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Original article

PKCβII modulation of myocyte contractile performance

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ABSTRACT

Significant up-regulation of the protein kinase CβII (PKCβII) develops during heart failure and yet divergent functional outcomes are reported in animal models. The goal here is to investigate PKCβII modulation of contractile function and gain insights into downstream targets in adult cardiac myocytes. Increased PKCβII protein expression and phosphorylation developed after gene transfer into adult myocytes while expression remained undetectable in controls. The PKCβII was distributed in a peri-nuclear pattern and this expression resulted in diminished rates and amplitude of shortening and re-lengthening compared to controls and myocytes expressing dominant negative PKCβII (PKCβDN). Similar decreases were observed in the Ca^{2+} transient and the Ca^{2+} decay rate slowed in response to caffeine in PKCβII-expressing myocytes. Parallel phosphorylation studies indicated PKCβII targets phosphatase activity to reduce phospholamban (PLB) phosphorylation at residue Thr17 (pThr17-PLB). The PKCβ inhibitor, LY379196 (LY) restored pThr17-PLB to control levels. In contrast, myofilament protein phosphorylation was enhanced by PKCβII expression, and individually, LY and the phosphatase inhibitor, calyculin A each failed to block this response. Further work showed PKCβII increased Ca^{2+}-activated, calmodulin-dependent kinase IIδ (CaMKIIδ) expression and enhanced both CaMKIIδ and protein kinase D (PKD) phosphorylation. Phosphorylation of both signaling targets also was resistant to acute inhibition by LY. These later results provide evidence PKCβII modulates contractile function via intermediate downstream pathway(s) in cardiac myocytes.

1. Introduction

Protein kinase C (PKC) modulates cardiac function and there is evidence isoforms of PKC target proteins in Ca^{2+} cycling and the contractile apparatus of myocytes [1,2]. Increased cardiac PKC isoform expression is associated with contractile dysfunction and a variety of pathological conditions [2–5]. However, it remains difficult to identify the role played by each PKC isoform in modulating contractile function or dysfunction. In particular, it is known that PKCβII expression and activity increases during the development of cardiac hypertrophy and the progression to heart failure [3,6–8]. While up-regulation of this isoform is linked to cardiac dysfunction in humans [3,7,8], the functional role played by PKCβII in modulating contractile function remains uncertain.

In earlier work with transgenic mice expressing PKCβII, cardiomyocyte contractility increased in one and decreased in a second animal model [4,9]. More recently, investigators reported little difference in the ventricular response to ischemia or pressure overload after PKCβ knockout [10]. The explanation for these different phenotypes is not well understood, and may result from a number of

Abbreviations: ANOVA, Analysis of variance; Ab, antibody; AU, arbitrary units; BSA, bovine serum albumin; CalA, calyculin A; CaMKIIδ, Ca^{2+}-mediated calmodulin-dependent kinase IIδ; pCaMKIIδ, phospho-CaMKIIδ; cTnI, cardiac troponin I; cTnT, cardiac troponin T; CX-43, connexin-43; DMEM, Dulbecco's modified Eagle's medium; ERK1/2, extracellular signal regulated kinase 1 and 2; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; JMEM, Joklik-modified minimal essential media; LY, LY379196; MOI, multiplicity of infection; cMyBP-C, cardiac myosin binding protein C; MHC, myosin heavy chain; MLC_2, myosin light chain 2; NCX, Na^+/Ca^{2+} exchange; P/S, penicillin and streptomycin; PLB, phospholamban; PKC, protein kinase C; PKCβDN, dominant negative protein kinase CβII; SERCA2A, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase 2A; SR, sarcoplasmic reticulum; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM, standard error of the mean; TR, Texas Red; TTD, time to Ca^{2+} decay; TTP, time to peak; TTR, time to re-lengthening; VAD, ventricular assist device.

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possibilities. For example, there may be divergent localization of PKCβII in response to up-regulation, as the wildtype isoform is expressed in one transgenic model while a constitutively active form is utilized in the other [4,9]. Differences in the developmental expression and/or compensatory adaptations to load also may contribute to the divergent functional outcomes in these models [4]. Thus, questions remain about the role PKCβII plays in modulating contractile function in cardiac myocytes.

One step toward understanding PKCβII modulation of function is to identify the targets for phosphorylation. Biochemical and animal model studies identified several targets for PKCβII, including proteins with a direct role in Ca^{2+} handling and myofilament proteins involved in contractile function. For example, *in vitro* activation of PKCβII phosphorylated the regulatory protein, cardiac troponin I (cTnI) [11]. Enhanced cTnI phosphorylation also developed in wildtype PKCβII transgenic mouse hearts with impaired contractile performance [9]. Additional biochemical studies indicated PKCβII activation phosphorylates the sarcoplasmic reticulum (SR) protein, phospholamban (PLB) which modulates sarcoplasmic reticulum (SR) Ca^{2+} uptake via the SR Ca^{2+}-ATPase, SERCA2A [12]. PKCα, the other major classical isoform expressed in mammalian heart also modulates PLB phosphorylation [2]. Given PKC-α and -β both increase in failing hearts [3,7,13], the influence of PKCβII on myofilament and Ca^{2+} cycling targets continues to be of interest.

Efficient gene expression in intact cardiac myocytes can be used to acutely increase expression using adenoviral-mediated gene transfer. This approach is utilized here to gain insights into the role of PKCβII in modulating cardiac myocyte contractile function, and serves as an important adjunct to earlier findings in animal models by determining the acute influence of PKCβII up-regulation on cellular contractile function. In addition, the present study is designed to determine whether the PKC targets identified in earlier biochemical studies [14–16] are phosphorylated in intact cells and correlates with the functional response. Our study also set out to determine whether this isoform targets other signaling pathways in intact myocytes.

