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Hybrid and non-hybrid actomyosins reconstituted with actin, myosin and tropomyosin from skeletal and catch muscles

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ABSTRACT

In this study, we investigated hybrid and non-hybrid actomyosin models including key contractile proteins: actin, myosin, and tropomyosin. These proteins were isolated from the rabbit skeletal muscle and the catch muscle of the mussel *Crenomytilus grayanus*. Our results confirmed literature data on an unusual ability of bivalve's tropomyosin to inhibit Mg–ATPase activity of skeletal muscle actomyosin. We have shown that the degree of inhibition depends on the environmental conditions and may vary within a wide range. The inhibitory effect of mussel tropomyosin was not detected in non-hybrid model (mussel myosin + mussel actin + mussel tropomyosin). This effect was revealed only in hybrid models containing mussel tropomyosin + rabbit (or mussel) actin + rabbit myosin. We assume that mussel and rabbit myosins have mismatched binding sites for actin. In addition, mussel tropomyosin interacting with actin is able to close the binding sites of rabbit myosin with actin, which leads to inhibition of Mg–ATPase activity.

1. Introduction

Myosin, actin, and tropomyosin are the key contractile proteins; they are found in all known muscles. Myosin and actin in isolated and purified form build a complex (synthetic actomyosin), which reveals mechanochemical activity in the presence of ATP. This activity resembles muscle activity and can be recorded at ATP splitting (Mg–ATPase activity), at changes in the optical density of the actomyosin suspension (clearing and superprecipitation), in the sliding speed of filaments (in vitro motility assay) and by other methods. Synthetic actomyosin is the simplest contractility model. The properties of such models obtained from different muscles may differ quantitatively, but are qualitatively similar. More complicated models include also other muscle proteins that regulate and modulate the actin-myosin (A-M) interaction in accordance with the functional features of the muscle. In this case, we can expect a certain matching of model properties to the properties of the original muscle.

The simple contractile models as well as the models including regulatory proteins can be hybrid, i.e. they can contain proteins from different muscles. In many cases, we cannot do without hybrid models because of the lack of the methods for isolation of proteins from the muscle of interest or because of the lack of the material for isolation of such proteins. Actin is one of these proteins. Straub-type actin can be readily isolated from skeletal and smooth muscles of vertebrates, but it is virtually impossible to isolate it from other muscles. Therefore, actin from skeletal muscles of vertebrates is traditionally used as an actin component in hybrid models. Skeletal muscles of vertebrates are often also used as a source of myosin for hybrid models, because this myosin is Ca^{2+}-insensitive unlike most other myosins. Therefore, introduction of skeletal muscle myosin into the models allows us to investigate the nature of Ca^{2+}-sensitivity of thin filaments in muscles with a double regulation [1].

In this study, we investigated hybrid and non-hybrid contractile models containing "mandatory" proteins: actin (FA), myosin (MY), and tropomyosin (TM). The proteins were isolated from the rabbit skeletal muscle and the catch muscle of the mussel *Crenomytilus grayanus*. These muscles are very different in their function. The rabbit muscle, like most muscles, can be in two states - relaxed and contracted, while smooth muscles of bivalves can be in three states – relaxed, contracted, and catch. The catch mechanism is unclear

Abbreviations: FA, F-actin; nFA, "natural" F-actin; MY, myosin; TM, tropomyosin; rab, rabbit; mus, mussel; PMSF, phenylmethylsulphonil fluoride; LPN, leupeptin; DTT, dithiothreitol.

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and now under intensive study, also with the use of hybrid models [2–5]. Some hybrid models, including proteins of the catch muscle of bivalves, possess unusual properties; molluscan tropomyosin - as a component of such models - inhibits their ATPase activity [6,7]. Also, hybrid models incorporating native mussel thin filaments have low ATPase activity [8]. It is assumed that these unusual properties are associated with peculiarities of the Ca^{2+}-regulatory system of bivalve's muscle thin filaments, which belongs to the "accelerator type" compared with the vertebrate skeletal muscle system that has the "brake-type" property [9].

