Published in final edited form as:

Heart Rhythm. 2016 March ; 13(3): 743–752. doi:10.1016/j.hrthm.2015.10.037.

JPH-2 INTERACTS WITH Ca_i -HANDLING PROTEINS AND ION CHANNELS IN DYADS: CONTRIBUTION TO PREMATURE VENTRICULAR CONTRACTION-INDUCED CARDIOMYOPATHY

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Abstract

Background—In a canine model of premature ventricular contraction-induced cardiomyopathy (PVC-CM), Cav1.2 is downregulated and misplaced from t-tubules. Junctophilin-2 (JPH-2) is also downregulated.

Objective—To understand the role of JPH-2 in PVC-CM, and to probe changes in other proteins involved in dyad structure and function.

Methods—We quantify t-tubule contents (di-8-ANEPPS fluorescence in live myocytes), examine myocyte ultra-structures (electron microscopy), probe JPH-2 interacting proteins (co-immunoprecipitation), quantify dyad and non-dyad protein levels (immunoblotting), and examine subcellular distributions of dyad proteins (immunofluorescence/confocal microscopy). We also test direct JPH-2 modulation of channel function (vs indirect modulation through dyad formation) using heterologous expression.

Results—PVC myocytes have reduced t-tubule contents but otherwise normal ultra-structures. Among nineteen proteins examined, only JPH-2, bridging-integrator-1 (BIN-1) and Cav1.2 are highly downregulated in PVC hearts. However, statistical analysis indicates a general reduction of dyad protein levels when JPH-2 is downregulated. Furthermore, several dyad proteins, including Na/Ca exchanger, are missing or shifted from dyads to peripheral surface in PVC myocytes. JPH-2 directly or indirectly interacts with Ca_i -handling proteins, Cav1.2 and KCNQ1, although

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Disclosures

None for MJ, MZ, MH, YHW, RCB, and GNT.

not BIN-1 or other scaffolding proteins tested. Expression in mammalian cells, that do not have dyads, confirms direct JPH-2 modulation of I_{CaL} (Cav1.2/Cav β 2) and I_{Ks} (KCNQ1/KCNE1).

Conclusion—JPH-2 is more than a ‘dyad glue’: it can modulate Ca_i -handling and ion channel function in the dyad region. Downregulation of JPH-2, BIN-1 and Cav1.2 plays a deterministic role in PVC-CM. Dissecting the hierarchical relationship among the three is necessary for the design of therapeutic interventions to prevent the progression of PVC-CM.

Keywords

Premature ventricular contractions; cardiomyopathy; dyad; t-tubules; sarcoplasmic reticulum; excitation-contraction coupling

INTRODUCTION

Dr. Huizar and his colleagues have created a canine model of premature ventricular contractions (PVCs)¹. They showed that chronic (> 3 months) and frequent (one extrasystole after every sinus beat) PVCs applied to structurally normal hearts could cause a gradual decline in the left ventricular ejection fraction (LVEF)¹. This is PVC-induced cardiomyopathy (PVC-CM). The lack of apoptosis, fibrosis or inflammation, together with a full recovery in 2-4 weeks after terminating PVCs suggests that PVC-CM is functional in nature¹. This is consistent with clinical reports of improvements in patients’ cardiac function after PVC ablations². For patients with PVCs that are not candidates for ablations, it is important to find therapeutic strategies to prevent the progression of PVC-CM. This task requires the knowledge of mechanisms for PVC-CM at the molecular, cellular and organ levels.

Previously, we showed that left ventricular (LV) myocytes from hearts exposed to chronic PVCs (PVC hearts) have the following defects: decrease in the L-type Ca current (I_{CaL}) density, displacement of Cav1.2 (I_{CaL} pore-forming subunit) from the t-tubules, and reduction in Ca-induced Ca release (CICR)³. There is also a decrease in junctophilin-2 (JPH-2) protein level in PVC hearts³. JPH-2 functions as a bridge between t-tubules and junctional sarcoplasmic reticulum (jSR), the constituents of ‘dyad’⁴⁻⁶. Therefore our data point to ‘dyad disarray’ as the major culprit of progression in PVC-CM.

