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Study of reciprocal effects of cardiac myosin and tropomyosin isoforms on actin–myosin interaction with *in vitro* motility assay

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ABSTRACT

Interaction of myosin with actin in striated muscle is controlled by Ca^{2+} via thin filament associated proteins: troponin and tropomyosin. In cardiac muscle there is a whole pattern of myosin and tropomyosin isoforms. The aim of the current work is to study regulatory effect of tropomyosin on sliding velocity of actin filaments in the *in vitro* motility assay over cardiac isomyosins. It was found that tropomyosins of different content of α- and β-chains being added to actin filament effects the sliding velocity of filaments in different ways. On the other hand the velocity of filaments with the same tropomyosins depends on both heavy and light chains isoforms of cardiac myosin.

1. Introduction

Tropomyosin is one of regulatory proteins of the thin filament of striated muscle. In mammals genes TPM1, TPM2 and TPM3 encode corresponding isoforms of α-, β- and γ-chains of tropomyosin. α- and β-tropomyosins are in the heart and fast skeletal muscles while γ-tropomyosin is presented only in slow skeletal muscle fibers [1]. All isoforms contain 284 aminoacid residues, α-tropomyosin is 87% identical to the β-tropomyosin. Individually both α- tropomyosin and β-tropomyosin sequences are highly conserved in human and all other studied mammalian species [2].

Expression of tropomyosin isoforms in the heart depends upon species and age of the animal [1,3]. α-Tropomyosin prevails in adult murine, rabbit and human hearts. β-Tropomyosin is mainly expressed during fetal development in murine hearts. Changes in the expression of these isoforms are also associated with cardiac pathologies.

In particular, studies by Izumo et al. demonstrated that an increase in the expression of β-tropomyosin occured during pressure-overloaded hypertrophy in the adult rat heart [4]. The experiments with overexpression of β-tropomyosin in the adult murine hearts showed that the increase of β-chain up to 50–60% increased calcium sensitivity of "pCa-force" relationship, decreased the maximum rate of relaxation and resulted in diastolic dysfunction [5]. At the same time no changes in the heart beat rate or maximal systolic and diastolic pressure of the left ventricle were observed (*ibidem*). Further increase of β-tropomyosin expression up to 75–80% led to animal death shortly after birth [3].

In myocardium of mammals not only tropomyosin isoform ratio but also that of myosin heavy chains (MHC) changes during ontogenesis. In myocardium of mammals there are two isoforms of heavy chains: α and β [6]. In ventricle together with ventricular isoform of light chains they form two main isomyosins: V1 and V3, homodimers consisting of α- and β-heavy chains, respectively [6]. In atria α- and β-heavy chains together with atrial light chains form A1 (αα) and A2 (ββ) isomyosins [7].

The identity of amino acid sequence between α- and β-MHC is 93% [8], with nonidentical residues located in functionally important domains associated with actin binding and ATPase activity [9].

It is known that isoforms of cardiac myosin, both ventricular and atrial, affect mechanical characteristics of acto–myosin complex. In particular, it was shown that sliding velocity of both F-actin [10–12] and the thin filament [13] in the *in vitro* motility assay depends on the ratio of isomyosins. Force developed by cross-bridges of cardiac isomyosins is also different [12,14].

On the other hand, tropomyosin participates in control of actin–myosin interaction. According to published data tropomyosin directly affects mechanical characteristics of acto–myosin complex [15] and this influence depends on the ratio of tropomyosin isoforms [16].

The aim of this work is to investigate the regulatory effect of tropomyosin on actin–myosin interaction. For this we studied the dependence of the sliding velocity of actin and actin–tropomyosin filaments in the *in vitro* motility assay on myosin and tropomyosin isoforms.

Abbreviations: MHC, myosin heavy chains; ELC, essential light chains; RLC, regulatory light chains; Tm, tropomyosin; FHC, familial hypertrophic cardiomyopathies.

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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. Obtaining of hyper- and hypothyroid rabbits

Shift to predominate V1 or V3 cardiac myosin isoform expression was produced in hyper- and hypothyroid rabbits. Hyperthyroidism was induced by intramuscular injections of L-thyroxine (0.2 mg/kg) in 2-month-old rabbits during two weeks. Animals were rendered hypothyroid with propylthiouracyl-supplemented drinking water (0.8 mg/ml) for three weeks [10]. Then atria and left cardiac ventricles were isolated and rapidly frozen in liquid nitrogen. Samples were kept at −86 °C.