2. Materials and methods

2.1. Proteins isolation

The source of proteins in this study was the posterior adductor of the sea mussel *Crenomytilus grayanus*, as well as the muscles of the back and hind limbs of rabbit. Mussel thin filaments, mussel myosin and mussel tropomyosin were prepared as described previously [8,10]. The rabbit skeletal muscle actin of Straub type was prepared according to Rees and Yang [11], rabbit skeletal muscle myosin was prepared according to Margossian and Lowey [12]. Rabbit skeletal muscle tissues were donated from the vivarium of the G.B. Elyakov Pacific Institute of Bioorganic Chemistry (Vladivostok). All procedures were approved by the Animal Care Committee of A.V. Zhirmunsky Institute of Marine Biology, Far Eastern Branch of the Russian Academy of Sciences (Protocol N 21 from 08.09.2014).

2.1.1. Mussel "myofibrils"

All procedures were done on ice. 100 g of fresh minced muscle were rigorized for 24 h with 4 volumes of glycerol solution (50% glycerol, 75 mM NaCl, 2 mM $MgCl_2$, 2 mM EGTA, 3 mM NaN_3, 0.1 mg/ml trypsin inhibitor, 0.5 mM PMSF, 1 mM DTT, 20 mM Imidazol-HCl, pH 6.5) with constant agitation with an overhead stirrer. Upon completion, glycerinated muscles were ground in a meat grinder and homogenized with Polytron PT 2500E at 5000 *rpm* for 10 min in 1 L of washing solution containing 75 mM KCl, 1 mM $MgCl_2$, 0.2 mM EGTA, 1 mM NaN_3, 0.5 mM DTT, 10 mM phosphate buffer, pH 6.5. The homogenate was diluted up to 4 L with the washing solution and centrifuged at 5000 *rpm* for 20 min. The pellet was resuspended in 2 L of washing solution and recentrifuged as above. The procedure was repeated with 1 L of washing solution, and the pellet was used as crude "myofibrils".

2.1.2. Mussel "natural" F-actin

The last washing pellet («myofibrils») was suspended in 400 ml of extraction solution: 75 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 15 mM ATP, 5 mM PPi, 0.5 mM DTT, 0.1 mM PMSF, 2.5 μg/ml LPN, 10 mM imidazol-HCl, pH 7.0. The extraction was conducted for one hour with stirring and the extract was obtain by centrifugation at 10 000 *rpm* for 30 min (Beckman rotor F0685). The extract was clarified by centrifugation at 40 000 *rpm* for 30 min (Beckman rotor type 50.2). The clarified extract was centrifuged at 40 000 *rpm* for 90 min (Beckman rotor type 50.2). The pellets was washed with extraction solution and suspended in 20 ml of this solution with the use of a Microman pipette. 3 M KCl were added to the solution to 0.6 M, the solution was stirred at a low rate for 30 min and ultracentrifuged at 50 000 *rpm* for 120 min (Beckman rotor type 70.1). The pellets were suspended in a suitable volume of the suitable solution, and dialized against this solution with stirring inside and outside the dialysis bag. If necessary, the solution of "natural" F-actin (nFA) was clarified by low-speed centrifugation. No contaminating proteins were detected by SDS PAGE in the nFA preparations.

2.2. Mg–ATPase assay

Myosin, F-actin, and tropomyosin dissolved in 0.5 M KCl were mixed at required ratios and diluted with a combination of two solutions: (1), 2.0 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM $CaCL_2$, and 10 mM imidazole–HCl (pH 7.0) and (2), the same solution with an addition of 75 mM KCl. Dilution with the first solution allowed us to set the desired ionic strength (75 mM KCl), and dilution with the second solution achieved the required concentration of proteins. The reaction was started by adding Mg-ATP to 0.3 mM and terminated after 10 min by adding trichloroacetic acid to 5 mM. Inorganic phosphate was determined colorimetrically. Reliability of the obtained data was checked by triple repetition of experiments. Two repetitions of each experiment were performed using proteins obtained from various preparations.

3. Results

The curves of activation of the Mg–ATPase activity of rabbit myosin by mussel thin filaments and by mussel "natural" F-actin in the presence and the absence of Ca^{2+} were compared (Fig. 1). "Natural" F-actin is filamentous actin obtained through removal of actin-binding proteins from thin filaments. The curve of activation of rabbit myosin by "natural" F-actin does not depend on Ca^{2+}, thus showing the absence of proteins of a Ca^{2+}-sensitive complex in this preparation, unlike mussel thin filaments that possess.

