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Effects of myosin light chain phosphorylation on length-dependent myosin kinetics in skinned rat myocardium

Hannah C. Pulcastro, Peter O. Awinda, Jason J. Breithaupt, Bertrand C.W. Tanner*

Department of Integrative Physiology and Neuroscience, Washington State University, Pullman, WA 99164-7620, USA

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ABSTRACT

Myosin force production is Ca^{2+}-regulated by thin-filament proteins and sarcomere length, which together determine the number of cross-bridge interactions throughout a heartbeat. Ventricular myosin regulatory light chain-2 (RLC) binds to the neck of myosin and modulates contraction via its phosphorylation state. Previous studies reported regional variations in RLC phosphorylation across the left ventricle wall, suggesting that RLC phosphorylation could alter myosin behavior throughout the heart. We found that RLC phosphorylation varied across the left ventricle wall and that RLC phosphorylation was greater in the right vs. left ventricle. We also assessed functional consequences of RLC phosphorylation on Ca^{2+}-regulated contractility as sarcomere length varied in skinned rat papillary muscle strips. Increases in RLC phosphorylation and sarcomere length both led to increased Ca^{2+}-sensitivity of the force-pCa relationship, and both slowed cross-bridge detachment rate. RLC-phosphorylation slowed cross-bridge rates of MgADP release (~30%) and MgATP binding (~50%) at 1.9 μm sarcomere length, whereas RLC phosphorylation only slowed cross-bridge MgATP binding rate (~55%) at 2.2 μm sarcomere length. These findings suggest that RLC phosphorylation influences cross-bridge kinetics differently as sarcomere length varies and support the idea that RLC phosphorylation could vary throughout the heart to meet different contractile demands between the left and right ventricles.

1. Introduction

The actin-myosin cross-bridge interaction uses chemical energy from ATP hydrolysis to generate the force and shortening that is required for the heart to pump blood throughout the body. Cross-bridge formation is Ca^{2+}-regulated by the thin-filament proteins (troponin and tropomyosin) and spatially-regulated by sarcomere length, both of which influence the number of potential cross-bridge interactions that are available to generate force throughout a heartbeat (for reviews see [15,26,71]). Myosin regulatory light chain (RLC) is a 19 kD protein that binds to the neck of myosin, and post-translational phosphorylation of RLC is thought to play an important role in cardiac contraction by promoting or stabilizing the actin-myosin cross-bridge interaction to augment force production [14,49,51,60,62,63].

In the cardiac ventricle, endogenous levels of RLC phosphorylation vary from <10% to 60%, depending upon species and region of the heart being sampled [17,21,34,35,51,58]. RLC phosphorylation increases with heart rate during exercise [21] and may increase with adrenergic stimulation [5,13,57], suggesting that RLC phosphorylation contributes to cardiac inotropy as contractile demands on the heart increase. Although little is known about differences in RLC phosphorylation between the left and right ventricles, a transmural gradient of RLC has been observed in the left ventricles of rats and mice [17,34,58]. It has been suggested that this RLC phosphorylation gradient may influence regional differences in force production, wall stress, and ventricular torsion to augment cardiac function throughout a heartbeat [17,58]. In contrast, decreases in RLC phosphorylation accompany the progression of heart failure [13,19,56,58,73,76], and heritable RLC mutations associated with cardiac hypertrophy and sudden cardiac death also appear to compromise RLC phosphorylation [1,38,65,77]. These observations suggest that modulation and control of RLC phosphorylation in the heart is important for maintaining healthy cardiac function.

Cross-bridge behavior is modulated by Ca^{2+}-flux and changes in

Abbreviations: AP, Alkaline Phosphatase; MLCK, Myosin Light Chain Kinase; RLC, ventricular myosin regulatory light chain-2.