These findings led to three main questions. First, what are the functional roles of JPH-2 in canine ventricular myocytes? Other proteins have been suggested to engage in t-tubule formation (BIN-1 and caveolin-3) and dyad maintenance (telethonin and triadin)^{4, 5, 7}. Indeed, it has been suggested that downregulation of BIN-1, instead of JPH-2, is the culprit for dyad disarray in diseased hearts^{8, 9}. This controversy leads to the second question: what happen to other proteins important for the structure and function of dyads in PVC-CM? Protein functions are determined not only by their levels, but also by their locations. This is critical for dyad proteins, because signal transmission, such as dyadic [Ca] transients, requires spatial control^{4, 5, 10}. This leads to the third question: does PVC-CM alter the distribution patterns of dyad proteins?

To address these questions, we have performed extensive immunoprecipitation, immunoblotting, and immunofluorescence/confocal experiments on LV samples from control (CON), SHAM, and PVC canine hearts. On-line Table S1 lists the proteins we have examined, their functions, subcellular distribution patterns, and the sources of antibodies used in our experiments.

METHODS

Animal models and myocyte isolation

This study conforms to the Guide for the Care and Use of Laboratory Animals, and was approved by IACUC at McGuire Research Institute. A total of 29 mongrel dogs (> 10 months old; weight, 35 to 45 lbs) were divided into 3 groups: control (7), sham (7) and PVC (15). The procedures of pacemaker implantation and pacing algorithm were as described previously¹. Myocytes were isolated from LV free wall irrigated by the first branch of left circumflex coronary artery using collagenase and mechanical trituration, as described previously³.

COS-7 culture, transfection, patch clamp and biotinylation experiments

Cell culture, transfection, patch clamp and biotinylation experiments were as described previously¹¹.

Immunoprecipitation and immunoblotting experiments

Preparation of whole-cell and whole-tissue lysates were as described previously³, except that for immunoprecipitation the triton X-100 concentration was lowered to 0.5% to minimize perturbations of non-covalent protein-protein interactions. All immunoprecipitation reactions were performed on freshly prepared lysates to minimize non-specific protein bindings during freezing.

Immunofluorescence and confocal experiments

Immunofluorescence and confocal experiments on LV myocytes were as described previously¹².

Detailed methods are provided in on-line *Supplemental materials*.

RESULTS

PVC myocytes have reduced t-tubule contents but otherwise normal ultra-structures

Fig. 1A depicts orthogonal views of live SHAM and PVC myocytes stained with a membrane fluorescent dye, di-8-ANEPPS. We use such z-stacks of di-8-ANEPPS images to quantify t-tubule contents in myocyte volumes. Relative to SHAM myocytes, PVC myocytes have significantly lower t-tubule contents (Fig. 1B). On the other hand, transmission electron microscopy shows that the myocytes' ultra-structures, in terms of myofilament organization and mitochondria morphology, are not altered except a modest shortening of sarcomere lengths in PVC myocytes (Fig. 1C). Therefore, the major structural change at the single cell level in PVC-CM is a loss of t-tubules.

Previously we reported a decrease in the JPH-2 protein level in a limited set of CON and PVC samples³. We now have extended this quantification to a larger set of CON, PVC, and SHAM samples (Fig. 1D). JPH-2 protein level is significantly reduced in PVC hearts, but there is no statistically significant difference between CON and SHAM hearts.

JPH-2 interacts with many dyad proteins

We probe native proteins that interact with JPH-2 (JPH-2 ‘interactome’) by co-immunoprecipitation from canine LV whole-cell and whole-tissue lysates. Fig. 2A, lower right panel (labeled ‘Self’), confirms the efficiency of JPH-2 immunoprecipitation under our experimental conditions. We probe the JPH-2 immunoprecipitates (IPs) with antibodies targeting 16 proteins of three functional groups (listed on top of Fig. 2A). To guard against false-positive and false-negative results, in addition to JPH-2 IPs, we include the following in the immunoblot experiments: the original lysates (direct input, DI), negative control (eluent from protein G-beads incubated with lysates without JPH-2 Ab, (-)), and the supernatant after immunoprecipitation (Super). For quantification purpose, we control the amounts of protein loading: 15 ug of lysate proteins in DI and Super lanes, and eluents equivalent to those from protein G-beads incubated with 150 ug of lysate proteins in (-) and IP lanes. Results from whole-cell and whole-tissue lysates are comparable, and the data are pooled.

