Biochemical and Biophysical Research Communications 390 (2009) 125–129

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Caldesmon inhibits the rotation of smooth actin subdomain-1 and alters its mobility during the ATP hydrolysis cycle

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ARTICLE INFO

Article history:
Received 15 September 2009
Available online 24 September 2009

Keywords:
Muscle contraction
Actin subdomains
Caldesmon
ATP analogs
Actomyosin intermediate states
Ghost muscle fibers
Fluorescence polarization

ABSTRACT

Smooth muscle thin filaments have been reconstituted in muscle ghost fibers by incorporation of smooth muscle actin, tropomyosin and caldesmon. For the first time, rotation of subdomain-1 and changes of its mobility in IAEDANS-labeled actin during the ATP hydrolysis cycle simulated using nucleotides and non-hydrolysable ATP analogs have been demonstrated directly. Binding of caldesmon altered the mobility and inhibited the rotation of actin subdomain-1 during the transition from AM**·ADP·Pi to AM state, resulting in inhibition of both strong and weak-binding intermediate states. These new results imply that regulation of actomyosin interaction by caldesmon during the ATPase cycle is fulfilled via the inhibition of actin subdomain-1 rotation toward the periphery of the thin filament, which decreases the area of the specific binding between actin and myosin molecules and is likely to underlie at least in part the mechanism of caldesmon-induced contractility suppression.

Introduction

Smooth muscle caldesmon is a flexible, asymmetric molecule which is thought to be axially arranged on thin filaments [1] with the tendency of N-terminus to project outward [2]. It is well established that caldesmon binds to myosin, actin, tropomyosin and Ca^{2+}-binding proteins; caldesmon is capable of inhibiting actomyosin ATPase, the sliding of actin filaments on immobilized myosin in the *in vitro* motility assay as well as relaxation of skinned skeletal and smooth muscle fibers. It is believed that caldesmon either inhibits the binding of myosin to actin or slows down the rate-limiting stage of the ATPase cycle. For reviews, see [3–5] and references therein.

Caldesmon molecule contains several actin-binding segments, mostly distributed in the 10 kDa C-terminal region; two of these segments located on the extreme of caldesmon C-terminus consist of two clusters of multiple point contacts with the C-terminal region of actin. They are responsible for inhibition of weak binding type of interaction between actin and myosin and hampering of the actomyosin ATPase activity (see [4] and references therein). This is consistent with the observation that caldesmon overlaps the weak-binding sites of myosin on actin filaments [1].

Polarized fluorimetry is a highly sensitive method for studying conformational changes of myosin, F-actin, tropomyosin and other proteins in muscle fibers (for a review, see [6]). Using this method, we have shown previously the on/off switching of the actin filaments and the ordered/disordered arrangement of the myosin heads in the muscle fiber at strong and weak states of binding of actin to myosin [7], as well as inhibition of alterations of F-actin structure and flexibility which causes the "freezing" of the thin filament structure at binding of caldesmon [8]. Interestingly, caldesmon inhibited the "rolling" of tropomyosin on the surface of the thin filament during the ATP hydrolysis cycle, drastically decreased the affinity of the whole tropomyosin molecule to actin and "freezed" tropomyosin in the position characteristic of weak binding of myosin to actin [9]. It was shown that S1 binding to actin in nucleotide-dependent displace of the C-terminal part of caldesmon molecule in thin filament and changes in its mobility [10], showing that the inhibition of the actomyosin ATPase activity may be due to changes in caldesmon position on the thin filament and its interaction with actin.

Further evidence exists that the inhibition ATPase activity can result from reducing of the binding between myosin heads and actin [11]. These views were confirmed by use of tryptic [12] or recombinant [1] fragments of the C-terminal part of caldesmon. Other authors [13] claimed that S1 treatment due to competitive

Abbreviations: S1, myosin subfragment-1; LC_{20}, 20,000-dalton light chain of smooth muscle myosin; 1,5-IAEDANS, N-iodoacetyl-N′-(5-sulfo-1-naphtylo)ethylenediamine; DTT, dithiothreitol.

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doi:10.1016/j.bbrc.2009.09.079

binding displaces whole caldesmon from thin filaments. However, according to the 3D reconstruction of the caldesmon–F-actin complex, caldesmon only partially overlaps the strong myosin-binding sites [1,14].

It is well known that caldesmon significantly affects the character of the actin–myosin interaction, as well as the structural state of F-actin during the transition of the muscle fiber from the relaxed state to rigor. It was suggested that caldesmon is able of regulating muscle contraction via inhibiting of the strong binding between myosin cross-bridges and actin [15,16]. However, fine molecular events taking place in actin at binding of caldesmon still remain unclear; the results presented in this paper may shed more light on this problem.

