Journal of Molecular and Cellular Cardiology 82 (2015) 93–103

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

A novel phosphorylation site, Serine 199, in the C-terminus of cardiac troponin I regulates calcium sensitivity and susceptibility to calpain-induced proteolysis

Paul J.M. Wijnker [a,b,*,1], Yuejin Li [c,1], Pingbo Zhang [c], D. Brian Foster [c], Cris dos Remedios [d], Jennifer E. Van Eyk [e], Ger J.M. Stienen [a,f], Anne M. Murphy [c], Jolanda van der Velden [a,g]

[a] Department of Physiology, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands
[b] Department of Experimental Pharmacology and Toxicology, Cardiovascular Research Center, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
[c] Department of Pediatrics, Division of Cardiology, Johns Hopkins University School of Medicine, Baltimore, USA
[d] Muscle Research Unit, Bosch Institute, The University of Sydney, Sydney, Australia
[e] The Advanced Clinical Biosystems Research Institute, The Heart Institute, Department of Medicine, Cedars Sinai Medical Center, Los Angeles, CA, USA
[f] Department of Physics and Astronomy, VU University, Amsterdam, The Netherlands
[g] ICIN—Netherlands Heart Institute, Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 26 September 2014
Received in revised form 4 March 2015
Accepted 5 March 2015
Available online 11 March 2015

Keywords:
Cardiomyocyte
Heart failure
Protein phosphorylation
Proteolysis
Cardiac troponin I
Myofilament function

ABSTRACT

Phosphorylation of cardiac troponin I (cTnI) by protein kinase C (PKC) is implicated in cardiac dysfunction. Recently, Serine 199 (Ser199) was identified as a target for PKC phosphorylation and increased Ser199 phosphorylation occurs in end-stage failing compared with non-failing human myocardium. The functional consequences of cTnI-Ser199 phosphorylation in the heart are unknown. Therefore, we investigated the impact of phosphorylation of cTnI-Ser199 on myofilament function in human cardiac tissue and the susceptibility of cTnI to proteolysis. cTnI-Ser199 was replaced by aspartic acid (199D) or alanine (199A) to mimic phosphorylation and dephosphorylation, respectively, with recombinant wild-type (Wt) cTn as a negative control. Force development was measured at various [Ca^{2+}] and at sarcomere lengths of 1.8 and 2.2 μm in demembranated cardiomyocytes in which endogenous cTn complex was exchanged with the recombinant human cTn complexes. In idiopathic dilated cardiomyopathy samples, myofilament Ca^{2+}-sensitivity (pCa_{50}) at 2.2 μm was significantly higher in 199D ($pCa_{50} = 5.79 \pm 0.01$) compared to 199A ($pCa_{50} = 5.65 \pm 0.01$) and Wt ($pCa_{50} = 5.66 \pm 0.02$) at ~63% cTn exchange. Myofilament Ca^{2+}-sensitivity was significantly higher even with only 5.9 ± 2.5% 199D exchange compared to 199A, and saturated at 12.3 ± 2.6% 199D exchange. Ser199 pseudo-phosphorylation decreased cTnI binding to both actin and actin-tropomyosin. Moreover, altered susceptibility of cTnI to proteolysis by calpain I was found when Ser199 was pseudo-phosphorylated. Our data demonstrate that low levels of cTnI-Ser199 pseudo-phosphorylation (~6%) increase myofilament Ca^{2+}-sensitivity in human cardiomyocytes, most likely by decreasing the binding affinity of cTnI for actin-tropomyosin. In addition, cTnI-Ser199 pseudo-phosphorylation or mutation regulates calpain I mediated proteolysis of cTnI.

1. Introduction

Cardiac troponin I (cTnI) is an essential regulator of contraction and relaxation of the heart. cTnI is the "inhibitor" of the trimeric cardiac troponin (cTn) complex, which together with cardiac troponin C (cTnC, the Ca^{2+}-sensor) and cardiac troponin T (cTnT), controls the position of tropomyosin (Tm) on the thin actin filament in response to Ca^{2+} [1]. In diastole (low intracellular [Ca^{2+}]), cTnI binds actin at multiple sites maintaining Tm at the outer domain of actin, and thereby blocks myosin-binding sites and prevents force development (blocked, B-state). In systole (high intracellular [Ca^{2+}]), Ca^{2+} binds to cTnC and induces a conformational change in the cTn complex. This results in