Fig. 2A depicts representative IB images. Cav1.2 is highly abundant in the IP lane, but very faint in the Super lane. This indicates that a large portion of Cav1.2 in canine ventricular myocytes is bound to JPH-2, so that it is almost totally co-immunoprecipitated with JPH-2. Several other proteins are detected in the IP lanes, but a considerable portion remains in the Super lanes. These proteins either bind JPH-2 only partially, or dissociate from JPH-2 following co-immunoprecipitation. There are also proteins that are not detectable in the IP lanes despite strong signals in the Super lanes, indicating that they do not bind JPH-2. We use the ratio of band intensities in IP vs Super lanes (IP:Super) as a measure of avidity of protein binding to JPH-2. Fig. 2B summarizes the values of IP:Super ratios for proteins detected in JPH-2 immunoprecipitates. Proteins that are not detectable in JPH-2 immunoprecipitates are listed in Fig. 2C.

A distinct pattern of JPH-2-interacting proteins emerges. JPH-2 can interact (directly or indirectly) with SR proteins involved in Ca release (RyR2), Ca uptake (SERCA2a), RyR2 modulators (triadin, and calsequestrin 2 [CSQ2]), and calnexin, a Ca-sensitive SR membrane protein highly concentrated in dyads and peripheral junctional-membrane complexes (see below). JPH-2 also interacts with Cav1.2 and KCNQ1, the pore-forming subunits of L-type Ca (I_{CaL}) and slow delayed rectifier (I_{Ks}) channels. On the other hand, JPH-2 does not interact, or only very weakly, with the six scaffolding proteins tested, including BIN-1, caveolin-3 and telethonin, that are involved in t-tubule formation and dyad maintenance^{4, 5}.

JPH-2 directly modulates I_{CaL} and I_{Ks} channels

We test the effects of JPH-2 on recombinant I_{CaL} (Cav1.2/Cav β 2) and I_{Ks} (KCNQ1/KCNE1) expressed in COS-7 or HEK293 cells (Fig. 3). JPH-2 does not alter whole cell Cav1.2

protein level, but significantly reduces % of Cav1.2 on the cell surface while increasing the Cav1.2/Cav β 2 current amplitude. JPH-2 does not alter whole cell KCNQ1 protein level, but increases % of KCNQ1 on the cell surface while decreasing the KCNQ1 and I_{Ks} current amplitudes. Therefore, in both cases JPH-2's effects on cell surface channel protein levels are opposite to its effects on the current amplitudes, supporting the notion that the latter reflects direct interactions between JPH-2 and the channels' pore-forming subunits on the cell surface. Furthermore, while the I_{Ks} current-suppressing effect is mediated by KCNQ1, the voltage-shift effect requires the presence of KCNE1, because JPH-2 does not shift the activation curve of KCNQ1 expressed alone.

In canine ventricular myocytes, native JPH-2 does not interact with Kv4.3 or ERG1 (Fig. 2). However when overexpressed in COS-7 cells, JPH-2 can be co-immunoprecipitated with Kv4.3 and hERG (Fig. 4Aa and 4Ba). Despite such 'forced' interactions, JPH-2 does not alter the current amplitude or gating kinetics of Kv4.3/KChIP2 or hERG (Fig. 4A and 4B, b-d). Together, these observations suggest that JPH-2 is a positive modulator of I_{CaL} but a negative modulator of I_{Ks}, by directly interacting with their pore-forming subunits.