70-80% Ca^{2+}-regulation (Fig. 1). Unexpectedly, in comparison with "natural" F-actin, thin filaments appeared to activate rabbit myosin 4- to 5-folds weaker even in the presence of Ca^{2+}. As these preparations differ only in the presence of actin-binding proteins, it is reasonable to suggest that the actin-binding proteins of thin filaments include proteins inhibiting activation of myosin. In addition, we compared activation of mussel and rabbit myosins by mussel thin filaments. It was found that mussel thin filaments in presence of Ca^{2+} activated their "own" myosin 6-7 times stronger than the "foreign" rabbit myosin (not shown).

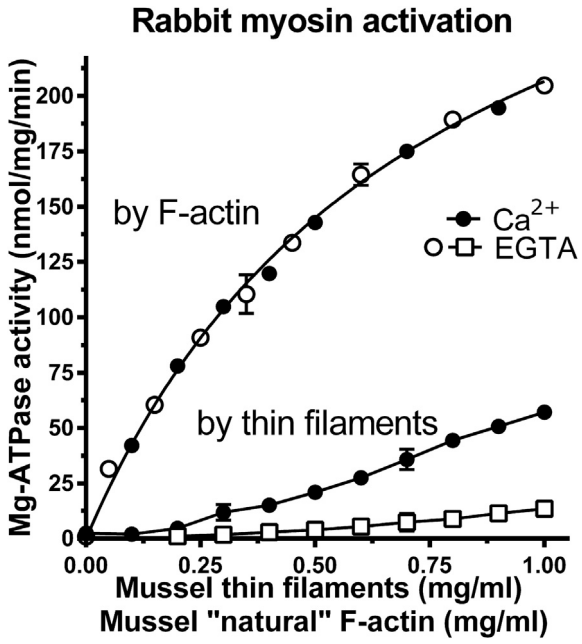

Fig. 1. Activation of rabbit myosin by mussel "natural" F-actin and by mussel thin filaments. Conditions: 75 mM KCl, 2 mM $MgCl_2$, 20 mM imidazole-HCl (pH 6.5), 0.1 mM $CaCl_2$ or 1 mM EGTA. Rabbit myosin, 0.1 mg/ml. Error bars shows variability of the data.

So, the situation appears to be more complicated: the differences in the degree of activation might be attributed to both the myosin component and the actin component. The actin-binding proteins of mussel thin filaments are tropomyosin, calponin, troponin components and a few non-identified minor polypeptide [8,13]. Testing of the proteins listed above has revealed that the inhibiting effect is exerted by mussel tropomyosin.

Fig. 2 shows the effects of mussel tropomyosin and rabbit tropomyosin on Mg-ATPase activity of rabbit actomyosin. These tropomyosins appeared to exert diametrically opposed effects: the rabbit tropomyosin enhanced the ATPase activity by some 25%, while the mussel tropomyosin reduced this activity by 85–90%. In both cases, the influence of tropomyosin achieved its maximum at A/TM = 7 (mol/mol).

The degree of inhibition of Mg–ATPase activity of rabbit myosin by mussel tropomyosin depends on the parameters of the medium: the ionic strength, pH, and $MgCl_2$ concentration. Fig. 3 demonstrates the influence of ionic strength on the degree of inhibition. The curve of the dependence has its maximum at 70 mM KCl, rapidly decreases with decreasing ionic strength and intersects the X-axis at 30 mM KCl. The level of inhibition also decreases at reduced pH of the solution and at decreased concentration of $MgCl_2$ (not shown). The above dependences allowed choosing the parameters of the medium so that mussel tropomyosin did not inhibit the Mg–ATPase activity of rabbit actomyosin.

The simplest contractile model is synthetic actomyosin, formed only from myosin and actin. This actomyosin is capable of activating myosin Mg–ATPase and also exhibits some mechano–chemical activity. We formed four variants of this model, assembled from myosin (MY) and actin (FA) of rabbit skeletal muscle (rab) and mussel adductor muscle (mus). Two models of them were non-hybrid - rab(MY + FA) and mus(MY + FA), and two models were hybrid - (rabMY + musFA) and (musMY + rabFA). The models containing rabbit myosin had a significantly higher activity than the models with mussel myosin. Furthermore, it was found that the rabbit actin activated both myosins stronger than mussel actin did. Thus, the activity of these models decreased in the order: (rabMY + rabFA); (rabMY + musFA); (musMY + rabFA); and (musMY + musFA). The differences among these activities were not

