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Original article

The multifunctional Ca^{2+}/calmodulin-dependent protein kinase II delta (CaMKIIδ) phosphorylates cardiac titin's spring elements

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ABSTRACT

Titin-based passive stiffness is post-translationally regulated by several kinases that phosphorylate specific spring elements located within titin's elastic I-band region. Whether titin is phosphorylated by calcium/calmodulin dependent protein kinase II (CaMKII), an important regulator of cardiac function and disease, has not been addressed. The aim of this work was to determine whether CaMKIIδ, the predominant CaMKII isoform in the heart, phosphorylates titin, and to use phosphorylation assays and mass spectrometry to study which of titin's spring elements might be targeted by CaMKIIδ. It was found that CaMKIIδ phosphorylates titin in mouse LV skinned fibers, that the CaMKIIδ sites can be dephosphorylated by protein phosphatase 1 (PP1), and that under baseline conditions, in both intact isolated hearts and skinned myocardium, about half of the CaMKIIδ sites are phosphorylated. Mass spectrometry revealed that both the N2B and PEVK segments are targeted by CaMKIIδ at several conserved serine residues. Whether phosphorylation of titin by CaMKIIδ occurs *in vivo,* was tested in several conditions using back phosphorylation assays and phospho-specific antibodies to CaMKIIδ sites. Reperfusion following global ischemia increased the phosphorylation level of CaMKIIδ sites on titin and this effect was abolished by the CaMKII inhibitor KN-93. No changes in the phosphorylation level of the PEVK element were found suggesting that the increased phosphorylation level of titin in IR (ischemia reperfusion) might be due to phosphorylation of the N2B element. The findings of these studies show for the first time that titin can be phosphoryalated by CaMKIIδ, both *in vitro* and *in vivo*, and that titin's molecular spring region that determines diastolic stiffness is a target of CaMKIIδ.

1. Introduction

Abnormal cardiac filling caused by increased diastolic chamber stiffness is an important factor in the pathophysiology of a range of cardiac diseases, including the highly prevalent heart failure with preserved ejection fraction (HFpEF) syndrome [1]. However, the mechanisms that determine the level of diastolic stiffness and its various tuning mechanisms are not completely understood. An important determinant of diastolic stiffness is the giant protein titin that spans from Z-disk to M-band of the cardiac sarcomere and has an elastic I-band region that functions as a diastolic stiffness generating molecular spring [2]. Titin's molecular spring region can be restructured through alternative splicing that gives rise to titin isoforms with spring elements that vary in length and generate different levels of passive stiffness [3]. However, changes in splicing are slow processes that require days to weeks to be accomplished and in recent years it has become apparent that phosphorylation-based mechanisms exist that rapidly tune stiffness. The two main spring elements of cardiac titin, the so-called N2B and PEVK elements, are both targeted by kinases. The PEVK spring element has been shown to be phosphorylated by protein kinase Cα (PKCα), a key player in contractile dysfunction and heart failure [4,5]; single molecule, single cell and myocardial tissue experiments in wildtype and PEVK KO mice have shown that PKCα phosphorylation of the PEVK element *increases* passive stiffness [6–8]. The N2B element of titin is also a kinase substrate whose mechanical properties change following phosphorylation. Protein kinase A (PKA), which is stimulated by the β-adrenergic pathway, phosphorylates the large unique sequence of the N2B element which *reduces* passive stiffness [9,10]. Similar to PKA, protein kinase G (PKG), a cGMP-dependent kinase that is part of signaling cascades initiated by nitric oxide (NO) and natriuretic peptides (NPs), phosphorylates the unique sequence of the N2B element and reduces passive stiffness; the PKG phosphorylation site in humans (S469) is also a residue targeted by PKA [11].