JPH-2, BIN-1 and Cav1.2 are uniquely downregulated in PVC-CM, but there is a general trend of reduction of other dyad proteins

We next probe the levels of 19 proteins in whole-tissue lysates prepared from CON, SHAM and PVC LVs (listed along the abscissa of the lower panel). Based on their subcellular distribution patterns, 17 proteins reside in dyads, and 2 proteins do not (KCNE1 and KCNE2). Fig. 5A depicts representative IB images and Fig. 5B summarizes densitometry quantification. Based on one-way ANOVA, only three proteins reach statistically significant changes: JPH-2, BIN-1 and Cav1.2. For each, the protein level in PVC samples is significantly less than those in CON and SHAM samples, but there is no significant difference between CON and SHAM samples.

Although the other sixteen proteins do not reach statistical significance based on ANOVA, some of them do show modest variations among CON, SHAM and PVC samples. We explore their relationship with JPH-2 in terms of the protein level. Data are pooled from 29 hearts. Protein levels are quantified as described for Fig. 5. The degree of correlation is quantified by two methods: linear regression statistics and Pearson correlation coefficient. Fig. 6A illustrates selective cases of linear regression between JPH-2 and eight proteins. The 'probability' value reports the probability that the linear relationship occurs by chance. Pearson correlation coefficient varies between +1 (totally and positively correlated), zero (totally unrelated), and -1 (totally and negatively correlated). Fig. 6B plots Pearson correlation coefficients against corresponding linear regression probability values. Cav1.2 and BIN-1 are highly correlated with JPH-2 based on the linear regress statistics. However, their Pearson correlation coefficients plateau at 0.7-0.8. Data for 15 other proteins roughly fall on a line with a negative slope: a larger positive Pearson correlation coefficient is associated with a smaller probability value (or a higher significance of linear relationship). Therefore, the two correlation analyses are consistent with each other, and show that there is a general trend of decrease in dyad proteins when the JPH-2 protein level is reduced. On the other hand, the two non-dyad proteins, KCNE1 and KCNE2, are not correlated with JPH-2.

Some dyad proteins have altered distribution patterns in PVC myocytes

Fig. 7a and 7b show that RyR2 and CSQ2 maintain the same striation pattern in CON and PVC myocytes, even in areas where t-tubules (WGA-stained striations) and JPH-2 appear missing. These observations indicate that in PVC myocytes the organization of junctional SR is intact, despite the reduction in t-tubule contents and downregulation of JPH-2. Cav-3 maintains the same distribution pattern in CON and PVC myocytes (Fig. 7c), indicating that the caveolar subdomains are preserved in PVC-CM.

In PVC myocytes, the reduced immunofluorescence signals of JPH-2, BIN-1 and Cav1.2 are consistent with the immunoblot data (Fig. 5). Fig. 7d, bottom, shows that in this PVC myocyte where t-tubules are clearly present (WGA-stained striations), both BIN-1 and JPH-2 are dim and dispersed. It has been shown that t-tubules in ventricular myocytes are reinforced by extracellular basal lamina proteins, transmembrane integrins, and intracellular cytoskeleton (dystrophin, spectrin, and F-actin)^{13, 14}. This may explain why residual t-tubules are present in areas of PVC myocytes where both BIN-1 and JPH-2 are absent. Fig. 7e, bottom, depicts a PVC myocyte with dim JPH-2 signals in striations coinciding with those of WGA. However, the Cav1.2 signals are dim and appears in random vesicles. This indicates that in PVC myocytes even when JPH-2 and t-tubules are still in place, downregulation of BIN-1 causes Cav1.2 to be ‘mis-targeted’.

Although the protein levels of NCX and calnexin are not significantly different between CON and PVC samples, their distribution patterns are affected by PVC. In CON myocytes both NCX and calnexin are distributed along the dyads and the peripheral sarcolemma (Fig. 7f). They shift from dyads to peripheral sarcolemma in PVC myocytes. The functional implications will be discussed below.

DISCUSSION

Our major findings can be summarized as the following: (1) Canine LV myocytes from PVC-CM hearts have reduced t-tubule contents but otherwise normal ultra-structures. (2) JPH-2 can be co-immunoprecipitated with proteins involved in Ca_i -handling, Cav1.2 and KCNQ1, but not or only weakly with six scaffolding proteins examined. Heterologous expression experiments show direct positive modulation of Cav1.2/Cav β 2 (I_{CaL}) and negative modulation of KCNQ1/KCNE1 (I_{Ks}) by JPH-2. (3) Among 19 proteins examined, only JPH-2, BIN-1 and Cav1.2 are significantly downregulated in PVC hearts. Correlation analysis indicates that JPH-2 downregulation is associated with modest reduction of many other dyad proteins. (4) In PVC myocytes NCX and calnexin shift from dyads to the lateral surface, despite similar total protein levels. The above are also depicted in the cartoons of the Graphical Abstract.