Materials and methods

Smooth muscle actin, caldesmon and tropomyosin were isolated from fresh duck gizzard smooth muscle [17]. Skeletal myosin subfragment-1 (S1) devoid of regulatory light chains was prepared according to [18]. G-actin was labeled at Cys374 with 1,5-IAEDANS (Molecular Probes) as described in [10].

Glycerinated muscle fibers were obtained from rabbit psoas muscle and ghost fibers were prepared as described earlier [19]. The proteins were incorporated into the ghost fibers in the following order: G-actin-AEDANS, S1, tropomyosin, caldesmon by incubation of the fibers in the solution containing 20 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 10 mM Tris–HCl, pH 6.8, and 2.0–2.5 mg/ml protein [16]. The measurements were carried out in the buffer containing 10 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 6.7 mM phosphate buffer, pH 7.0, in the absence or presence of 2.5 mM ADP, 25 mM AMP-PNP, 15 mM ATPγS or 5 mM ATP [36]. The molar ratio of tropomyosin and caldesmon to actin in the resultant fibers was 1:6.5 (±2) and 1:14 (±2). In the absence of the nucleotides and in the presence of ADP, AMP-PNP, ATPγS, and ATP the molar ratio of S1 to actin was 1:5 (±2), 1:5 (±2), 1:8 (±2), 1:12 (±2), and 1:14 (±2), respectively. Caldesmon decreased the S1:actin molar ratio on average by 20%.

Fluorescence polarization from 1.5-IAEDANS-labeled actin was registered at 500–600 nm after excitation at 365 ± 5 nm. To estimate the changes in a probe orientation, the helix plus isotropic model [20,21] was used. The intensities of the four components of polarized fluorescence were measured by photometer [22] in parallel ($_{\parallel}I_{\parallel}$,$_{\parallel}I_{\perp}$) and in perpendicular ($_{\perp}I_{\perp}$,$_{\perp}I_{\parallel}$) of the fiber axis relative to the polarization plane of the exiting light. Fluorescence polarization ratios were defined as: $P_{\parallel} = (_{\parallel}I_{\parallel} - _{\parallel}I_{\perp})/(_{\parallel}I_{\parallel} + _{\parallel}I_{\perp})$ and $P_{\perp} = (_{\perp}I_{\perp} - _{\perp}I_{\parallel})/(_{\perp}I_{\perp} + _{\perp}I_{\parallel})$. The ratios of fluorescence intensities were considered as functions of angles Φ_A, Φ_E, and N, where Φ_A, Φ_E are angles between the fiber axis and the absorption and emission dipoles of fluorophores, respectively; N is the relative number of disordered fluorophores.

The position of the maximum of AEDANS-actin fluorescence spectrum was the same in all the experiments and the polarized fluorescence was proved to be independent of S1 content in the fiber in the range of myosin head to actin molar ratios from 1:3 to 1:20. Based on these data all changes in the parameters of fluorescence ($P_{\parallel}$, $P_{\perp}$, Φ_A, Φ_E and N) were interpreted in terms of the changes in orientation and mobility of the absorption and emission dipoles of the probe, located in actin subdomain-1 at binding of S1, TM and caldesmon in the absence or presence of nucleotides. In our steady-state experiments, the polarized fluorescence of F-actin reflected the average structural state of the protein molecule in the population as a whole.

Since in all experiments the values of Φ_A were very close to that of Φ_E, only Φ_E and N values are described. The statistical reliability of the changes was evaluated using Student's t-test.

Results and discussion

To study the effect of caldesmon on the conformational states of actin subdomain-1 during the sequential steps of the actomyosin ATPase cycle, we used a well-organized model system of thin filaments reconstituted in ghost fibers from fluorescently labeled exogenous smooth muscle G-actin, tropomyosin and caldesmon. The polarized fluorescence from the fluorescent probe covalently bound to Cys374 of actin subdomain-1 was measured at various simulated intermediate stages of the ATPase cycle. As it was impossible to resolve multiple conformational sub-states in our steady-state experiments, we proceeded with the assumption that in the absence of the nucleotides and in the presence of MgADP, MgAMP-PNP, MgATPγS or MgATP predominantly actomyosin states A·M, A^·M^·ADP, A′·M′·ADP, A*·M*·ATP and A**·M**·ADP·Pi, respectively, were simulated [23,24], where A, A^, A′, A*, A** and M, M^, M* and M** are various conformational states of the actin and myosin head, respectively.

According to the results presented in Table 1 and Fig. 1, nucleotides altered the polarized fluorescence of AEDANS-actin decorated by S1 in the absence or presence of caldesmon. Since modification of actin does not significantly affect the functional properties of actin [10], the observed changes in actin conformational state are likely to reflect those occurring during the muscle contraction. The degree of fluorescence polarization from AEDANS-actin in ghost fibers devoid of other proteins was a high positive value when a fiber was oriented parallel to the polarization plane of the exciting light ($P_{\parallel}$) and a lower positive value when the fiber was oriented perpendicularly to this plane ($P_{\perp}$) (Table 1). This indicated that the absorption and emission oscillators of the probe on actin molecules were predominantly oriented parallel to the fiber axis. By using the model-dependent method, the angle Φ_E was found to be close to 50° (Fig. 1A), and N to 0.358 rel. units (Fig. 1B), indicating a well-ordered arrangement of the probe.

