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Original Article

Protein kinase A mediated modulation of acto-myosin kinetics

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Abstract

The effects of protein kinase A (PKA) mediated phosphorylation on thin filament and cross-bridge function is not fully understood. To delineate the effects of troponin I (TnI) phosphorylation by PKA on contractile protein performance, reconstituted thin filaments were treated with PKA. With the use of the in vitro motility assay, PKA treated thin filament function was assessed relative to non-phosphorylated thin filaments in a calcium-regulated system. At maximal calcium activation, unloaded shortening velocity and force did not differ between the groups. However, at submaximal activation, an increase in calcium sensitivity of the thin filament was observed for velocity but a decrease in calcium sensitivity was observed for force. Activation of the thin filament by myosin strong-binding did not elicit a calcium-independent effect. The rightward shift in calcium sensitivity for force and the leftward shift in calcium sensitivity for velocity indicate that PKA phosphorylation of TnI directly modulates the kinetics of the myosin cross-bridge. In addition, the altered velocity dependence on thin filament length implicates reduced myosin cross-bridge binding with PKA treatment. These data highlight the importance of TnI serine 23 and 24 phosphorylation in the modulation of cardiac function.

Keywords: Thin filament regulation; Calcium sensitivity; Protein kinase A; Myocardial contractility

1. Introduction

Myocardial β-adrenergic receptor stimulation is mediated through the cyclic adenosine monophosphate (cAMP) dependent kinase, protein kinase A (PKA), ultimately resulting in enhanced ventricular performance. PKA is known to phosphorylate several key proteins involved in cardiac contractility, including phospholamban, the ryanodine receptor, the L-type calcium channel and the myofilament proteins, myosin binding protein C (MyBP-C), titin, and troponin I (TnI). A decrease in PKA activity has been implicated in the altered myofilament function found in failing human myocardium [1,2], underscoring the importance of this kinase in myocardial function.

At the myofilament level, titin phosphorylation by PKA is known to reduce passive tension, which may also affect active tension development [3]. MyBP-C is known to influence factors that govern unloaded shortening [4] and contractility [5]. Phosphorylation of MyBP-C by PKA appears to affect actin and myosin interactions [6], but has no discernable effect on the calcium sensitivity of myofibrillar ATPase [7]. PKA phosphorylation of TnI is known to decrease the calcium affinity of troponin C (TnC) [8,9], which is likely the result of altered interactions between the C-terminus of TnC and the N-terminal segment of TnI with phosphorylation of serine 23 and 24 on TnI [10]. Decreased thin filament calcium sensitivity for force and ATPase has been demonstrated with PKA mediated phosphorylation in skinned fibers [7,11]. These findings suggest that the reduced calcium sensitivity for force is the direct result of a reduced number of cross-bridges interacting with the thin filament due to the decrease in thin filament calcium affinity after PKA treatment [9]. In contrast, PKA mediated phosphorylation has been shown to accelerate relaxation, and in some studies increase slack shortening of skinned fibers and single myocytes (containing the PKA targets TnI, MyBP-C and titin) [12,13]. These β-adrenergic induced changes in relaxation and sarcomere

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shortening suggest that PKA mediated phosphorylation of the myofilament may directly affect kinetic transitions of the myosin cross-bridge [14]. However, an effect of PKA on myofiber relaxation rates [15] and cross-bridge cycling [16] has not consistently been observed, raising uncertainty as to the specific effects of PKA mediated myofibrillar phosphorylation on myosin cross-bridge kinetics in general.

In the present study, the specific effects of TnI phosphorylation by PKA on in vitro motility and force was assessed using fully calcium-regulated contractile proteins. The advantage of this in vitro system lies in the fact that isometric force and unloaded shortening velocity as a function of free calcium concentration can be determined under identical experimental conditions, and without the potentially confounding effects of PKA-mediated titin and MyBP-C phosphorylation. Through this approach, we directly investigated the effects of PKA phosphorylation of TnI on the mechanical parameters of acto-myosin function.