JPH-2 interactome suggests that it is more than a ‘dyad glue’

It has been shown that JPH-2 can be co-immunoprecipitated with Cav1.2¹⁵ and RyR2^{15, 16}. Our data are consistent with these previous findings. Since we probe JPH-2 IPs with a wide range of Abs, and the experiments are designed to quantify the avidity of protein binding to JPH-2, our data reveal a broader pattern of JPH-2 interacting proteins in myocytes. JPH-2

directly or indirectly interacts with proteins involved in SR Ca release (RyR2), Ca reuptake (SERCA2a), and RyR2 modulators (triadin and CSQ2). JPH-2 also directly interacts with the pore-forming subunits of I_{CaL} and I_{Ks} . Thus, as has been shown previously^{16, 17}, JPH-2 appears capable of doing more than simply gluing the t-tubule and jSR membranes together. I_{CaL} and I_{Ks} work in concert to ensure the proper response to β -adrenergic stimulation: enhanced I_{CaL} increases contractility, while enhanced I_{Ks} shortens action potential duration¹⁸. The latter is important in ensuring sufficient diastole for chamber filling during β -adrenergic-induced tachycardia. JPH-2 modestly increases I_{CaL} but significantly reduces I_{Ks} , accompanied by a positive shift in the voltage-dependence of I_{Ks} activation. This will tilt the balance toward increase in contractility.

BIN-1, caveolin-3, and telethonin have been proposed to be involved in t-tubule biogenesis and dyad maintenance^{4, 5}. Ankyrin B, dystrophin and α -actinin are sub-sarcolemmal adaptor proteins and F-actin anchors that help stabilizing the t-tubule structures in ventricular myocytes, to withstand the constant forces of contraction-relaxation cycles^{13, 14}. JPH-2 does not interact (or only very weakly) with these scaffolding proteins, indicating that JPH-2's function as a 'dyad glue' does not rely on interactions with these proteins.

PVC-CM is associated with a broad range of changes in dyad proteins

Fig. 6B indicates that when JPH-2 is reduced, more than a dozen of dyad proteins are also reduced although to different degrees. The mechanisms are likely multifaceted. Some of these proteins directly or indirectly bind JPH-2 (triadin, CSQ2 and calnexin), while others do not. In the latter cases, structure changes in dyads that disrupt protein targeting or retention may be the cause.

Changes in the distribution patterns of dyad proteins can have significant functional consequences. A case in point is Na/Ca exchanger (NCX). Under normal conditions, NCX in the t-tubule membrane contributes to removing Ca ions from the dyadic spaces during systole. In PVC myocytes, NCX shifts from dyads to the lateral surface. Losing NCX in the dyad regions may be a compensatory mechanism initially (enabling higher and longer [Ca] elevations in the dyadic spaces for more sustained CICR), that can turn mal-adaptive (calpain-1 activation and cleavage of JPH-2¹⁵, Cav1.2¹⁹, and probably other dyad proteins).

JPH-2, BIN-1 and Cav1.2 play deterministic roles in the progression of PVC-CM

The protein levels of JPH-2, BIN-1 and Cav1.2 are uniquely downregulated in PVC hearts, indicating that they are the major players in structural and functional impairments in PVC-CM. Our working hypothesis is diagrammed in Fig. 8. This hypothesis is built upon data presented here and those in the literature. JPH-2 downregulation in PVC-CM can be due to increase in autophagy and lysosomal degradation³, calpain cleavage¹⁵, and/or miR24 repression²⁰. Loss of JPH-2 weakens the linkage between t-tubule and jSR membranes⁶, resulting in decreased t-tubule contents²¹. However, loss of JPH-2 *per se* does not reduce Cav1.2 protein level or I_{CaL} amplitude¹⁶. Loss of BIN-1 in PVC-CM can cause mis-targeting of Cav1.2 away from t-tubules²², decreasing cell surface Cav1.2 protein and I amplitude²³. However, loss of BIN-1 *per se* does not reduce t-tubule contents, disrupt t-tubule organization¹⁴, or reduce the total Cav1.2 protein level²³. Orphaning of RyR2, in

