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# Effects of cardiac myosin binding protein-C on the regulation of interaction of cardiac myosin with thin filament in an *in vitro* motility assay

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ABSTRACT

Modulatory role of whole cardiac myosin binding protein-C (cMyBP-C) in regulation of cardiac muscle contractility was studied in the *in vitro* motility assay with rabbit cardiac myosin as a motor protein. The effects of cMyBP-C on the interaction of cardiac myosin with regulated thin filament were tested in both *in vitro* motility and ATPase assays. We demonstrate that the addition of cMyBP-C increases calcium regulated Mg-ATPase activity of cardiac myosin at submaximal calcium. The Hill coefficient for '*p*Ca–velocity' relation in the *in vitro* motility assay decreased and the calcium sensitivity increased when cMyBP-C was added. Results of our experiments testifies in favor of the hypothesis that cMyBP-C slows down cross-bridge kinetics when binding to actin.



## 1. Introduction

According to recent studies about a half of familial hypertrophic cardiomyopathies (FHC) occur due to mutations in genes encoding cardiac myosin binding protein-C (cMyBP-C) [1]. This means cMyBP-C may play an important role in the maintenance of myocardium contractility. Numerous data report cMyBP-C contribution not only to the thick filament structure but also to the regulation of the actomyosin interaction. However, specific mechanism by which cMyBP-C may affect the regulation is still unclear.

cMyBP-C is a large (140–150 kDa) thick filament-associated protein. It represents a single polypeptide chain of 40 nm long and consists of 11 domains (8 immunoglobulin I-like and three fibronectin 3-like) called C0–C10 from N- to C-terminus. The C-terminus (domains C7–C10) binds rod part of myosin molecule and titin [2,3], the N-terminus (domains C1–C2) binds myosin subfragment 2 [4,5]. There are indications that cMyBP-C binds actin by Pro–Ala rich region between C0 and C1 domains and by C0 domain [6,7]. A phosphorylable motif between C1 and C2 domains consisting of 100 amino acid residues has 3 and 4 sites of phosphorylation which play a role in regulation of contraction [8].

Whole heart studies have shown that mouse models with cMyBP-C knockout resulted in a dilated cardiomyopathy [9] which is accompanied by a reduced ejection and maximal left ventricle end-systolic elastance despite preserved maximal rate of pressure rise [9].

Studies on muscle fibers of wild type animals [10,11] and data obtained from cMyBP-C knockout mice [12] have shown the modulatory role of cMyBP-C in muscle regulation. It has been found in muscle fiber experiments that cMyBP-C changed calcium sensitivity and the Hill coefficient of '*p*Ca-force' curves [10,12–14].

A few biochemical studies demonstrated that cMyBP-C enhanced actin-activated ATPase activity of cardiac myosin in a concentration-dependent manner [15,16]. cMyBP-C affected actin-activated ATPase activity of cardiac myosin, notably it increased the maximal hydrolysis rate ($V_{max}$) and decreases ATPase $K_m$ [15,16]. At the same time cMyBP-C did not affect myosin ATPase activity in the absence of actin as well as the activity of myosin filaments [15]. Recently, Shaffer et al. reported that addition of N-terminal peptides of cMyBP-C resulted in stimulating effect on calcium regulated $Mg^{2+}$-ATPase activity of skeletal myosin at submaximal calcium [17].

Recent studies in the *in vitro* motility assay have shown that the addition of either whole cMyBP-C or its N-terminal domain fragment resulted in an increase of calcium sensitivity of interaction of skeletal myosin with thin filament [18,19]. At high calcium levels the addition of any of these proteins depressed sliding velocity of regulated thin filament for myosin and its proteolytic subfragments (*ibid*). It should be mentioned that latter studies were done with skeletal myosin and therefore need to be confirmed with cardiac one.

There is only one published work where cardiac MyBP-C was used with cardiac myosin in the *in vitro* motility assay [20]. The results of that work substantially differ of those obtained with skeletal myosin. Notably both the sliding velocity of actin filaments and the relative isometric cross-bridge force in the *in vitro* motility assay were lower in the absence of cMyBP-C than that in its presence.