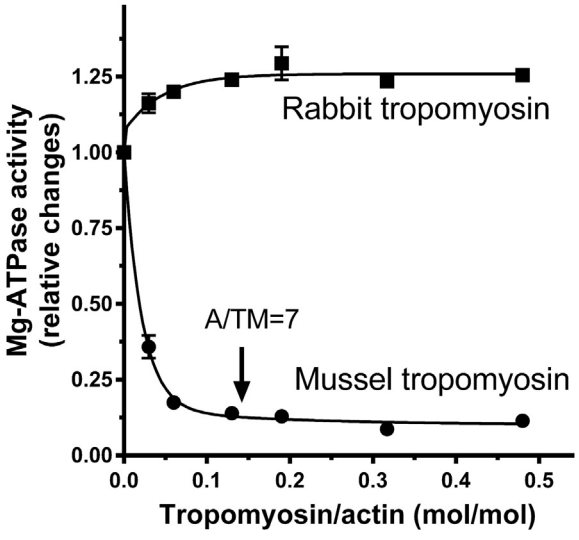

Fig. 2. Influence of rabbit tropomyosin and mussel tropomyosin on Mg–ATPase activity of rabbit actomyosin. Conditions: 75 mM KCl, 2 mM $MgCl_2$, 2 mM NaN_3, 20 mM imidazole-HCl (pH 7.0), 0.1 mM $CaCl_2$. Rabbit actin, 0.1 mg/ml; rabbit myosin, 0.1 mg/ml. Error bars shows variability of the data.

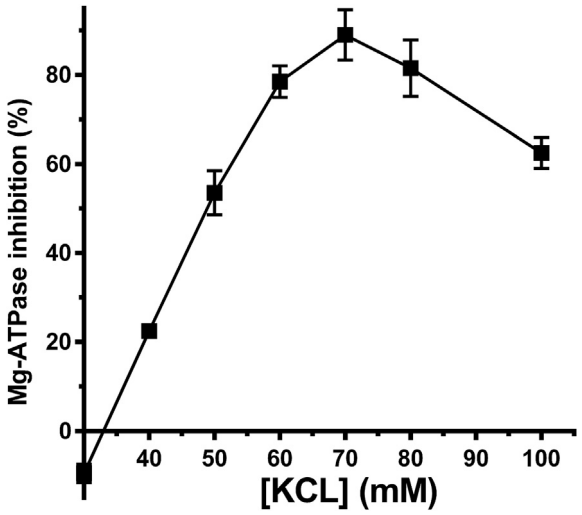

Fig. 3. Influence of ionic strength of the solution on the degree of inhibition of Mg–ATPase activity of rabbit actomyosin by mussel tropomyosin. Conditions: 0–100 mM KCl, 2 mM $MgCl_2$, 2 mM NaN_3, 20 mM imidazole-HCl (pH 7.0), 0.1 mM $CaCl_2$. Rabbit actin, 0.1 mg/ml; rabbit myosin, 0.1 mg/ml; mussel tropomyosin, 0.03 mg/ml. Error bars shows variability of the data.

associated with hybrid models but meet the functional characteristics of the original muscle [14].

Furher, we complicated the models by introducing different amounts of the regulatory proteins - rabbit tropomyosin and mussel tropomyosin. As a result, we obtained eight models, two of them non-hybrid (rabMY + rabFA + rabTM) and (musMY + musFA + musTM), and the remaining six models were hybrid, as each of them contained two proteins from one muscle, and one protein from the other. Fig. 4 presents a comparison of Mg–ATPase activities of all these models with dependence on the contents of rabbit and mussel tropomyosins. Six of the eight models were similar in the effect of tropomyosin on them: Mg–ATPase activity increased more or less with an increase in the content of tropomyosin. But in the case of two models containing rabbit myosin and mussel tropomyosin (Fig. 4C), we observed a strong inhibition of the ATPase activity, and this was low dependent of the source of actin. When we replacedthe rabbit myosin with mussel myosin in these models (Fig. 4B, C), then inhibition was replaced by activation, again regardless of the origin of actin. It should be noted, however, that although the properties of models did not generally depend on the origin of actin, the curves of activation of Mg–ATPase activity with different actins do not really coincide.