2. Materials and methods

2.1. Contractile protein isolation

Chicken pectoralis myosin was purified and stored in high salt buffer (600 mM KCl, 20 mM imidazole pH 6.8, 2 mM dithiothreitol (DTT) and 50% glycerol) at –20 °C [17]. To assess thin filament function, calcium-regulated reconstituted thin filaments were used. Skeletal actin was purified by standard techniques with further purification obtained through gel filtration chromatography [17]. Beef cardiac troponin and tropomyosin were isolated by the methods of Potter [18]. Final purification of tropomyosin was achieved through the use of hydroxyapatite chromatography. Actin, troponin and tropomyosin were reconstituted in low salt buffer (25 mM KCl, 25 mM imidazole, 5 mM $MgCl_2$, 10 mM DTT and 2 mM EGTA, pH 7.4) as previously reported [19]. Reconstituted thin filaments were labeled with rhodamine-phalloidin at a 1:1 actin/phalloidin ratio prior to use in the motility assay.

2.2. Protein kinase A (PKA) treatment

Reconstituted thin filaments containing predominantly dephosphorylated troponin [20] were treated with PKA per the method of Karczewski et al. [21] with slight modification. Specifically, reconstituted thin filaments (2.0 μM actin, 0.5 μM troponin, and 0.5 μmol/l tropomyosin) were treated with the catalytic subunit of PKA (Sigma Ltd.) in PKA treatment buffer (50 mM MOPS, 20 mM NaF, 1 mM DTT, 10 mM $MgCl_2$, 200 μmol/l ATP, 750 U/ml PKA) at 30 °C for 20 min and then on ice overnight prior to use in the motility assay. Control thin filaments were handled similarly with the exception of PKA. To quantify the in vitro phosphorylation of TnI by PKA, the PKA treatment protocol was repeated using ATP-γP^{32} as previously reported [20]. Specifically, treated thin filaments were run on SDS-PAGE (12.5%). TnI was excised from the gels after Coomassie blue staining. Incorporated radioactivity was quantified with a Tri-Carb (Packard Instruments) liquid scintillation analyzer.

2.3. In vitro motility assay

The in vitro motility assay has been previously described in detail [22]. In brief, myosin (100 μg/ml in loading buffer (300 mM KCl, 25 mM imidazole, 5 mM $MgCl_2$, 10 mM DTT and 2 mM EGTA, pH 7.4)) was plated onto a nitrocellulose-coated glass coverslip. Fluorescently labeled thin filaments moved across the myosin surface in the presence of ATP at 30 °C. To probe thin filament regulation, free calcium was varied in the motility solutions (*p*Ca 10–4.0). Motility was observed with epifluorescence microscopy, recorded on videotape, and subsequently analyzed using the Motion Analysis System VP110 (Motion Analysis Corporation). Typically >400 individual filament velocities were averaged to determine the mean velocity for each *p*Ca value. PKA treatment and *p*Ca–velocity experiments were conducted three times with the means fit to the Hill equation.

2.4. Force measurements

Relative isometric force was determined by loading actin filaments with the actin binding protein, α-actinin. This experimental approach has been previously described in detail [17,23]. As α-actinin avidly binds to actin, the motion of the thin filament is impeded by α-actinin that is adhered to the motility surface. By repeat experimentation, the amount of α-actinin on the motility surface was successively increased until thin filament motility was completely arrested. Relative isometric force was defined as the minimum amount of α-actinin needed to completely impede thin filament movement. Therefore, to determine force at a single *p*Ca value a minimum of five force assay experiments were conducted. Force was determined at eight different free calcium concentrations. PKA treatment and *p*Ca–force experiments were repeated two times, with the means fit to the Hill equation. For these experiments, α-actinin (Sigma Ltd.) was dialyzed into low salt buffer prior to use. Force was normalized to maximal force for the control thin filaments.

2.5. Length dependence of thin filament velocity

In order to determine the relative effect of PKA phosphorylation of TnI on cross-bridge binding to the thin filament; thin filament velocity as a function thin filament length was determined. It has been previously demonstrated at low surface myosin concentrations that thin filament velocity is dependent on the length of the thin filament (i.e. the number of cross-bridges interacting with the thin filament) [22,24]. These experiments were conducted at near half-maximal calcium activation (*p*Ca 6.25) with a loading buffer myosin concentration of 37.5 μg/ml. Myosin binding to the motility