conjunction with decrease in I_{CaL} , impairs CICR, while APD prolongation and heterogeneous changes in I_{CaL} and I_{Ks} may increase the risk for arrhythmia. Future experiments should test whether JPH-2 downregulation and cellular remodeling are reversed when the extrasystoles are stopped.

CONCLUSION

We propose that in canine ventricular myocytes JPH-2 is involved not only in maintaining the dyad structure, but also in modulating Ca_i -handling as well as I_{CaL} and I_{Ks} channel function. JPH-2, BIN-1 and Cav1.2 are uniquely and significantly downregulated in PVC hearts. This suggests that they are the major players in the progression of PVC-CM. Dissecting the temporal or hierarchical relationship among the three will help the design of therapeutic interventions to prevent the progression of PVC-CM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

Confocal microscopy was performed at the Virginia Commonwealth University – Department of Neurobiology & Anatomy Microscopy Facility, supported in part by NIH-NINDS Center Core Grant 5P30NS047463. We thank Drs. Jose Miguel Eltit and Karoly Kaszala, as well as Adithi Krishnamoorthy and Carl Beglinger, for their contributions to this project, and Dr. Hiroshi Takeshima for the mouse JPH-2 plasmid and antiserum.

FUNDING

This study was supported by the following funds: RO1 HL107294 from NIH/NHLBI (GNT), SDG9310032 from AHA/National Center (JFH), and RO1 HL105713 from NIH/NHLBI (RCB).

JFH and AT receive research support from Boston Scientific (BS), and Biotronik (BTK).

Abbreviations

PVC hearts	hearts exposed to PVC myocytes isolated from PVC hearts
PVC myocytes	myocytes isolated from PVC hearts
IB	immunoblot
IP	immunoprecipitation
IF	immunofluorescence
jSR	junctional sarcoplasmic reticulum
JMC	junctional membrane complex
JPH-2	junctophilin-2
BIN-1	bridging integrator-1
RyR2	ryanodine receptor 2
SERCA2a	Sarcoplasmic reticulum Ca pump

CSQ2	calsequestrin 2
Cav-3	caveolin-3
NCX	Na/Ca exchanger
AnkB	ankyrin B
LTCC	L-type Ca channel
Cavβ2	voltage-gated Ca channel β subunit 2
I_{CaL}	L-type Ca channel current
I_{Ks}	slow delayed rectifier current
Q1	KCNQ1
E1	KCNE1
I_{Kr}	rapid delayed rectifier current
ERG1	ether-a-go-go related gene 1
hERG	human ether-a-go-go related gene
I_{to}	Transient outward current
KChIP2	K channel interacting protein 2

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CLINICAL PERSPECTIVES

Chronic and frequent PVCs applied to normal canine hearts causes marked downregulation of JPH-2, BIN-1 and Cav1.2. Loss of JPH-2 and BIN-1 destabilizes the t-tubule structures along with proteins localized in the dyad regions. These changes, along with decrease in I_{CaL} , impair EC coupling and contribute to the decline in LVEF. While the decrease in t-tubule contents has been observed in diseased human hearts and animal models of heart diseases, the decrease in I_{CaL} appears to be specific for the canine model of PVC-CM. We now know that ventricular action potential profile/duration can be influenced not only by the expression levels/distribution patterns/functional status of ion channels that contribute inward or outward currents during action potentials, but also by the degree of t-tubule membrane folding induced by BIN-1 binding. The interplay of changes in these factors could upset the normal pattern of action potential heterogeneity, increasing the risk for arrhythmia. In terms of patient care, the first line of treatment should be to eliminate PVCs with either antiarrhythmics or ablation when possible. However, it is important to explore alternative and/or additional therapeutic options for all patients particularly those that are not candidates for either approach. One possibility is to restore JPH-2 expression level. According to our data, this intervention may stop dyad disruption, restore contractility and reduce action potential variability. To translate our discoveries into clinical practice, we need to test whether JPH-2 gene therapy can restore dyad structure and function in the canine model of PVC-CM.