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However, effect(s) of cMyBP-C on the regulation of contraction was not examined in that study.

Unlike all prior studies, the purpose of the present work was to assess the modulatory role of whole cMyBP-C in the regulation of cardiac muscle. The data clarify some regulatory mechanisms of myocardium contractility.

## 2. Materials and methods

### 2.1. Solution

AB buffer was composed of 25 mM KCl, 25 mM imidazole, 4 mM $MgCl_2$, 1 mM EGTA, and 10 mM DTT, pH 7.5.

### 2.2. Preparation of proteins

Actin was obtained from rabbit skeletal muscle according to standard procedure [21]. Cardiac troponin and tropomyosin were isolated from left ventricle of bovine heart as described [22,23]. Myosin from left ventricles of rabbit hearts was obtained according to standard technique [24] and stored in 50% glycerol at −20 °C. On the day of experiment ATP-insensitive myosin molecules were removed by ultracentrifugation [25]. Usage of contractile and regulatory proteins extracted from various species to combine in the motility assay is that of common practice [17–19,25,40].

cMyBP-C was purified from frozen chicken hearts according to Hartzell and Glass [26] with the following modification. After extraction the protein was precipitated by addition of $(NH_4)_2SO_4$ to a final saturation of 55%, then dissolved in a buffer consisting of 70 mM KC1, 10 mM MES, 2 mM $NaN_3$, 0.1 mM EDTA, 3 mM 2-mercaptoethanol, pH 6.45 and finally dialyzed overnight against this buffer. The dialyzed crude cMyBP-C was purified on a 5-ml Hi-Trap Q HP column using AKTA basic 10 FPLC (Amersham Biosciences) with NaCl gradient from 70 to 300 mM NaC1 in buffer containing 10 mM MES, 2 mM $NaN_3$, 0.1 mM EDTA, 3 mM 2-mercaptoethanol, pH 6.45. The major protein peak eluting between 90–130 mM NaCl was concentrated by addition of $(NH_4)_2SO_4$ to a final saturation of 55% and following centrifugation at 15,000*g* for 15 min. The precipitated protein was dissolved in AB buffer containing 80 mM KCl with dialysis overnight. Purity of all proteins was determined by 10% SDS–PAAG [27].

### 2.3. Reconstitution of the thin filaments

The thin filaments were reconstructed from actin, troponin, and tropomyosin by mixing these proteins in the following concentrations: 400 nM rhodamine-phalloidine labeled F-actin, 100 nM troponin and 100 nM tropomyosin at 4 °C in AB buffer. Protein ratio in the thin filaments was checked by 10% SDS-PAAG [27].

### 2.4. Preparation of myosin filaments

Myosin filaments were obtained by overnight dialysis against AB buffer with 100 mM KCl [28]. Myosin filaments and cMyBP-C were mixed and preincubated for 5 min at 4 °C just before ATPase measurements.

### 2.5. $Ca^{2+}$-regulated $Mg^{2+}$-ATPase measurements

The $Ca^{2+}$-regulated $Mg^{2+}$-ATPase activity of myosin filaments with or without cMyBP-C at 28 °C was determined by $P_i$ release [29]. The $Ca^{2+}$-regulated $Mg^{2+}$-ATPase activity was measured in AB buffer containing 0.05 μM myosin heads, 10 μM thin filaments (by actin), 1 mM ATP and appropriate Ca–EGTA ratio. The ATPase activity experiments were carried out five times.

