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JPH-2 interacts with Ca_i-handling proteins and ion channels in dyads: Contribution to premature ventricular contraction–induced cardiomyopathy

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BACKGROUND In a canine model of premature ventricular contraction–induced cardiomyopathy (PVC-CM), Cav1.2 is downregulated and misplaced from transverse tubules (T tubules). Junctophilin-2 (JPH-2) is also downregulated. Q5

OBJECTIVES The objectives of this study were 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 ultrastructures (electron microscopy), probe JPH-2–interacting proteins (co-immunoprecipitation), quantify dyad and nondyad 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 ultrastructures. Among 19 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 in dyad protein levels when JPH-2 is downregulated. Furthermore, several dyad proteins, including Na/Ca exchanger, are missing or shifted from dyads to the peripheral surface in PVC myocytes. JPH-2 directly or indirectly interacts with Ca_i-handling proteins, Cav1.2 and KCNQ1, although not BIN-1 or other scaffolding proteins tested. Expression in mammalian cells that do not have dyads confirms direct JPH-2 modulation of the L-type Ca channel current (Cav1.2/voltage-gated Ca channel β subunit 2) and slow delayed rectifier current (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 3 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

ABBREVIATIONS BIN-1 = bridging integrator-1; **$Cav_{\beta}2$** = voltage-gated Ca channel β subunit 2; **CICR** = Ca-induced Ca release; **CON** = control; **CSQ2** = calsequestrin 2; **I_{CaL}** = L-type Ca channel current; **I_{Ks}** = slow delayed rectifier current; **IP** = immunoprecipitate; Q6 **JPH-2** = junctophilin-2; **jSR** = junctional sarcoplasmic reticulum; **LV** = left ventricular; **NCX** = Na/Ca exchanger; **PVC hearts** = hearts exposed to PVC myocytes isolated from PVC hearts; **PVC myocytes** = myocytes isolated from PVC hearts; **RyR2** = ryanodine receptor 2; Q7 **SR** = sarcoplasmic reticulum; **Super** = supernatant after immunoprecipitation; **T tubule** = transverse tubule; **WGA** = Q8 wheat germ agglutinin

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This study was supported by the National Institutes of Health/National Heart, Lung, and Blood Institute (grant no. RO1 HL107294, to Dr Tseng, and grant no. RO1 HL105713, to Dr Balijepalli) and American Heart Association National Center (grant no. SDG9310032, to Dr Huizar). Also, confocal microscopy was performed at the Microscopy Facility, Department of Neurobiology and Anatomy, Virginia Commonwealth University, supported in part by the National Institutes of Health–National Institute of Neurological Disorders and Stroke Institutional Center Core Grant 5P30NS047463. Dr Huizar and Dr Tan have received research support from Boston Scientific and Biotronik. **Address reprint requests and correspondence:** Dr Gea-Ny Tseng, Department of Physiology and Biophysics, Virginia Commonwealth University, 1101 E. Marshall St, Sanger 3-042a, Richmond, VA 23298. E-mail address: gtseng@vcu.edu. Q4

Introduction

Huizar et al[1] have created a canine model of premature ventricular contractions (PVCs). They showed that chronic (≥3 months) and frequent (1 extrasystole after every sinus beat) PVCs applied to structurally normal hearts could cause a gradual decline in the left ventricular (LV) ejection fraction.[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

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that PVC-CM is functional in nature.[1] This is consistent with clinical reports of improvements in patients' cardiac function after PVC ablations.[2] For patients with PVCs who 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 LV myocytes from hearts exposed to chronic PVCs (PVC hearts) have the following defects: decrease in the L-type Ca channel current (I_{CaL}) density, displacement of Cav1.2 (I_{CaL} pore-forming subunit) from transverse tubules (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 hearts.[3] 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 main culprit of progression in PVC-CM.

These findings led to 3 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 (bridging integrator-1 [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 of 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. Online Supplemental 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-month-old; weight 35–45 lb) were divided into 3 groups: CON (n = 7), SHAM (n = 7), and PVC (n = 15). The procedures of pacemaker implantation and pacing algorithm were as described previously.[1] Myocytes were isolated from LV free wall irrigated by the first branch of left circumflex coronary artery using collagenase and mechanical trituration, as described previously.[3]

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

Cell culture, transfection, patch clamp, and biotinylation experiments were conducted as described previously.[11]

Immunoprecipitation and immunoblotting experiments

Whole-cell and whole-tissue lysates were prepared as described previously,[3] except that for immunoprecipitation the Triton X-100 concentration was lowered to 0.5% to minimize perturbations of noncovalent protein-protein interactions. All immunoprecipitation reactions were performed on freshly prepared lysates to minimize nonspecific protein bindings during freezing.

Immunofluorescence and confocal experiments

Immunofluorescence and confocal experiments on LV myocytes were conducted as described previously.[12]

Detailed methods are provided in Online Supplemental Materials.

Results

PVC myocytes have reduced T-tubule contents but otherwise normal ultrastructures

Figure 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 (Figure 1B). In contrast, transmission electron microscopy shows that the myocytes' ultrastructures, in terms of myofilament organization and mitochondria morphology, are not altered except a modest shortening of sarcomere lengths in PVC myocytes (Figure 1C). Therefore, the major structural change at the single cell level in PVC-CM is loss of T tubules.

Previously we reported a decrease in JPH-2 protein level in a limited set of CON and PVC samples.[3] We have now extended this quantification to a larger set of CON, PVC, and SHAM samples (Figure 1D). The 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. Figure 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 3 functional groups (listed on the top of Figure 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), negative control (eluent from protein G beads incubated with lysates without JPH-2 Ab), and the supernatant after immunoprecipitation (Super). For quantification purposes, we control the amounts of protein loading: 15 μg of lysate proteins in direct input and Super lanes, and eluents equivalent to those from protein G beads incubated with 150 μg of lysate proteins in negative control and IP

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