* Corresponding author. Room 255 Vet/Biomed Research Building 1815 Ferdinand's Lane, Department of Integrative Physiology and Neuroscience, Washington State University, Pullman, WA 99164-7620, USA.
E-mail address: btanner@vetmed.wsu.edu (B.C.W. Tanner).

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sarcomere length throughout a heartbeat, allowing the heart to dynamically modulate contractility on a beat-to-beat basis, while the balance of RLC phosphorylation and dephosphorylation occurs on a slower timescale (minutes to hour(s) [13,21,35]). Although Ca^{2+}-activation, sarcomere length, and RLC phosphorylation are known to influence cross-bridge formation and actin-myosin cross-bridge cycling rates, the combined influences of these factors on cardiac contractility remain largely unclear. Recent observations from our laboratory suggested that cross-bridge cycling kinetics slow with increases in sarcomere length due to reduced rate of MgADP dissociation from myosin cross-bridges [67]. Prior studies have proposed that RLC phosphorylation repositions myosin cross-bridges nearer to thin-filaments to promote cross-bridge attachment rate [14,43,60,64], and other studies have suggested that RLC phosphorylation may slow cross-bridge detachment [14,16,22,28,51,54,60]. We hypothesized that effects of RLC phosphorylation on cross-bridge cycling and Ca^{2+}-regulated tension development may vary between long and short sarcomere lengths due to physical changes in the myofilament lattice, such as thick-to-thin filament overlap, thick-to-thin filament spacing, and steric constraints on repositioning of myosin heads upon RLC phosphorylation. To test this hypothesis, we measured tension-pCa relationships and myosin cross-bridge kinetics as RLC phosphorylation varied at short and long sarcomere length in skinned papillary muscle strips from rats. We find that RLC phosphorylation increases Ca^{2+}-sensitivity of the tension-pCa relationship more greatly at long vs. short sarcomere length, and that increases in sarcomere length and RLC phosphorylation both slow cross-bridge detachment rate. Biochemical analysis of RLC phosphorylation also showed regional heterogeneities in RLC phosphorylation across the left ventricle free wall and between the right and left ventricles. Thus, RLC phosphorylation may enhance cardiac contractility more greatly at longer sarcomere lengths and could represent a mechanism to accommodate regional heterogeneities in contractile demands between the right and left ventricles.

2. Materials and methods

2.1. Animal models

All procedures were approved by the Institutional Animal Care and Use Committee at Washington State University and complied with the *Guide for the Use and Care of Laboratory Animals* published by the National Institutes of Health. Adult male Sprague–Dawley rats (17–29 weeks old) were acquired from Simonsen Laboratories (Gilroy, CA). Rats were anesthetized by isoflurane inhalation (3% volume in 95% O_2–5% CO_2 flowing at 2 L/min), following which hearts were immediately excised and placed in dissecting solution on ice.

2.2. Solutions for biochemical quantification of RLC phosphorylation

Methods and solutions were adapted from Hidalgo et al. [34], and all concentrations are listed in mM unless otherwise noted. Dissecting solution: 50 BES, 30.83 K propionate, 10 Na azide, 20 EGTA, 6.29 $MgCl_2$, 6.09 ATP, 1 DTT, 20 BDM, 50 μM Leupeptin, 275 μM Pefabloc, and 1 μM E-64. Running buffer (10X): 1.22 M Glycine, 200 Trizma base, pH 8.6. Sample preparation buffer: 8M urea, 80 Tris, 488 Glycine, 0.1% Bromophenol blue, 100 DTT, 15 μM E-64, 140 μM Leupeptin, 743 μM Pefabloc, with 50% Glycerol. Acrylamide Stock: 29.22% Acrylamide, 0.78% Bis-acrylamide. Urea glycerol stacking gel: 20 Tris-Glycine, 5% Acrylamide stock, 20% Glycerol, 0.1% TEMED, 0.03% Ammonium persulfate. Urea glycerol resolving gel: 20 Tris-Glycine, 10% Acrylamide, 40% Glycerol, 0.1% TEMED, 0.02% Ammonium persulfate. Transfer buffer: 48 Tris, 39 Glycine, 0.1% SDS, 20% Methanol. PBS-Tween: 137 NaCl, 2.7 KCl, 4.3 Na_2HPO_4, 1.4 KH_2PO_4, 0.05% Tween 20, pH 7.4.