### 2.6. In vitro motility assay

The *in vitro* motility assay was performed as described previously [30]. In brief, a 50-μl flow cell was constructed with nitrocellulose surface. First 50 μl of myosin in concentration 300 μg/ml in AB buffer containing 0.5 M KCl was loaded in the flow cell. After 2 min the flow cell was rinsed first with a high and then with a low ionic strength AB buffer to remove unbound myosin. Then 50 μl of 0.5 mg/ml bovine serum albumin (BSA) in AB buffer was added for 60 s to block any exposed nitrocellulose surface. Further 50 μg/ml of non-labeled F-actin in AB buffer with 2 mM ATP was added and incubated for 5 min to block nonfunctional myosin heads. The flow cell was rinsed three times with AB buffer. Then 50 μl of 10 nM rhodamine-phalloidine labeled F-actin (or thin filaments) in AB buffer was added for 5 min. When regulated thin filaments were used, AB buffer contained 100 nM troponin and 100 nM tropomyosin to prevent dissociation of regulatory proteins. Unbound actin was washed out with AB buffer. Finally the cell was washed with AB buffer containing 0.5 mg/ml BSA, 3.5 mg/ml glucose, 0.02 mg/ml catalase, 0.15 mg/ml glucose oxidase, 20 mM DTT, 2 mM ATP and 0.5% methylcellulose. For the regulated thin filaments final solution contained additionally 100 nM troponin, 100 nM tropomyosin and free calcium. Required concentration of free calcium was obtained by appropriate Ca–EGTA ratio [30].

The cMyBP-C was added together with myosin. Used concentration of cMyBP-C was expressed as molar ratio to myosin. All experiments were performed at 28 °C.

Fluorescent labeled actin filaments were visualized by Axiovert 200 inverted epifluorescence microscope equipped with 100×/1.45 Oil alpha Plan-Fluar objective (Carl Zeiss) and an EMCCD iXon-897BV camera (Andor Technology). Typically 10 fields by 30 s each were recorded in every flow cell.

### 2.7. Recording the 'pCa–velocity' relationship

To obtain the '*p*Ca–velocity' relationship the sliding velocities of the thin filaments on the myosin- or myosin with cMyBP-C-coated surface for various *p*Ca (4–8) were measured. Data was analysed using GMimPro software [31]. Typically velocities of >100 individual filament were averaged to determine the mean and SD for each *p*Ca. The '*p*Ca–velocity' experiments were carried out three times and the means of individual experiments were fitted to the Hill equation:

$$v = v_{max}(1 + 10^{n(pCa - pCa_{50})})^{-1},$$

where v and $v_{max}$ are velocity and maximal velocity obtained at saturating calcium concentration, respectively, $pCa_{50}$ is *p*Ca at which half maximal velocity is achieved (*i.e.* calcium sensitivity), and *n* is the Hill coefficient.

### 2.8. Statistical analysis

All values are expressed as mean ± SD. All comparisons were performed by paired *t*-test or Mann Whitey at a 0.05 level of significance ($p < 0.05$).

## 3. Results

### 3.1. Effect of cMyBP-C on the motility of actin filaments and thin filaments over cardiac myosin

Fig. 1A shows effect of two concentrations of cMyBP-C (1:5 and 1:2 molar ratio of cMyBP-C/myosin) on the sliding velocity of actin filaments over cardiac myosin in the motility assay. Particularly

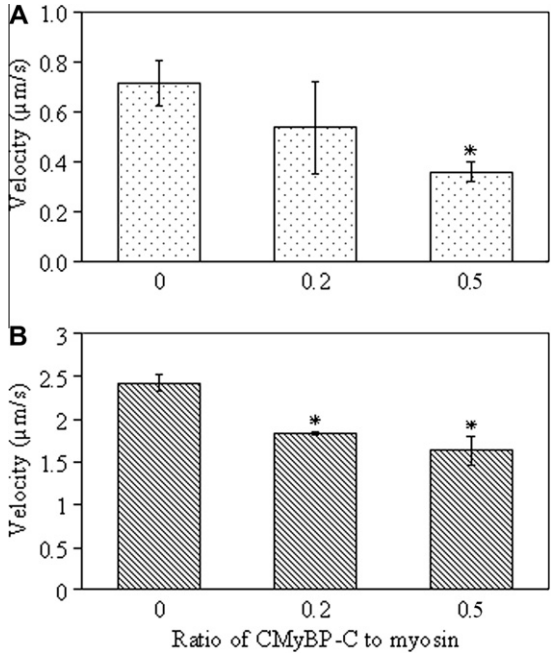


**Fig. 1.** Dependence of sliding velocities of F-actin (A) and the thin filaments at *p*Ca 4 (B) on cMyBP-C concentration expressed as molar ratio to concentration of cardiac myosin. The columns and error bars are mean ± SD. Asterisks indicate significant difference from control (without cMyBP-C), $p < 0.05$.

cMyBP-C in proportions 1:5 reduced the velocities of actin filaments from 0.7 ± 0.1 to 0.5 ± 0.1 μm/s (~29% decrease). At higher cMyBP-C concentration the velocity of F-actin decreased by ~60%.