2. Methods

2.1. Adenoviral constructs

Recombinant PKCβII and dominant negative PKCβII (PKCβDN) adenoviruses were kind gifts from Jeffery Molkentin (Cincinnati Children's Hospital) and were originally generated by Ohba et al. [17,18]. PKCβ was cloned into the Kpn1/Xba1 site of pEGFP-1 (Clontech Laboratories, Inc, Mountain View, CA), subcloned into the pACCMVpLpA shuttle plasmid, and then co-transfected with pJM17 in HEK 293 cells to generate the PKCβGFP recombinant adenovirus. High titer stocks of each viral construct were prepared as described earlier [19].

2.2. Myocyte isolation and gene transfer

Adult rat cardiac myocytes were isolated as described in earlier studies [19]. Briefly, myocytes were isolated from heparinized rats with collagenase and hyaluronidase to digest the heart, and then cells were made Ca^{2+} tolerant over 15 min. Isolated myocytes were plated on laminin-coated coverslips for 2 h in DMEM plus penicillin (50 U/ml), streptomycin (50 μg/ml; P/S), and 5% FBS. Two hours later, gene transfer was carried out with high titer PKCβII, PKCβDN or PKCβGFP (10 MOI) recombinant adenovirus [19]. At this MOI, ~80% of cardiac myocytes expressed GFP 2 days after gene transfer (unpublished results). Myocytes were electrically paced in M199 plus P/S media 24 h after plating, with subsequent media changes every 12 h [20].

A similar protocol was used to isolate adult myocytes from New Zealand male rabbits (2.2–2.6 kg) with the following modifications. Isolated hearts were immersed in an ice-cold 50:50 mixture of Joklik-modified MEM (JMEM) and Hank's Balanced Salt Solution plus 15 mM HEPES and P/S. Hearts were initially perfused with Ca^{2+}-free DMEM plus 15 mM HEPES and P/S at 37 °C, followed by DMEM supplemented with 10 mM HEPES, P/S, collagenase (250 U/ml), and hyaluronidase (0.1 mg/ml) for 10 min, Protease type XIV (0.2 mg/ml) was added to the perfusate for an additional 15 min. Isolated myocytes were made Ca^{2+} tolerant in DMEM plus 10 mM HEPES, 2.5 mg/ml BSA, P/S and 1.25 μM $CaCl_2$, with re-addition of Ca^{2+} to a final concentration of 1.80 mM over 1 hr, and then plated in DMEM supplemented with 5% FBS and P/S. Two hours later, gene transfer was carried out with recombinant adenovirus diluted in serum-free DMEM plus P/S for 1 h, followed by the addition of fresh serum-free media. Myocytes were then cultured in M199 plus P/S within 24 h after plating. All animal procedures followed the guidelines and were approved by the University Committee on Use and Care of Animals at the University of Michigan.

2.3. Contractile function and Ca^{2+} transient measurements

Sarcomere shortening was measured in isolated myocytes 2–3 days after gene transfer, as described previously [21]. Briefly, coverslips were transferred to a 37 °C temperature-controlled chamber, perfused with M199 plus P/S and paced at 0.2 Hz. Sarcomere shortening under basal conditions was measured using a video-based microscope camera system (Ionoptix, Beverly, MA). Resting sarcomere length, peak shortening amplitude, shortening and re-lengthening rate, and time to 50% of peak amplitude ($TTP_{50\%}$) plus time to 25%, 50%, and 75% re-lengthening ($TTR_{25\%}$, $TTR_{50\%}$, and $TTR_{75\%}$) were determined for each myocyte. Calcium transients and sarcomere shortening were measured simultaneously in a subgroup of myocytes. These cells were loaded with Fura-2 AM, as described earlier [21]. Signal averaged measurements of resting and peak Fura-2 ratios, the rates of Ca^{2+} rise and Ca^{2+}decay, and time to 50 and 75% decay ($TTD_{50\%}$, $TTD_{75\%}$) along with the sarcomere shortening measurements described above were collected from each myocyte.

2.4. Western analysis

PKCβ expression and phosphorylation in myocytes were measured by Western analysis after gene transfer. Myocyte proteins were separated with 12% SDS-PAGE, as described earlier [20,21]. Separated protein bands were transferred onto PVDF membrane for 2000 V-h, and expression was detected on milk-blocked membranes using PKCβII primary antibody (Ab) (1:400; BD Biosciences, San Jose, CA). Anti-phospho-PKCα/β antibody (1:1000; Cell Signaling Technology, Inc, Danvers, MA) was used to measure phospho-PKC on BSA-blocked membranes. Both primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence. Quantitative analysis of protein expression on blots was determined with Quantity One software and normalized to actin expression (5C5; Sigma) and/or a silver-stained band on the SDS-PAGE.

PKCβII expression was assessed in tissue homogenates from failing human ventricular heart samples. Biopsy samples from non-failing donor hearts (non-failing; n = 3) were compared to discarded explant tissue collected at the time of ventricular assist device (VAD) implantation in 15 patients with heart failure (n = 15; 3 female, 12 male; 13 Caucasian, 2 African-American, Mean ± SEM for age = 41 ± 3 year; ejection fraction = 11 ± 1.2%, time from initial diagnosis to VAD implantation: 2.7 ± 0.9 year). All samples were frozen in liquid N_2 at the time of explant and stored at −80 °C. Homogenates were prepared from tissue ground to a powder in liquid N_2 and then resuspended in sample buffer. Protein concentrations were determined for each sample prior to protein separation and Western detection of PKCβII (1:200; C-18; Santa Cruz Biotechnology, Santa Cruz, CA) by ECL. The protocol used to