilities.

**NCX localizes to the cardiac dyad.** In cardiomyocytes, NCX is critical for maintaining intracellular Ca\(^{2+}\) homeostasis, mainly serving as a Ca\(^{2+}\) removal mechanism during relaxation. The possibility that NCX exists within dyad has proven controversial. Diffraction-limited colocalization studies by Scriven and Moore (22) suggested little colocalization between RyRs and NCX in rat cardiac myocytes. A more recent confocal immunofluorescence study suggested that ~40% of total

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**Fig. 6. Pharmacological inhibition of NCX increases calcium spark width.** A: representative tracings of fluorescence correlating to cardiomyocyte Ca\(^{2+}\) levels following stimulation with 10 mM caffeine (Caff) and 1 mM Cd\(^{2+}\). NT, normal Tyrode solution. Vertical scale bar, 2 F/F\(_0\); and horizontal scale bar, 5 s. B: representative line scans of Ca\(^{2+}\) sparks in cells with addition of vehicle (top) and Cd\(^{2+}\) (bottom). Vertical scale bar, 8 \(\mu\)M; and horizontal scale bar, 1 s. C: bar graph demonstrating mean Ca\(^{2+}\) reuptake (\(\tau\)) in cells with vehicle (black) and 1 mM Cd\(^{2+}\) (white). D: bar graph demonstrating mean Ca\(^{2+}\) spark width in cells with vehicle (black) and Cd\(^{2+}\) (white). Numbers in within bar graph denote number of cells analyzed and number of mice analyzed (in parentheses). *\(P < 0.05\); **\(P < 0.01\).
NCX localizes within CRUs and that over 50% of CRUs are NCX positive (10). Given the direct interaction of JPH2 and RyR2, it is possible that RyR2 and NCX similarly colocalize to the dyad. In addition, the so-called “fuzzy space” theory strongly suggested that both Na⁺ channels and NCX are either within, or in close proximity to, the cardiac dyad (13, 14). This possibility is supported by superresolution microscopy, showing colocalization of RyR2 and NCX in some clusters in the cardiac dyad (Fig. 5). Taken together, these findings suggest that a proportion of NCX localizes to the junctional membrane complex within the cardiac dyad, which is reduced following loss of JPH2.

Limitations. While Ca²⁺ spark data were used to estimate SR Ca²⁺ leak in our study, recent evidence suggests that there may be a Ca²⁺ spark-independent mechanism that contributes to SR Ca²⁺ leak. We were unable to test for this possibility via standard leak protocols, because MCM-shJPH2 myocytes were very sensitive to the 0Na⁺/0Ca²⁺ solution, which induced a significant elevation in cytosolic Ca²⁺ and frequent Ca²⁺ waves, making baseline measurements impossible. Furthermore, as the Ca²⁺ sensitivity of RyR2 is increased with JPH2 knockdown, leakage measured with 0Na⁺/0Ca²⁺ conditions may falsely bias estimates. Moreover, increased RyR2 activity was observed in MCM-shJPH2 mice despite a reduced SR Ca²⁺ load, which can largely exclude the possibility that the difference in SR Ca²⁺ content could contribute to the observed difference in RyR2 activity (25).

The increase in spark width in MCM-shJPH2 was considerable and was only partially mimicked in experiments in which NCX activity was reduced. Thus it is possible that additional mechanisms contribute to the observed increase in apparent spark mass. We also note that Fluo indicators are nonratiometric, and therefore small changes in resting [Ca²⁺] may not be detected, leaving the possibility that some component of spark mass changes may reflect changes in indicator saturation. The permeabilization experiments suggest that differences between control and JPH knockdown are abolished upon change to a standard solution and the elimination of transmembrane fluxes, consistent with a role of NCX in shaping spark properties. The interpretation is complicated by the potential loss of diffusible cytosolic components and the possibility that the buffering required for permeabilization may mask remaining differences. We therefore suggest that our experiments are consistent with a mechanistic role of functional NCX in explaining the increase in spark diameter and mass but are unlikely to be the only source underlying these differences. Nevertheless, our results underline the complex changes accompanying a reduction in JPH and highlight the need to understand the feedback between JPH and other Ca²⁺-handling mechanisms. These changes may be mediated via feedback through intracellular Ca²⁺ and/or and gene regulation networks that may themselves be sensitive to Ca²⁺.

Conclusions. This study demonstrates a novel cellular mechanisms underlying enhanced SR Ca²⁺ leak, following JPH2 knockdown associated with HF. In addition to maintaining the ultrastructure of the cardiac dyad, JPH2 negatively regulates RyR2 activity and helps retain NCX within the dyadic cleft to regulate local Ca²⁺ signaling. Loss of JPH2 promotes RyR2-mediated SR Ca²⁺ leak and simultaneously impairs NCX function due to subcellular redistribution of NCX, which fur-
ther drives Ca\(^{2+}\) leakage. This multifunctional role for JPH2 in cardiomyocytes may have implications for the development of novel therapeutic approaches for HF and cardiac arrhythmias.

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**DISCLOSURES**

X. H. T. Wehrens is a founding partner of Elex Biotech, a company that develops new drugs targeting intracellular calcium leak.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