Under $Ca^{2+}$-regulated assay conditions addition of cMyBP-C into the flow cell at the same concentrations also slowed down sliding velocity of the thin filaments at maximal calcium (*p*Ca 4; Fig. 1B). cMyBP-C in proportion 1:5 to myosin decreased the velocity by 25%, and by 33% in proportion 1:2.

Our data correspond well to recent studies showing that both whole cMyBP-C and N-terminal domain fragments reduced actin filament velocity in the motility assay in a concentration-dependent manner for whole skeletal myosin and its proteolytic fragments [18,19].

### 3.2. Effect of cMyBP-C on the 'pCa–velocity' relation for cardiac myosin

A series of experiments was performed to obtain the dependence of movement velocity of thin filaments on calcium concentration (*p*Ca 5–*p*Ca 8) for cardiac myosin both with and without cMyBP-C. The Hill cooperativity coefficient and calcium sensitivity taken as $pCa_{50}$ were estimated from the '*p*Ca–velocity' relations.

Addition of cMyBP-C at about physiological proportion (1:5 molar ratio of cMyBP-C/myosin) to the motility assay led to a decrease in the sliding velocity of thin filaments at high calcium levels (Fig. 2). The velocity at saturating calcium (*p*Ca 5) was 1.7 ± 0.1 μm/s (+cMyBP-C) *vs.* 2.1 ± 0.2 μm/s (−cMyBP-C). At *p*Ca 7 all filaments were immobile in the absence of cMyBP-C. However, the addition of cMyBP-C led to their movement (0.7 ± 0.1 μm/s). Besides the presence of cMyBP-C shifted $pCa_{50}$ from 6.3 ± 0.01 to 6.7 ± 0.2. The Hill cooperativity coefficient of the '*p*Ca–velocity' curve was found to be most sensitive to cMyBP-C: 3.7 ± 0.5 (−cMyBP-C) *vs.* 0.7 ± 0.1 (+cMyBP-C).

Razumova et al. [19] observed similar change in the shape of the '*p*Ca–velocity' curve for skeletal myosin using N-terminal domains of cMyBP-C. Besides, addition of whole cMyBP-C at 1:2 molar ratio of cMyBP-C/myosin resulted in 25% slowing down of the sliding

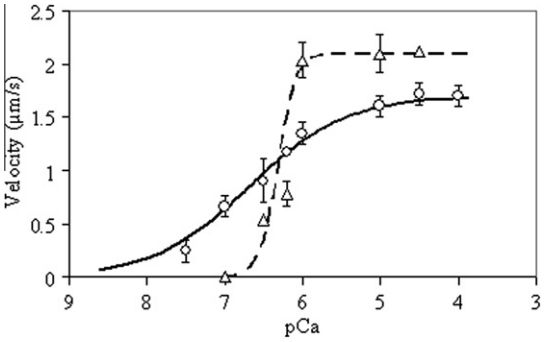


**Fig. 2.** The '*p*Ca–velocity' relationships for regulated thin filaments in the presence (circles, solid line) and the absence (triangles, dashed line) of cMyBP-C. Myosin concentration was 300 μg/ml (0.65 μM), concentration of cMyBP-C was 20 μg/ml (0.13 μM). The data were fit with the Hill equation (see text for detail). Each data points represent mean ± SD of three experiments.

velocity of thin filaments over skeletal myosin at maximal calcium and accelerated the motion at submaximal calcium [18]. However, the presence of cMyBP-C did not affect the cooperativity of thin filament activation for velocity (*ibid*).