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In this study we focused on the Ca^{2+} and calmodulin dependent serine/threonine kinase (CaMKII) that is activated by increases in cellular Ca^{2+}. Four isoforms have been described (α, β, δ, γ) of which CaMKIIδ is the predominant isoform in the heart [12]. CaMKIIδ phosphorylates several Ca^{2+}-handling proteins including phospholamban (PLB) [13,14], ryanodine receptor (RyR2) [15–17], and L-type Ca^{2+} channel (LTCC) [18], as well as myofilament proteins including TnT [19] and MyBP-C [20]. Our aim was to determine whether CaMKIIδ also phosphorylates titin, and to use phosphorylation assays and mass spectrometry to study which of titin's spring elements might be phosphorylated by CaMKIIδ. We found that CaMKIIδ phosphorylates titin in mouse LV skinned fibers, that the CaMKIIδ sites can be dephosphorylated by protein phosphatase 1 (PP1), and that about half of the CaMKIIδ sites are phosphorylated under baseline conditions in isolated hearts and skinned myocardium. We also found that the N2B and PEVK segments are targeted by CaMKIIδ at several conserved sites. Whether phosphorylation of titin by CaMKIIδ occurs *in vivo* was tested in several conditions, including ischemia reperfusion (IR), that is known to activate CaMKIIδ [21]. IR induced an increase in the phosphorylation level of CaMKIIδ sites on titin and this was abolished by the CaMKII inhibitor KN-93. Findings of this work have been presented previously in abstract format [22].

2. Material and methods

2.1. In vitro phosphorylation assay of skinned myocardium

All experiments were performed on 3 month old male C57BL/6J mice and were approved by the University of Arizona IACUC and followed the U.S. National Institutes of Health "Using Animals in Intramural Research" guidelines for animal use. Skinned fibers isolated from the left ventricular (LV) wall [23] were incubated for 2 h at 30 °C with 0.05 U/μl human CaMKIIδ (expressed in insect cells, Invitrogen, CA USA), in kinase buffer (25 mM BES, 1 mM $CaCl_2$, 5 μM calmodulin, 4 mM NaATP, 4 mM $MgCl_2$, 1 mM DTT, 50 μM protein kinase A inhibitor (Sigma), 5 mM NaF, 1 mM Na_3VO_4 pH 7.0), and 10 μCi of [γ-^{32}P]ATP (specific activity 3000 Ci/mmol, Perkin-Elmer). Some fibers were dephosphorylated by incubation with 0.75 U/μl of protein phosphatase 1 (PP1; recombinant rabbit muscle α-isoform, Calbiochem), 2 h at 30 °C, followed by extensive washing and then incubation with CaMKIIδ. The reaction was stopped by adding solubilization buffer (6 M urea, 1.5 M thiourea, 2.25% SDS, 56.25 mM DTT, 0.0225% bromophenol blue, 35% glycerol, 0.0825 mg/ml leupeptin, 1 mM E-64, 0.015 mM PMSF, 37.5 mM Tris–HCl pH 6.8) and the proteins were separated on 2–7% SDS-PAGE gel gradient. The gels were stained with Coomassie blue, dried, scanned, and exposed to X-ray film and analyzed. The titin optical density (OD) of the autoradiograph was normalized to that of the Coomassie blue-stained gel, to normalize for protein loading. In a subset of experiments Western Blots were used to detect phosphorylation of S26 and S170 in the PEVK region of the N2B cardiac titin isoform. Skinned fibers were solubilized and proteins were separated on 0.8% agarose gel and transferred to PVDF membrane (Millipore). The membranes were stained with Ponceau S (Sigma) to determine the level of transferred proteins. The membranes were probed with phospho-specific rabbit polyclonal antibodies against titin's p-S26 and p-S170 [24]. Secondary antibodies conjugated with fluorescent dyes (Biotium, Hayward, Ca, USA) with infrared excitation spectra were used for detection and membranes scanned and analyzed using an Odyssey Infrared Imaging System (Li-Cor Biosciences).

