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Invited Perspective

Pacsin 2 is required for the maintenance of a normal cardiac function in the developing mouse heart

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ABSTRACT

The Pacsin proteins (Pacsin 1, 2 and 3) play an important role in intracellular trafficking and thereby signal transduction in many cells types. This study was designed to examine the role of Pacsin 2 in cardiac development and function. We investigated the development and electrophysiological properties of Pacsin 2 knockout (P2KO) hearts and single cardiomyocytes isolated from 11.5 and 15.5 days old fetal mice. Immunofluorescence experiments confirmed the lack of Pacsin 2 protein expression in P2KO cardiac myocytes in comparison to wildtype (WT). Western blotting demonstrates low expression levels of connexin 43 and T-box 3 proteins in P2KO compared to wildtype (WT). Electrophysiology measurements including online Multi-Electrode Array (MEA) based field potential (FP) recordings on isolated whole heart of P2KO mice showed a prolonged AV-conduction time. Patch clamp measurements of P2KO cardiomyocytes revealed differences in action potential (AP) parameters and decreased pacemaker funny channel (I_f), as well as L-type Ca^{2+} channel (I_{CaL}), and sodium channel (I_{Na}). These findings demonstrate that Pacsin 2 is necessary for cardiac development and function in mouse embryos, which will enhance our knowledge to better understand the genesis of cardiovascular diseases.

1. Introduction

Understanding the factors and networks of signals that regulate the formation and function of the heart can provide significant insight into both development and disease. The Pacsin (protein kinase C and casein kinase 2 substrate in neurons) proteins, also called syndapins, comprise a subfamily of the Bin-Amphiphysin-Rvs- (BAR-) protein superfamily mediating membrane deformation required for the generation of carriers in intracellular transport processes and/or regulating the transport of specific cargo proteins (for review see Safari and Suetsugu [1]). Of the three family members, termed Pacsin 1–3, Pacsin 2 shows a broad tissue distribution, while Pacsin 1 is mainly expressed in neuronal tissue and Pacsin 3 restricted to muscle, heart and lung [2]. All Pacsins share an N-terminal Fes-Cip4 homology-BAR (F-BAR) domain, followed by an unstructured linker region and a C-terminal SH3 domain, which is crucial for binding to proline-rich regions of specific interaction partners. Thus, all Pacsins bind to dynamin and to the neuronal Wiskott-Aldrich-syndrome protein (N-WASP) to activate the Arp 2/3 complex, linking actin remodeling processes to endocytic sites [3]. Two recent studies elucidated a role for Pacsin 2 in caveolin-dependent endocytosis [4,5]. Pacsin 2 binds directly to caveolin1 via its F-BAR domain and is thought to recruit dynamin to caveolae scission sites. In addition to its localization at caveolae and at vesicles, Pacsin 2 is also found at tubular structures [2,6].

Pacsin 1 and 2 also bind to Eps15 homology domain (EHD) proteins, which are dynamin-like ATPases, with an N-terminal G-domain and a C-terminal EH-domain [7]. EHD proteins interact via the EH domain with the NPF motifs present in the linker regions of Pacsin 1 and 2. EHD proteins were previously shown to participate in distinct clathrin-dependent and independent endocytic and recycling processes [8,9]. Furthermore, in a recent study EHD proteins were identified as direct interactors of ankyrin B in cardiomyocytes which regulate its localization and thereby membrane excitability [10].

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Previous studies on Pacsin 2 concentrated on its functional characterization in other tissues and cells, however, the function of Pacsin 2 in cardiomyogenesis has not yet been investigated and no information is available about the exact role of this protein in heart function and the biologic networks in which it participates. In the present study, we attempted to define the role of Pacsin 2 protein in embryonic mouse heart during development.

We report that Pacsin 2 is expressed in both, atrial and ventricular cardiomyocytes (CMs) where it appears to participate in the generation and propagation of the electrical impulse required for heart contraction. Thus, the absence of Pacsin 2 results in significant electrophysiological alterations, at least during late stage mouse embryonic development. Taken together, our results suggest that Pacsin 2 is required for proper heart development and function.

2. Material and methods

An expanded methods section is available in the Online Data Supplement.

2.1. Mouse lines

In this study embryos of Pacsin 2-deficient mice (P2KO; manuscript on generation and molecular characterization in preparation) backcrossed to C57BL6 background and wild-type C57BL6 animals (C57 black) were used. Briefly, to generate P2KO mice, *Pacsin 2*-floxed animals were crossed to Cre deleter mice [11], yielding ubiquitous disruption of the *Pacsin 2* gene and homologous recombination controlled by Southern blotting. The lack of Pacsin 2 protein was verified by immunoblotting.

All mice were housed in a specific pathogen free (SPF) environment and under light-, temperature- and humidity-controlled conditions. Food and water were available *ad libitum*. The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals and with the regulations of the "Tierschutzgesetz" issued by the "Bundesjustizministerium", Germany, on July 4, 2013. All animal procedures were approved by the local government authorities ("Bezirksregierung Köln", Cologne, Germany; Permit Number: AZ 2010.A218) and the "Tierschutzbeauftragte" of the University of Cologne.