2.3. Preparation of proteins

Actin is very conservative protein [17]. Therefore the skeletal actin isoform is used traditionally in the motility assay with cardiac isomyosins [10,12,13]. In our experiments actin was obtained from rabbit skeletal muscle according to standard procedure [18]. Actin filaments were polymerized and labeled with TRITC phalloidine (molar ratio 1:2).

To obtain different ratio of α- and β-tropomyosin we used cardiac tropomyosin from left ventricle of bovine or rabbit and skeletal tropomyosin from rabbit *psoas* muscle. The tropomyosins were isolated as described [19]. Tropomyosin was stored frozen at −86 °C. Myosin from atria and left ventricles of rabbit hearts was obtained according to standard technique [20] and stored in 50% glycerol at −20 °C. Before experiment ATP-insensitive myosin molecules were removed by ultracentrifugation [21]. Usage of contractile and regulatory proteins extracted from various species to combine in the motility assay is that of common practice [10,12,13,21].

2.4. Gel electrophoresis

Determination of α- and β-chains content of tropomyosin was performed using 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with 4 M urea [22]. Gels were stained with Coomassie Brilliant Blue R 250. Heavy chains composition of cardiac myosin was determined using SDS–PAGE with silver staining [23]. Content of light chains of atrial and ventricular myosins was analyzed by SDS–PAGE [24]. Gels were scanned with a Bio-Rad densitometer and percent ratio of α- and β-heavy chains myosin and α- and β-chains of tropomyosins was determined.

Tropomyosin extracted from rabbit myocardium is a α-chain homodimer (Fig. 1A). Bovine ventricular tropomyosin contains 10% β-chain while tropomyosin from rabbit *psoas* muscle has 40% β-chain. Myosin from atria and left ventricles of hyperthyroid rabbits predominantly contains α-heavy chain (Fig. 1B). Myosin from left ventricles of hypothyroid rabbit predominantly contains β-heavy chain (Fig. 1B). Atrial and ventricular myosins contain different essential light chains (ELC) and regulatory light chains (RLC) isoforms (Fig. 1C).

2.5. Reconstitution of the actin–tropomyosin filaments

The actin–tropomyosin filaments were reconstructed from actin and tropomyosin by mixing these proteins in the following concentrations: 400 nM rhodamine phalloidine labeled F-actin and 100 nM tropomyosin at 4 °C in AB buffer.

2.6. In vitro motility assay

The *in vitro* motility assay was performed as described previously [25]. In brief, 50 μl of myosin in concentration 300 μg/ml in AB buffer containing 0.5 M KCl was loaded in the flow cell with nitrocellulose surface. To avoid the effect of myosin concentration on mobility of actin–tropomyosin filament we used saturating myosin concentration on the surface of flow cell [15]. After 2 min the flow cell was rinsed first with AB buffer containing 0.5 M KCl to remove unbound myosin and then with AB buffer. Then 50 μl