2.2. In vitro kinase assay of titin recombinant proteins

The human titin recombinant fragments N2B, PEVK (from cardiac N2B isoform), Ig8-15, Ig84-91 and the murine recombinant N2B were expressed in *Escherichia coli* as describe [25]. The purified recombinant proteins were incubated with 0.05 U/μl human recombinant CaMKIIδ (Invitrogen, CA, USA), kinase buffer (see above), and 10 μCi of [γ-^{32}P]ATP (Perkin-Elmer), 2 h at 30 °C. The proteins were solubilized (0.5 M Tris–HCl pH 6.8, 10% glycerol, 2% SDS, 0.1 mM 2-mercaptoethanol, 0.01% bromophenol blue) and separated on 4–20% gradient or 12% SDS-PAGE. The gels were Coomassie blue stained, dried, scanned, exposed to X-ray film, and analyzed as described above. We also probed the phosphorylation levels of PEVK's S26 and S170 in recombinant protein using Western blots. The human titin recombinant fragment PEVK expressed in *E. coli* including WT, mutants S26A, S170A, and S26A/S170A [24] was incubated for 2 h at 30 °C with 0.05 U/μl human recombinant CaMKIIδ (Invitrogen, CA, USA) in kinase buffer (see above), and 10 μCi of [γ-^{32}P]ATP (Perkin-Elmer). The proteins were solubilized (0.5 M Tris–HCl pH 6.8, 10% glycerol, 2% SDS, 0.1 mM 2-mercaptoethanol, 0.01% bromophenol blue) and separated on 10% SDS-PAGE. The gels were Coomassie blue stained, dried, scanned, exposed to X-ray film, and analyzed as described above. Additionally some gels were also transferred to PVDF membrane (Millipore) and blotted with phospho-specific antibodies against p-S26 and p-S170 (for details, see above).

2.3. Tandem mass spectrometry coupled to liquid chromatography

Nonphosphorylated and CaMKIIδ-phosphorylated human and murine titin N2B recombinant proteins (see above) were electrophoresed and Coomassie blue stained. The N2B bands were excised from the gels and digested with trypsin or chymotrypsin. Tandem mass spectrometry coupled to liquid chromatography (LC–MS/MS) analyses were carried out (LTQ Orbitrap Velos Thermo Scientific Inc., MA USA) and tandem MS spectra of peptides were analyzed with TurboSEQUEST. Various possible modifications such as alkylation of cysteine residues and of methionine residues and phosphorylation were included in the search parameters.

2.4. Isoproterenol stimulation of intact cardiac myocytes

Cells were isolated as described previously [26,27]. In brief, the heart was cannulated via the aorta and perfused for 4 min with perfusion buffer (in mM: 113 NaCl, 4.7 KCl,0.6 KH_2PO_4, 0.6 Na_2HPO_4, 1.2 $MgSO_4$, 12 $NaHCO_3$, 10 $KHCO_3$, 10 HEPES, 5.5 glucose, 5 BDM, 10 taurine, 20 Creatine, 5 Adenosine, 5 Inosine adjust pH to 7.4 at 37 °C), followed by perfusion with digestion buffer (perfusion buffer plus 0.06 mg/ml of TM liberase [Liberase TM research grade medium thermolysin concentration; Roche Applied Science, IN, USA], and 12.5 μM $CaCl_2$) for 8–10 min. The left ventricle was cut into small pieces that were triturated several times with a transfer pipette and then filtered through a 300-μm nylon mesh filter. The cells were gravity pelleted and Ca^{2+} was reintroduced to a final concentration of 1.8 mM. Some of the cells were solubilized and electrophoresed [28,29] to determine titin content. Activation of CaMKIIδ was determined indirectly by the measurement by Western Blot of the phosphorylation level of the specific CaMKIIδ target Thr17 on phospholamban, PLB [30,31]. The cardiomyocytes were stimulated as described by Erickson et al. [32] with some modifications. Freshly prepared calcium tolerant cardiomyocytes in minimum essential medium α (MEM α) (Invitrogen) supplemented with 1.8 mM calcium chloride were transferred to Petri dish coated with 0.01% poly-L-lysine (Sigma). The myocytes were allowed to adhere for 30 min at 37 °C, 5% CO_2 in a CO_2 incubator (Thermo Scientific). The cell suspension was paced (1 Hz) and 1 μM Okadaic acid (Sigma) was added and the cells incubated for 10 min. The cells were electrically stimulated (paced) using a 6 well C-Dish electrode assembly in conjunction with a C-Pace EP cell culture stimulator (IonOptix). 1 μM isoproterenol (Sigma) was added to the cardiomyocytes and the cells paced at 1 Hz for 20 min. One set of cells was pre-incubated with 1 μM of the CaMKIIδ inhibitor KN93 (Calbiochem) for 10 min before the isoproterenol was added. Then the frequency of stimulation was raised to 4 Hz and the cells were paced for an additional 20 min. The medium was removed and the cells were solubilized by adding solubilization buffer (see above) and heated at 65 °C for 5 min. The proteins were separated by SDS-PAGE (15%) and transferred to PDVF membrane. The membranes were blotted with