### 3.3. Effect of cMyBP-C on $Ca^{2+}$-regulated $Mg^{2+}$-ATPase activity of cardiac myosin

Effect of different concentrations of cMyBP-C on $Ca^{2+}$-regulated $Mg^{2+}$-ATPase activity of cardiac myosin filaments at maximal (*p*Ca 4) and submaximal (*p*Ca 7) calcium is shown in Fig. 3. Addition of cMyBP-C at maximal calcium did not affect appreciably the ATPase at increased cMyBP-C concentration (Fig. 3A).

On the contrary at submaximal calcium cMyBP-C increased ATPase activity of cardiac myosin in 2.5-fold (Fig. 3B). This agrees with the data of Shaffer and colleagues who observed an increase

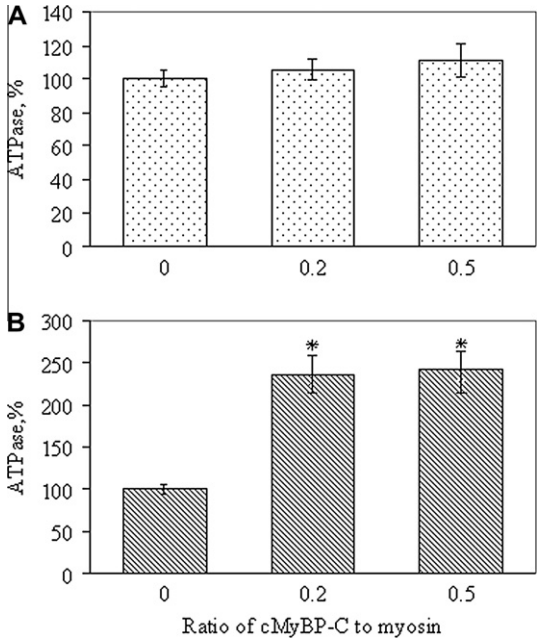


**Fig. 3.** Dependence of $Ca^{2+}$-regulated ATPase of myosin filaments on cMyBP-C at saturated (A) *p*Ca 4) and subsaturated (B) *p*Ca 7) calcium concentration. The final assay mixture contained 0.05 μM myosin filaments (by myosin), cMyBP-C from 0.01 to 0.1 μM, thin filament consisted of 10 μM actin, 2 μM tropomyosin, and 2 μM troponin. The abscissa is a molar ratio of cMyBP-C concentration to myosin. The columns and error bars are mean ± SD. Asterisks indicate significant difference from control (without cMyBP-C), $p < 0.05$.

in the ATPase activity of skeletal myosin S1 with addition of N-terminal peptides of cMyBP-C at submaximal calcium level [17].

## 4. Discussion

Lecarpentier et al. [20] studied the effects of cardiac MyBP-C on interaction of cardiac myosin with actin filaments using the *in vitro* motility assay. They found that both the sliding velocity of actin filaments and relative isometric cross-bridge force were lower in the absence of cMyBP-C than in its presence. These results obtained in the absence of calcium with non-regulated filaments and cardiac myosin are quite different from those obtained with myosin from skeletal muscle [18,19]. This implies that the data with regulated thin filament collected from cardiac myosin might also differ from those from skeletal myosin. It is known that functional characteristics of cardiac myosin are different of those of skeletal one. For these reasons it seems important to use just cardiac myosin for studying the effect(s) of cMyBP-C on regulation of contractility of heart muscle.

The aim of the present study was to assess the modulatory role of cMyBP-C in the regulation of contractility of cardiac muscle. The *in vitro* motility assay with cardiac myosin in the capacity of a motor protein was used as a model for studying regulation of muscle proteins interaction. The effects of cMyBP-C on the movement of either actin filament or regulated thin filament over the surface coated by cardiac myosin as well as on the ATP hydrolysis were studied.

Despite a number of studies aimed to disclose the mechanism by which cMyBP-C affects the interaction of contractile proteins in sarcomere [10,18] it is still unsolved. Results of our work are supporting the idea that cMyBP-C slows down cross-bridge kinetics when binding to actin.