2.2. Embryonic heart and cardiomyocyte preparation

Murine embryonic hearts were obtained from superovulated WT and P2KO mice. Early (E11.5, EDS) and late (E15.5, LDS) developmental stage embryonic whole-mount mouse hearts and single myocytes – isolated and prepared as described in the Online Data Supplement – were used.

2.3. Real-time quantification PCR (qRT-PCR)

The RNA isolation, cDNA and RT-qPCR techniques were followed as previously reported [12]. Briefly, total RNA of atria and ventricles of wild and P2KO mice, were isolated using TRIzol reagent (Invitrogen, Darmstadt, Germany) and CHCl3 (Sigma, Steinheim, Germany) and further purified with the miRNeasy mini kit (Qiagen, Hilden, Germany). The quantification and quality control measurements were performed using a NanoDrop spectrophotometer (ND-1000, Thermo-Fisher, Langenselbold, Germany). For cDNA synthesis, 1000 ng of total RNA was used as the starting material with the Super Script Vilo cDNA synthesis kit (Invitrogen, Darmstadt, Germany) according to the kit instructions. cDNA was diluted with nuclease-free water, and 100 ng of cDNA was amplified by RT-qPCR. The primer sequences were taken from primer bank. (https://pga.mgh.harvard.edu/primerbank). Platinum SYBR Green qPCR SuperMix (Invitrogen, Darmstadt, Germany) was used for the PCR assays with the Applied Biosystems 7500 FAST cycler. The gene expression of target genes was normalized to the reference gene GAPDH. The mRNA expression values were represented as the fold change relative to the respective control. The primer sequences are listed in the supplementary Table S1.

2.4. Immunohistochemistry and western blotting

For immunocytochemistry analysis samples of WT and P2KO mice were fixed with 4% paraformaldehyde for 10 min at room temperature. After permeabilization with 0.3% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) for 20 min at room temperature cells were blocked with 5% bovine serum albumin in PBS for 1 h. Primary antibodies diluted in PBS were added and cells were stored at 4 °C overnight. Antibodies used were: Mouse monoclonal IgG_1 anti sarcomeric α-actinin (1:800, Sigma-Aldrich), mouse monoclonal IgG_1 anti-cardiac troponin T (1:500, Thermo Scientific), anti- Connexin 43 (1:5000, Sigma), anti- T- Box 3 (1:1000, Santa Cruz), rabbit polyclonal IgG α-HCN1(1:100), α-HCN2(1:200), α-HCN3 (1:200), and α-HCN4 (1:100) (Alomone Labs, Jerusalem, Israel), affinity-purified polyclonal rabbit serum specific for murine Pacsin 2 (1:250) [13]. Secondary antibodies used were goat-anti-mouse IgG-Alexa-Fluor® 555, goat-anti-mouse IgG Alexa-Fluor® 488, goat-anti-mouse IgG-Alexa-Fluor® 546 and goat-anti-rabbit IgG Alexa-Fluor® 488, (1:1000, all Invitrogen) were applied. Nuclei were counterstained with Hoechst 33432 (1:2000, Sigma-Aldrich). Cover glasses were mounted onto slides with ProLong Gold antifade reagent (Invitrogen). Samples were analyzed using an Axiovert Microscope (Zeiss Axiovert 200/ApoTome) equipped with the image processing software Axiovision 4.5 (Carl-Zeiss, Jena, Germany).

For Western blotting, cardiac lysates were prepared from the heart tissue of WT and P2KO embryonic mice at E15.5 developmental stage using RIPA buffer (Sigma). Analyses were performed with 10 μg of cardiac lysates. These were separated by SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride membranes (Thermo Scientific). After blocking the membranes with 5% non-fat milk suspended in T-PBS (0.1% Tween 20, Sigma–Aldrich), the membranes were incubated with the following primary antibodies in 1% non-fat milk at 4 °C overnight: anti- Connexin 43 (1:5000, Sigma), anti- T- Box 3 Box 3 (1:1000, Santa Cruz) and –ß-actin (1:5000, Sigma). The proteins were visualized using the ECL Pierce Fast Western Blot system (Thermo Scientific).

2.5. Extracellular field potential measurements and ECG parameters

The spontaneously beating murine hearts harvested from E11.5 and E15.5 embryo were directly plated and attached onto a MEA60 chamber. Extracellular recording was performed using the MEA data acquisition system (Multi Channel Systems, Reutlingen, Germany, http://www.multichannelsystems) as described [14–16]. Standard measurements were performed at 1–2 kHz (bandwidth 1–5 kHz) in Iscoveís modified Dulbeccoís medium (IMDM) without FCS. During recordings, temperature was kept at 37.0 °C. Data were analyzed off-line with a customized toolbox programmed with MATLAB (Mathworks, Natick, Mass, USA) as reported elsewhere [17]. ß-adrenergic and muscarinic receptor agonists/blockers were added as drops to the bath solution. Different parameters were defined according to the typical ECG-like shape of MEA recordings on E15.5 embryo heart as described in the Online Data Supplement.

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