Fig. 1. PVC myocytes have reduced t-tubule contents, lowered JPH-2 protein level but otherwise normal ultra-structures

(A) Orthogonal views of di-8-ANEPPS fluorescence signals from myocytes of SHAM and PVC LVs. (B) Quantification of t-tubule contents based on % of di-8-ANEPPS signals within cell borders. Small symbols represent data points from individual myocytes (2 animals each); large symbols are mean values with standard deviation bars pooled from all myocytes; numbers of myocytes examined in parentheses. (C) *Top and middle*: transmission electron microscope images of SHAM and PVC myocytes. *Bottom*: Summary of sarcomere lengths, same format as Fig. 1B. (D) *Top*: Immunoblot (IB) image of JPH-2 in whole tissue lysates from CON, SHAM and PVC LVs. *Bottom*: Coomassie blue stain confirming even protein loading among lanes.

Fig. 2. Probe JPH-2 ‘interactome’ in canine LV myocytes by co-immunoprecipitation (co-IP) JPH-2 goat Ab immunoprecipitates (IP) from canine LVs are probed with Abs targeting proteins of three functional groups (listed on top). **(A)** Representative immunoblot images of direct input ‘DI’, negative control ‘(-)’, supernatant ‘Super’, and IP, with protein targets and size marker positions noted on the left. The ratio of band intensities in IP vs Super lanes (IP:Super) reflects degree of protein binding to JPH-2. Lower right ‘Self’: IB with JPH-2 rabbit Ab to confirm high efficiency of immunoprecipitation. **(B)** Summary of ‘IP:Super’ values of proteins detected in JPH-2 IPs. **(C)** List of proteins not detected in JPH-2 IPs. (n): number of samples tested.

Fig. 3. Confirm direct JPH-2 modulation of I_{CaL} and I_{Ks} channels using mammalian cell heterologous expression

(A) **a**: JPH-2 decreases % Cav1.2 on cell surface without changing whole cell Cav1.2 protein level. *Left*: Representative IB images of whole-cell lysate (W) and biotinylated fraction (b) from COS-7 expressing cDNAs listed on top, probed with antibodies targeting Cav1.2 (top) and JPH-2 (bottom). Lack of JPH-2 band in 'b' lane confirms no cytoplasmic protein contamination. *Right*: summary from four experiments. Small symbols represent data from individual experiments, with data of '- JPH-2' and '+ JPH-2' linked by lines.

Large symbols represent data averaged from experiments with Cav β 2 coexpression. **b-d** JPH-2 increases I_{CaL} current density (pA/pF) without altering I_{CaL} gating kinetics. '– JPH-2' and '+ JPH-2' data are color-coded gray and black. **b**: I_{CaL} traces recorded from HEK293 cells transfected with cDNAs marked on top; voltage clamp protocol diagrammed in inset. **c**: average peak current density-voltage relationships. **d**: maximal current density (I_{max}, based on Boltzmann fit, see below) normalized by mean value of '– JPH-2' cells. **(B)** **a**: JPH-2 increases % Q1 on cell surface without changing whole cell Q1 protein level. Format and data analysis the same as 'Aa'. Lack of α -actin band in 'b' lanes confirms no cytoplasmic protein contamination. **b-e** JPH-2 reduces I_{Ks} current density and alters its gating kinetics. '– JPH-2' and '+ JPH-2' data are color-coded gray and black. 'Q1 alone' and 'Q1/E1' data are presented by open and closed symbols or histogram bars, respectively. **b**: current traces recorded by perforated patch clamp of COS-7 cells transfected with cDNAs listed on top; voltage clamp protocol diagrammed in inset. **c**: average activation curves. **d**: I_{max} based on Boltzmann fit and normalized by mean value of '– JPH-2' cells. **e**: summary of half-maximum activation voltage (V_{0.5}). To estimate I_{max} and V_{0.5} for each cell, the relationship between peak amplitudes of tail currents (I_{tail}) and test pulse voltages (V_t) is fit with a Boltzmann function: $I_{tail} = I_{max} / [1 + \exp(V_{0.5} - V_t)/k]$, where k is a slope factor. The ratio, I_{tail}/I_{max}, is expressed as 'fraction activated' in 'c'. In Ad and Bd, numbers in histogram bars are those of cells patch clamped from 3 independent expression experiments. t-test '+ JPH-2' vs '– JPH-2', * p < 0.05, ** p < 0.01.