2.3. Biochemical quantification of RLC phosphorylation throughout the ventricles

Tissue samples were taken from the sub-endocardial left ventricle free wall, sub-epicardial left ventricle free wall, transmural left ventricle free wall, transmural right ventricle wall, and septum, dissected down to about 30 mg, flash frozen in liquid nitrogen, and stored at −80 °C. Frozen tissue samples were weighed prior to homogenization. Frozen tissue was ground for 5 min using a glass homogenizer chilled periodically in liquid nitrogen. After tempering for 5 min at −20 °C, 40 μL of sample preparation buffer were added for each milligram of tissue sample. Samples were then homogenized in solution for 5 min at 60 °C. After centrifugation at 12000 rpm for 5 min the supernatant was aliquoted and stored at −80 °C for subsequent use. Phosphorylated and non-phosphorylated RLC were separated by charge using 1d isoelectric gel electrophoresis with urea-glycerol gels using 1X running buffer at 400 V, 14–16 mA, for 150 min at room temperature. Proteins were transferred to nitrocellulose membrane in transfer buffer, using a semidry blotter at 20V for 45 min. After blocking the membrane for 1 h in 5% BSA in PBS-Tween, and washing with PBS-Tween for 30 min, the membrane was incubated overnight in Myosin MAb primary antibody diluted 1:100 (ALX-BC-1150-S-L001, Enzo Life). After washing the membrane with PBS-Tween for 30 min, the membrane was incubated for 1 h with: NIF825 Peroxidase labelled anti-mouse secondary antibody (RPN2108, Amersham ELC western blotting analysis system, GE Healthcare) diluted 1:5000. Bands were detected using ELC chemiluminescent substrate solution and imaged with a BioRad ChemiDoc XRS + system. Densitometry analysis of digital western blot ImageLab images was done using ImageJ.

2.4. Solutions for skinned myocardial strips

Muscle mechanics solution concentrations were formulated by solving equations describing ionic equilibria according to Godt and Lindley [25], and all concentrations are listed in mM unless otherwise noted. Dissecting solution: same as described above for the biochemical quantification of RLC phosphorylation. Skinning solution: dissecting solution with 1% Triton-X100 wt/vol and 50% glycerol wt/vol. Storage solution: dissecting solution and 50% glycerol wt/vol. Relaxing solution: pCa 8.0 ($pCa = \log_{10}[Ca^{2+}]$), 5 EGTA, 5 MgATP, 1 Mg^{2+}, 0.3 P_i, 35 phosphocreatine, 300 U/mL creatine kinase, 200 ionic strength, pH 7.0. Activating solution: same as relaxing with pCa 4.8. Rigor solution: same as activating solution without MgATP. Alkaline phosphatase (AP) solution: same as relaxing solution with 6 U/mL recombinant AP from E-coli (P-4252). Myosin light chain kinase (MLCK) solution: was same as relaxing with pCa 6.0, 1.1 μM human skeletal MLCK, and 12 μM xenopus calmodulin.

2.5. Skinned myocardial strips

Left ventricular papillary muscles were dissected from five hearts and pared down to thin strips (~180 μm in diameter and 700 μm long). Strips were skinned overnight at 4 °C and stored at −20 °C for up to one week. Aluminum T-clips were attached to the end of each strip and strips were mounted between a piezoelectric motor (P841.40, Physik Instrumente, Auburn, MA) and a strain gauge (AE801, Kronex, Walnut Creek, CA), lowered into a 30 μL droplet of relaxing solution maintained at 17 °C, and

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