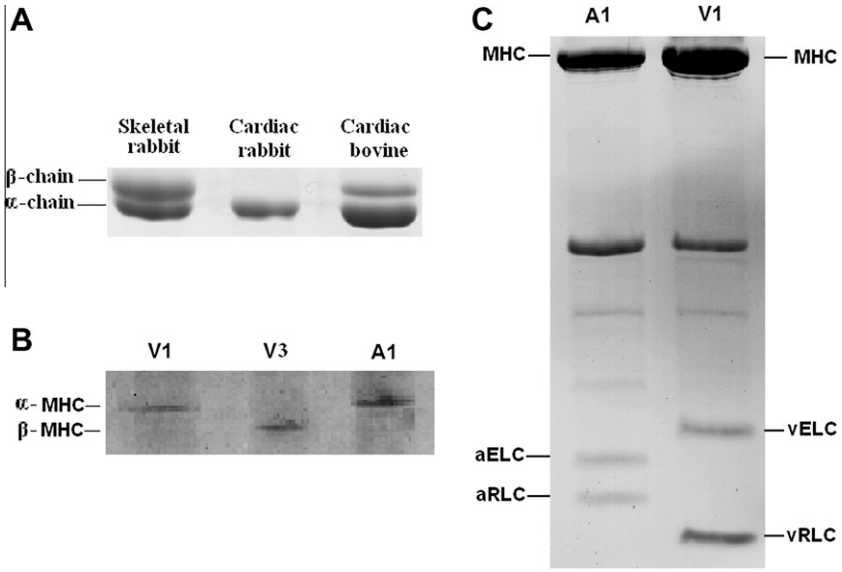

Fig. 1. (A) Gel electrophoresis of tropomyosins from left ventricle of bovine (*cardiac bovine*), left ventricle of rabbit (*cardiac rabbit*) and *psoas* rabbit muscle (*skeletal rabbit*). (B) Silver-stained gel electrophoresis of myosin heavy chains (MHC) of atrial (A1) and ventricular myosin from hyper- (V1) and hypothyroid (V3) rabbit. (C) Gel electrophoresis of atrial (A1) and ventricular (V1) myosin: MHC, myosin heavy chain; aELC, atrial essential light chain; aRLC, atrial regulatory light chain; vELC, ventricular essential light chain; vRLC, ventricular regulatory light chain.

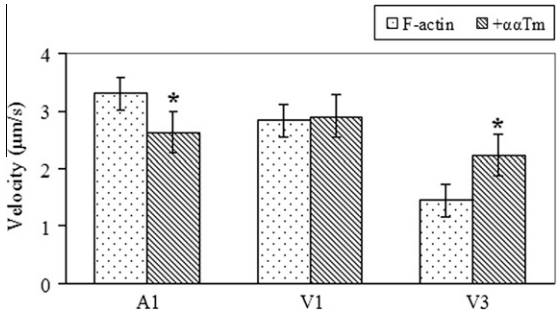

Fig. 2. The sliding velocity of actin and actin-Tm filaments consisting of cardiac rabbit ααTm over cardiac myosin isoforms in the *in vitro* motility assay. The columns and error bars are mean ± S.D. Asterisks indicate significant difference, $p < 0.05$.

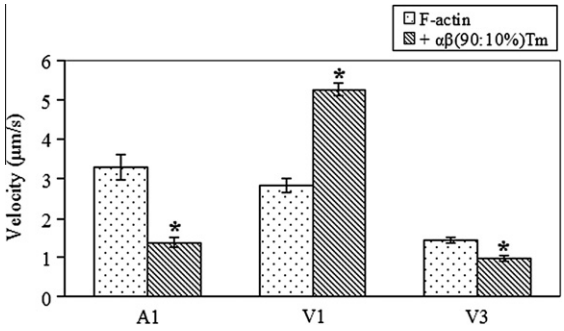

Fig. 3. The sliding velocity of actin and actin-Tm filaments containing cardiac bovine αβ (90:10%) Tm over cardiac myosin isoforms in the *in vitro* motility assay. The columns and error bars are mean ± S.D. Asterisks indicate significant difference, $p < 0.05$.

of 0.5 mg/ml bovine serum albumin (BSA) in AB buffer was added for 60 s. 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 actin – tropomyosin filaments) in AB buffer was added for 5 min. When actin–tropomyosin filaments were used, AB buffer contained 100 nM tropomyosin to prevent dissociation of tropomyosin. 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.

Fluorescent labeled actin filaments were visualized by Axiovert 200 inverted epifluorescence microscope equipped with 100x/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. Data were analyzed using GMimPro software [26]. Typically velocities of >100 individual filaments were averaged to determine the mean and SD of velocity.

2.7. Statistical analysis

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

3. Results

Fig. 2 shows the sliding velocity of both actin and actin-Tm filaments over different cardiac isoforms in the *in vitro* motility assay. Addition of cardiac rabbit tropomyosin (ααTm) to actin did not affect the velocity of the thin filament over V1 isomyosin (2.84 ± 0.32 μm/s vs. 2.91 ± 0.24 μm/s) and decreased its velocity over atrial myosin isoform A1 (3.3 ± 0.28 μm/s vs. 2.63 ± 0.36 μm/s). However in the case of myosins consisting of βMHC this led to a considerable increase in the filament velocity. Particularly, the velocity of actin filaments over cardiac isomyosin V3 was 1.45 ± 0.16 μm/s while for actin-Tm filaments it was 2.23 ± 0.13 μm/s.