Abbreviations: A, alanine; cMyBP-C, cardiac myosin-binding protein-C; cTn, cardiac troponin; cTnC, cardiac troponin C; cTnI, cardiac troponin I; cTnT, cardiac troponin T; D, aspartic acid; F_{max}, maximal force; F_{pas}, passive force; HF_{dys}, dyssynchronous pacing-induced heart failure; IDCM, idiopathic dilated cardiomyopathy; k_{tr}, rate of tension redevelopment; $k_{tr\text{-}max}$, maximal rate of tension redevelopment; MLC2, myosin light chain 2; pCa_{50}, $-\log_{10}$ of the calcium concentration at which 50% of maximal force is reached; nHill, steepness of the force–pCa relation; PKA, protein kinase A; PKC, protein kinase C; Ser, serine; Tm, tropomyosin; 199A, cTnI-Ser199 mutated into alanine (pseudo-dephosphorylated); 199D, cTnI-Ser199 mutated into aspartic acid (pseudo-phosphorylated).

* Corresponding author at: Department of Physiology, VU University Medical Center, Van der Boechorststraat 7, 1081BT Amsterdam, The Netherlands. Tel.: +31 20 4448110; fax: +31 20 4448255.
E-mail address: p.wijnker@vumc.nl (P.J.M. Wijnker).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.yjmcc.2015.03.006
0022-2828/

the release of cTnI from actin and a shift of Tm closer to the inner domain of actin (Ca^{2+}-activated, C-state), thereby enabling actin–myosin interactions (myosin-induced, M-state) and force development [2].

The functional properties of the cTn complex are regulated by phosphorylation of cTnI, which therefore has an important role in tuning cardiomyocyte performance. The best characterized cTnI phosphorylation sites are protein kinase A (PKA) sites Serine 23 (Ser23) and Serine 24 (Ser24). Phosphorylation of both sites by PKA, which is activated via the beta-adrenergic receptor pathway during stress and exercise, results in a decreased Ca^{2+}-sensitivity of force development and thereby improves relaxation of the heart [3–6]. In addition, PKA-mediated cTnI phosphorylation has been demonstrated to enhance the length-dependent increase in myofilament Ca^{2+}-sensitivity [7,8]. Myofilament length-dependent activation is the cellular basis of Frank–Starling's law of the heart, which describes the ability of the heart to adjust the force of its contraction (stroke volume) to changes in ventricular filling (end-diastolic volume). However, also less favorable effects of cTnI phosphorylation for cardiac performance have been reported. Protein kinase C (PKC)-mediated phosphorylation of cTnI at Ser42/44 and Thr143 has been implicated in myofilament dysfunction and cardiac disease [9,10].

Recently a new PKC phosphorylation site, Ser199, was identified in human myocardium at the C-terminus of cTnI [11]. Increased phosphorylation at Ser199 was demonstrated in human end-stage heart failure compared to non-failing donor myocardium and in a canine model of dyssynchronous pacing-induced heart failure (HF_{dys}) [10]. Ser199 is highly conserved among all the three TnI isoforms and across a wide range of species including human, dog, mouse, rat, chicken and frog, indicating a high selection pressure on its physiological function [12]. In the canine HF_{dys} model, resynchronization therapy reversed the phosphorylation status of Ser199 (in dog Ser198) [10]. Moreover, a mutation at the Ser199 position on cTnI that has been found in different families with hypertrophic cardiomyopathy has been associated with the occurrence of arrhythmias and sudden cardiac death [13]. Therefore, in this study, we examined the effects of phosphorylation of cTnI-Ser199 on human myofilament function.

Proteolysis is another post-translational modification of cTnI found in both physiological and pathological conditions [14–17]. The selective cleavage of 17 amino acid residues (a.a.) at the C-terminus of cTnI is the primary effect of cTnI proteolysis in ischemia/reperfusion injury and results in cardiac dysfunction [18–20]. While the truncation of the unique N-terminus of cTnI is present in normal human hearts and believed to be a compensatory response in microgravity [17]. The mechanism underlying the selective cleavage of cTnI is not clear. It was indicated that calpain I, a ubiquitous Ca^{2+} (1–20 μM) activated protease, is an active agent, especially in ischemia/reperfusion hearts [21–23]. Additionally, regulation of proteolysis by phosphorylation has been reported in both myofilament [24,25] and non-myofilament proteins [26,27]. Indeed, the sensitivity of cTnI to calpain I cleavage is depressed when cTnI is phosphorylated by PKA while promoted by PKC [28]. However, to date, there is no direct evidence to identify the specific phosphorylation sites on cTnI responsible for the cross-talk between its phosphorylation and proteolysis and whether the C-terminus phosphorylation sites influence proteolysis. Since Ser199 is a substrate of PKC-α and the only phosphorylation site within the C-terminal 17 a.a. fragment proteolysed during ischemia/reperfusion injury, we tested whether cTnI-Ser199 phosphorylation regulates cTnI proteolysis.