4. Discussion

In skeletal muscles, the equilibrium position occupied by tropomyosin on actin in the absence of troponin is very close to the position brought about by Ca^{2+}-saturated troponin [15]. Accordingly, introduction of skeletal TM in skeletal AM does not lead to a change in Mg-ATPase activity [16]. But introduction of smooth muscle TM into skeletal AM -instead of skeletal TM-forms a hybrid model and results in an increase in Mg-ATPase activity [16]. In the case of proteins from bivalve's muscles, introduction of musTM in the skeletal-muscle model, conversely leads to strong inhibition, similar to response of Ca^{2+}-regulatory system to the absence of calcium. With that, the origin of actin does not really matter. Hence, the inhibitory effect is determined by the properties of musTM, but is realized in cooperation with rabMY. These proteins do interact within the framework of Ca^{2+}-regulatory mechanism of the “actin”

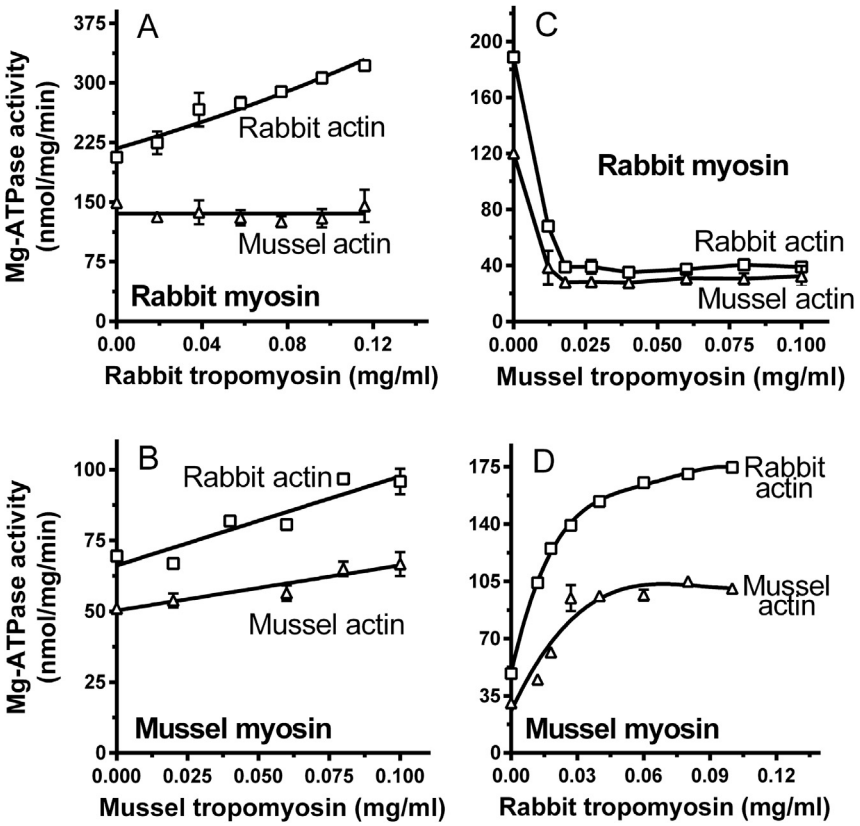

Fig. 4. Properties of actomyosin models reconstructed with myosin, actin, and tropomyosin from rabbit and mussel in various combinations. Conditions: 75 mM KCl, 2 mM $MgCl_2$, 2 mM NaN_3, 20 mM imidazole-HCl (pH 7.0), 0.1 mM $CaCl_2$. Rabbit actin, 0.1 mg/ml; rabbit myosin, 0.1 mg/ml; mussel actin, 0.1 mg/ml; mussel myosin, 0.1 mg/ml. Error bars shows variability of the data.

type. The acceptor of Ca2 + in this mechanism is troponin, which is able - according to the steric-blocking mechanism of muscle regulation - to close/to open the myosin-binding sites on actin by tropomyosin.

We believe that musTM itself blocks the rabMY binding sites on actin in the absence of troponin. When we used musMY in the pair together with musTM, we observed no resulting inhibitory effect (Fig. 4B); this indicates that the binding sites for musMY were open. Thus, it seems that the tested myosins have different binding sites on actin. Most likely that the inhibitory effect is not somehow linked to any kind of error in the models' preparation, since the effect is observed also at the use of native thin filaments (Fig. 1).

By changing the condition of the medium, in particular, by reducing the ionic strength to 30 mM KCl, the inhibitory effect of musTM disappeared (Fig. 3). Evidently, it is connected with a change of the musTM binding site on actin. At decreased ionic strength, this site changes it position and ceases to overlap the rabMY-binding site on actin. Such an adjustment normalizes the properties of hybrid models externally, however, it is unclear whether the functional properties of these models become the same as the properties of non-hybrid actomyosin.

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