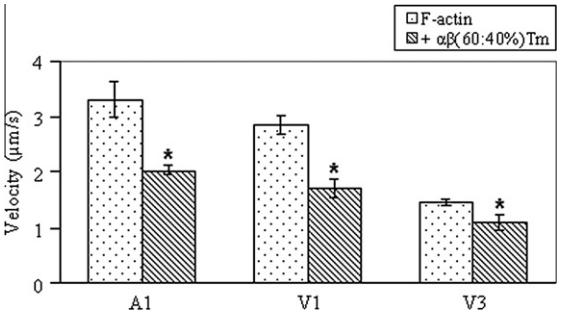

Fig. 4. The sliding velocity of actin and actin-Tm filaments consisting of αβ (60:40 %) Tm from *psoas* rabbit muscle over cardiac myosin isoforms in the *in vitro* motility assay. The columns and error bars are mean ± S.D. Asterisks indicate significant difference, $p < 0.05$.

The sliding velocity of both actin and actin-Tm filaments consisting of cardiac beef tropomyosin (αβ (90:10%) Tm) over the same myosins as those in Fig. 2 is shown in Fig. 3. Addition of αβTm to actin had an opposite effect on the velocity of actin-Tm filament over V1 and A1 isomyosins. The velocity of actin-Tm filament (5.27 ± 0.14 μm/s) over V1 was significantly higher than that of bare F-actin (2.84 ± 0.32 μm/s). But the velocity of actin-Tm filament (1.37 ± 0.15 μm/s) over A1 was lower than that of F-actin (3.3 ± 0.28 μm/s). Addition of αβTm to actin led the actin-Tm filament velocity over V3 isomyosin to decrease from 1.45 ± 0.16 μm/s to 0.9 ± 0.17 μm/s.

Fig. 4 shows the sliding velocity of both F-actin and actin-Tm filament consisting of skeletal rabbit tropomyosin (αβ (60:40%) Tm) over the same myosins as those in Figs. 2 and 3. Addition of αβTm to actin resulted in the reduction of the velocity of actin-Tm for V1 from 2.84 ± 0.32 μm/s to 1.72 ± 0.08 μm/s and A1 from 3.3 ± 0.28 μm/s to 2.03 ± 0.35 μm/s, respectively. Addition of αβTm to actin also reduced the velocity of actin-Tm filaments over isomyosin V3 from 1.45 ± 0.16 to 1.0 ± 0.18 μm/s.

4. Discussion

Tropomyosin plays an important role in regulation of actin–myosin interaction in striated muscle. In myocardium of mammals it is represented by either αα-homodimer or αβ-heterodimer. According to recent studies, mutations in the gene encoding cardiac tropomyosin α-chain result in FHC (Familial Hypertrophic Cardiomyopathy) and dilated cardiomyopathy [27–29]. Overexpression of the β-chains leads to a disturbance of diastolic function via an increase in calcium sensitivity of the "pCa–force" relationship [3,4].

To study the regulatory effect of tropomyosin on *in vitro* model of cardiac actin–myosin interaction we used the isoforms of tropomyosin with different composition of α- and β-chains obtained from cardiac and skeletal muscles. Tropomyosin extracted from rabbit myocardium is α-chain homodimer. Bovine ventricular tropomyosin along with α-chain contains 10% β-chain while tropomyosin from rabbit *psoas* muscle has 40% β-chain.

The results of the experiments showed that the addition of tropomyosins with different content of α- and β-chains to the actin

filament has varied effects on the sliding velocity of actin–tropomyosin filaments. In the other words the regulatory effect of tropomyosin depends on the proportion of its α- and β-chains. Noteworthy that the addition of tropomyosins with increased content of β-chains, as typical for cardiac pathologies, reduced the velocity of actin-Tm filaments over all types of cardiac isomyosin both atrial and ventrical. In turn different isoforms of myosin also affect the velocity of actin–tropomyosin filaments containing the same tropomyosins. This means that the interaction of myosin with F-actin is influenced by both myosin and tropomyosin isoforms. Exact mechanism of this influence is unknown but it can be explained for the reason of following data.

Tropomyosin on actin filament participates in control of actin-to-myosin interaction and according to published data directly affects mechanical characteristics of acto–myosin complex [15]. Using the *in vitro* motility assay it was shown that skeletal tropomyosin added to actin filaments increased force of skeletal muscle cross-bridges and inhibited velocity of the filaments at subsaturating myosin surface densities. At saturating myosin density neither cross-bridge force nor actin–tropomyosin velocity were affected by tropomyosin [15]. This data corresponds to those of Lehrer et al. [30] who measured Mg-ATPase of skeletal myosin and found that at low concentration of myosin S1 skeletal tropomyosin inhibited the ATPase and stimulated it at high concentration. The authors explained the results by both steric and allosteric effects of tropomyosin on interaction of myosin with actin [15,30]. Steric effect consists in that tropomyosin blocks myosin binding sites on actin filaments and so low myosin concentration is insufficient for activation of actin–tropomyosin filament. At high myosin concentration the filaments become fully activated and this demonstrates allosteric effect of tropomyosin.