Our results (data not shown here) as well as the data published by both Hartzell [15] and Margossian [16] indicated that cMyBP-C activated actomyosin ATPase. At the same time cMyBP-C inhibited movement of actin filaments over myosin in the motility assay (Fig. 1A). These effects can be explained if we assume that cMyBP-C linking myosin subfragment 2 with actin facilitates interaction of myosin S1 with actin but at the same time works as a brake. Besides cMyBP-C slightly stimulated ATPase of myosin filaments at saturating calcium concentration ($p$Ca5; Fig. 3A) and decelerated movement of regulated thin filaments by 30% at high proportion (1:2) with respect to myosin (Fig. 1B). This effect can be explained by activation of myosin ATPase activity by troponin–tropomyosin complex itself in the presents of calcium [32]. Activation by cMyBP-C in this case is inessential but it still hamper the movement.

Depending on the $Ca^{2+}$ concentration cMyBP-C exerted opposite effects on the movement of the regulated thin filaments (Fig. 2). In particular addition of cMyBP-C decreased sliding velocity of the filaments at maximal calcium but increased the velocity at submaximal calcium. As a result the Hill cooperativity coefficient for the '$p$Ca–velocity' relations decreased and calcium sensitivity estimated as $pCa_{50}$ increased when cMyBP-C was added with respect to its absence. Changes in the movement velocities were supported by ATPase measurements. Specifically, addition of cMyBP-C in near physiological proportions to myosin (1:5 and 1:2 molar ratio) at submaximal calcium significantly increased ATPase activity of cardiac myosin but the presence of cMyBP-C did not change the ATPase activity at maximal calcium. At a non-saturating calcium concentration cMyBP-C stimulated myosin ATPase activity and the movement of regulated thin filaments.

This effect can be explained assuming that myosin heads spending more time in the attached to actin state are able to increase an allosteric, or cooperative, cross-bridge binding along the thin filament. Such cooperative effects occurs as follows: (1) a myosin head attached to an $A_7$TnTm (actin–troponin–tropomyosin) regulatory unit can shift tropomyosin molecule [33] in this and neighbor regulatory unit(s) and thus promotes attachment of the heads in the units along the thin filament; (2) strong binding of cross-bridges in one $A_7$TmTn unit increases the $Ca^{2+}$ binding in that unit or in the nearest neighbor units [34,35]. The last mechanism was shown to play a key role in the regulation of contractility in intact myocardium [35–37]. We suggest that probably the same cooperative mechanisms underlie the activating effect of cMyBP-C on the thin filament movement velocity that we observed in the motility assay at non-saturating calcium. In fact the increase in the duration of the cross-bridge strong binding state leads to a rise of the total cross-bridge concentration and thus increases troponin C affinity to calcium according to the above mechanism of cooperativity. In other words the same calcium concentration activates the thin filaments greater when cMyBP-C is added.

Our interpretation of the obtained results is confirmed by recent studies which showed that Pro–Ala sequence of cMyBP-C that links C0 and C1 domains is identical to that of myosin essential light chains (ELC) [6]. The Pro/Ala sequence of ELC modulates actomyosin interaction. Namely it either slows down cross-bridge detachment kinetics when binding to actin [38] or functioning as a tether [39]. It is possible that Pro/Ala sequence of cMyBP-C plays a role similar to that of ELC and decelerates cross-bridge detachment kinetics [11,17] and thus cMyBP-C slows down overall cross-bridge cycle [14].

Difference in the motility assay data obtained by Lecarpentier et al. [20] from others can be explained by species-specificity of amino acid sequence of cardiac myosins. The sequences of such essential elements of myosin S1 structure as ATPase pocket and actin binding site are different in different animals that affects functioning of these proteins [40].

According to our data from cardiac myosin, cMyBP-C modulates interaction of myosin with actin. Its role in regulation of interaction of myosin with $Ca^{2+}$-regulated thin filament is even more important although detailed mechanism of the cMyBP-C regulatory effect is still not clear.

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