2. Materials and methods

2.1. Exchange of human cTn complex

Human recombinant cTn complex was prepared as described before [20]. Besides wild-type (Wt) cTnI, two different cTnI forms were made via site-directed mutations of Ser199. Ser199 was replaced by aspartic acid (D; 199D) to mimic phosphorylation or alanine (A; 199A) to mimic dephosphorylation (creating a non-phosphorylatable site). Exchange of these cTn complexes in human cardiomyocytes was done as described previously [5]. Briefly, single cardiomyocytes were mechanically isolated with a glass tissue homogenizer, and permeabilized by Triton X-100 (0.5%; v/v) for 5 min. They were subsequently incubated overnight at 4 °C in exchange solution (10 mM imidazole, 200 mM KCl, 5 mM $MgCl_2$, 2.5 mM EGTA, 1 mM DTT (pH 6.9)) containing recombinant human cTn complex at concentrations ranging between 0.0625 and 1.0 mg/mL with the addition of 4 mM $CaCl_2$, 4 mM DTT, 5 μl/mL protease inhibitor cocktail (Sigma, P8340) and 10 μl/mL phosphatase inhibitor cocktails 2 and 3 (Sigma, P5726 and P0044) (pH 6.9). The next day, the cardiomyocytes were washed twice in rigor solution and finally in relaxing solution (5.95 mM Na_2ATP, 6.04 mM $MgCl_2$, 2 mM EGTA, 139.6 mM KCl, 10 mM Imidazole, pH 7.0). This method results in a homogenous distribution of recombinant cTn complex within the exchanged cardiomyocyte [20]. Troponin exchange did not significantly affect maximal force development compared to control cardiomyocytes incubated overnight in exchange solution without cTn.

Exchange experiments were performed in cardiomyocytes from end-stage failing idiopathic dilated cardiomyopathy (IDCM) hearts (2 males/1 female, left ventricular ejection fraction 16.7 ± 4.4%, age 54.3 ± 1.9 years) or from non-failing donor myocardium obtained during heart transplantation surgery. The tissue was perfused with cold cardioplegic solution, transported to the laboratory and rapidly frozen and stored in small (~1 g vials) in liquid nitrogen. Samples were obtained after informed consent and with approval from the Human Research Ethics Committee of The University of Sydney (#2012/2814). The investigation conforms with the principles outlined in the Declaration of Helsinki (1997). The human cardiac samples used were extensively characterized (cardiomyocyte force characteristics and cTnI phosphorylation) in a previous study [29].

2.2. Determination of the degree of cTn exchange

To determine the degree of cTn exchange and to assess the protein phosphorylation status, part of the suspension of cells was treated with 2D-clean-up kit (GE Healthcare) as described by the manufacturer protocol after overnight cTn exchange. Subsequently, tissue pellets were homogenized in sample buffer containing 15% glycerol, 62.5 mM Tris (pH 6.8), 1% (w/v) SDS and 2% (w/v) DTT. Protein concentration measured with RCDC Protein Assay Kit II (BioRad) ranged between 2 and 4 mg/mL.

Immunoblotting was used to determine the degree of exchange of endogenous cTn by recombinant cTn complex. Recombinant cTnT was labeled with a Myc-tag to allow discrimination between endogenous and recombinant cTn complex. Proteins were separated on a one-dimensional 13% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane (Hybond) using the protocol supplied by the manufacturer in 1 h at 75 V. A specific monoclonal antibody against cTnT (Clone JLT-12, Sigma; dilution 1:1250) was used to detect endogenous and recombinant cTnT by chemiluminescence (ECL, Amersham Biosciences). We have previously demonstrated that the affinity of the cTnT antibody was the same for cTnT compared to cTnT-Myc and that cTnT loading was within the linear range [5].

2.3. Myofilament protein phosphorylation

Phosphorylation levels of sarcomeric proteins were determined before and after cTn exchange using ProQ-Diamond phospho-stained 1D-gels, as described previously [5]. The phosphorylation signals (cardiac myosin-binding protein-C, myosin light chain 2 and desmin) were normalized to the intensities of the SYPRO Ruby stained myosin light chain 2 bands to correct for small differences in protein loading. The PeppermintStick Phosphoprotein marker (Molecular Probes) was used to correct for differences in staining between gels [30]. The ratio of the intensities of ProQ-Diamond and SYPRO Ruby stained ovalbumin band was used to correct for inter-gel differences.