The hypothesis of allosteric effect of tropomyosin is also supported by a number of works [31–33] where it was shown that mutant tropomyosin with deletion of two or three quasi-repeats of inner part of the molecule has the same binding order of F-actin as a wild type of β-tropomyosin in the absence of myosin S1 and troponin complex. Addition of S1 to F-actin leads to a cooperative enhancement of β-tropomyosin binding with F-actin and an increase of binding constant. However mutant tropomyosin did not show such effect [31,32]. Recently it was shown [33] that chimeric yeast tropomyosins containing different fragments of skeletal β-tropomyosin variously effect myosin S1 binding with F-actin. Thus presence of the third quasi repeat in yeast tropomyosin intensified binding of S1 to actin. Other quasi repeats in tropomyosin molecule as well as wild type yeast tropomyosin did not affect the binding. Besides affecting the binding constants the mutant tropomyosin with deleted quasi repeats of the inner part modifies the mechanical properties of acto–myosin complex. In the *in vitro* motility assay the mutant tropomyosin slowed down the motility of regulated filaments at subsaturating Ca^{2+} [31]. In isolated myocardium strips this mutant led to a decrease in maximal isometric force [34,35]. Allosteric effect of tropomyosin can be explained by its ability to cause structural changes in F-actin. Rubenstein and colleagues using pyren-labeled actin have found a dependence of pyren fluorescence on the tropomyosin isoform type [36]. It was recently found by Moraczewska and colleagues [37] that the degree and cooperativity of myosin-induced shift of tropomyosin localization was different for various of tropomyosin isoforms. All these assume that different isoforms of tropomyosin differently affect the interaction of myosin with actin filament.

Activation of actin–tropomyosin filament depends on the amino acid sequence of both tropomyosin [37] and actin-binding domain of myosin [38]. According to Burghardt and others [38] an important role in the interaction of myosin with actin plays C-loop of myosin heavy chain. Exchanging the native amino acid sequence of the C-loop in smooth muscle myosin to that of cardiac or skeletal one they showed that such chimeric myosins differently interact with actin–tropomyosin filament in the *in vitro* motility assay. On the native myosin the addition of tropomyosin led to an increase in the filament velocity while there was no effect on the chimeric myosins. This observation demonstrates that a certain tropomyosin isoform with the native myosin possesses a positive allosteric effect, that is increases the moving velocity of F-actin, but with chimeric myosins the same isoform reveals negative or no effect.

It is known, that the formation of myosin cross-bridges causes conformational changes in actin molecule [39,40], and one can assume that the character of these changes may depend on different myosin isoforms.

From the results of our experiments we can conclude that the interaction of myosin with actin–tropomyosin filament is affected by amino acid sequence of both heavy and light chains of myosin. The effect of amino acid sequence of the myosin heavy chains is discussed above. The impact of the light chains is first shown in the current study for the case of homodimers of myosin α-heavy chains (α-MHC; A1 and V1 isoforms). We showed that the homodimers of myosin β-heavy chains with different composition of the light chains have different regulatory effects with respect to actin–tropomyosin filament.

Exact mechanism of this effect is unknown but it can be explained on the base of following data. It is known that in striated muscle the light chains participate in force generation by myosin head [41,42]. In particular, it was shown that extraction of the light chains from fast skeletal myosin result in myosin inability to move actin filaments in the *in vitro* motility assay [41,42]. Mutations of essential and/or regulatory light chains both in skeletal and cardiac myosins lead to a change in kinetics of actin–myosin interaction [43–45]. In cardiac muscle change in expression of the light chain isoforms resulted in a modification of contractile characteristics [46,47]. In particular, Morano with coworkers [48] have shown that atrial ELC (aELC) unlike ventricular one (vELC) does not interact with actin and therefore kinetics of the actin–myosin interaction can be different for the myosin isoforms containing either aELC or vELC.

Thus our results support reciprocal effects of myosin and tropomyosin on actin–myosin interaction in myocardium. This may play a significant role in maintenance of the effective heart work both during ontogenesis and in pathological conditions.

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