Fig. 4. JPH-2 does not modulate I_{to} or I_{Kr} channels in COS-7 cells despite co-immunoprecipitation of JPH-2 with Kv4.3 and hERG

(A) **a**: Probing JPH-2 immunoprecipitates from canine ventricular myocytes (left) or COS-7 cells (right, expressing Kv4.3/KChIP2 plus JPH-2) with Kv4.3 mouse mAb (top). The membrane is reprobed with JPH-2 rabbit Ab to confirm effective JPH-2 immunoprecipitation in both cases (bottom). DI, (-) and IP have the same meaning as in Fig. 2. **b-d** JPH-2 does not affect peak current density or voltage-dependence of inactivation of Kv4.3/KChIP2. (B) **a**: Probing JPH-2 immunoprecipitates for ERG1 (canine LV myocytes) or hERG (COS-7 cells) (top). The same membrane is reprobed with JPH-2 rabbit Ab (bottom). **b-d** JPH-2 does not affect peak tail current density or voltage-dependence of activation of hERG. In both panels, '-JPH-2' and '+ JPH-2' data are color-coded gray and black, and voltage clamp protocols are diagrammed in inset of 'd'.

Fig. 5. JPH-2, BIN-1, and Cav1.2 are uniquely downregulated in PVC-CM

(A) Representative immunoblot images. The original immunoblots have 11 -14 sample lanes, 5 of which are CON sample lanes (for data normalization, see below). For clarity, only two each of CON, SHAM, and PVC lanes are shown, and white lines denote where extra lanes have been removed. Protein targets and size marker positions are noted on the left. Dots on the right mark the bands used for densitometry quantification. Proteins are divided into three functional groups marked on top. (B) Summary of densitometry quantification. (n): numbers of LV samples examined. For every protein examined, each sample is represented by one data point mostly averaged from 2 – 6 independent IB experiments. To allow data averaging from separate IB experiments, for each immunoblot the band intensities are normalized by the mean value of band intensities in CON lanes. Statistics: for each protein, one-way ANOVA is used to evaluate whether there is any significant difference among the CON, SHAM, and PVC groups. If there is, Tukey's test is used for all-pairwise comparisons. * JPH-2, BIN-1 and Cav1.2 protein levels in PVC samples are significantly lower than respective levels in CON and SHAM samples.

Fig. 6. Positive correlation between JPH-2 and other dyad proteins

(A) Selected examples of linear regression between the proteins noted in the panels (normalized protein levels, as described for Fig. 5, plotted along Y-axis) and JPH-2 (X-axis). The probability values (of occurrence by chance) based on linear regression statistic are noted in the panels. (B) Pearson correlation coefficients are plotted against corresponding linear regression probability values for 17 proteins. KCNE2 is excluded. Cav1.2 and BIN-1 are uniquely highly correlated with JPH-2. The other 15 proteins fall along the dotted line with a negative slope.

Fig. 7. Dyad proteins distribution patterns in CON and PVC myocytes

Each panel shows confocal images of fluorescence signals from the same CON (top) or PVC (bottom) myocyte, representing immunofluorescence signals detected by Abs raised in different hosts (Table S1) targeting proteins noted in the panels, or Alexa647-conjugated wheat germ agglutinin (WGA). Horizontal lines point to transverse striations of dyads or t-tubules. Asterisks mark lateral surfaces. Open arrows point to JPH2⁺ vesicles.

Fig. 8.

Proposed mechanisms for the deterministic roles of JPH-1, BIN-1 and Cav1.2 in the progression of PVC- CM.