It had been shown earlier that skeletal muscle contraction was accompanied by a change in the orientation of actin monomers in the thin filaments [25,26]. It was suggested that each intermediate state of actomyosin during the ATPase cycle corresponded to a certain orientation of actin monomers and to a definite rigidity of the thin filaments [27]. In the present work, it has been shown that each actomyosin intermediate state is characterized by a certain spatial organization and mobility of subdomain-1 of smooth muscle actin in the muscle fiber.

According to Fig. 1, binding of S1 to thin filaments containing "pure" F-actin led to an increase of the Φ_E angle by 5.1°. As the

Table 1
The effect of nucleotides on polarization ratios of 1.5-IAEDANS bound to Cys374 of actin in ghost fibers in the absence or presence of caldesmon.

Nucleotide	S1	TM	CD	n	$P_{\parallel}$ ± SEM	$P_{\perp}$ ± SEM
	–	–	–	10	0.240 ± 0.001	0. 112 ± 0.001
	+	–	–	7	0.079 ± 0.001	0.207 ± 0.001
	–	–	+	8	0.257 ± 0.001	0.196 ± 0.002
	+	+	–	7	0.093 ± 0.001	0.231 ± 0.001
	+	+	+	7	0.136 ± 0.002	0.191 ± 0.001
ADP	+	+	–	7	0.097 ± 0.002	0.208 ± 0.001
	+	+	+	7	0.113 ± 0.001	0.211 ± 0.002
ANPPNP	+	+	–	7	0.179 ± 0.002	0.184 ± 0.002
	+	+	+	7	0.183 ± 0.002	0.200 ± 0.002
ATPγS	+	+	–	7	0.220 ± 0.002	0.168 ± 0.002
	+	+	+	7	0.191 ± 0.002	0.193 ± 0.002
ATP	+	+	–	6	0.234 ± 0.002	0.129 ± 0.002
	+	+	+	6	0.194 ± 0.002	0.140 ± 0.002

$P_{\parallel}$ and $P_{\perp}$ were calculated as described in Materials and methods. n is the number of fibers used in the experiments. S1, TM, CD and nucleotides have pronounced effect on the values of $P_{\parallel}$ and $P_{\perp}$, indicating the changes in the conformational state of actin subdomain-1 in ghost fibers ($p < 0.05$).

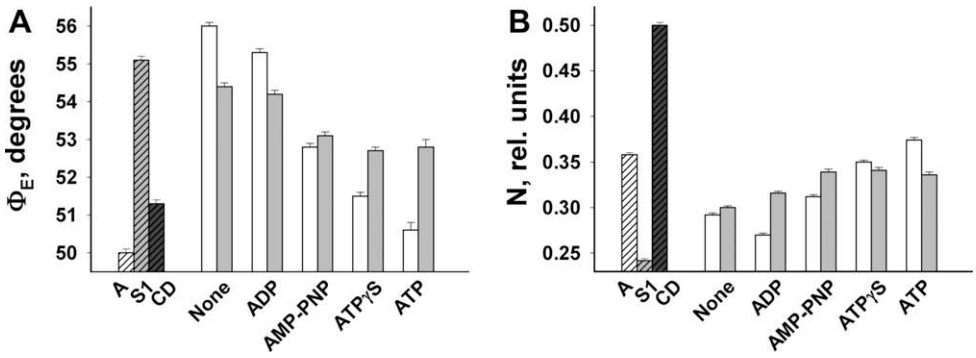

Fig. 1. The effect of caldesmon on the values of Φ_E (A) and N (B) of the polarized fluorescence of AEDANS-actin revealed in ghost fibers at simulating various steps of the ATPase cycle. The first group of bars represents the data for thin filaments containing "pure" F-actin, S1 and CD, respectively; in the next groups of bars the data for thin filaments, containing S1 and TM; S1, TM and CD, respectively, are shown. The data represent means of 6–10 ghost fibers for each experimental condition. The Φ_E and N values are significantly altered by S1, TM and CD in the absence and in the presence of the nucleotides ($p < 0.05$). Error bars indicate ± SEM.

probe is rigidly linked to the protein, the movement of the probe toward the periphery of the thin filament (an increase in Φ_E) can be interpreted [22] as a consequence of a rotation of the actin subdomain-1 to the periphery of the thin filament. The fraction of the disordered probes, N, decreased by 33%, which indicated a ordering of the dye dipoles on the filament through a decrease in the mobility of subdomain-1 [22]. It should be mentioned that immobilization of actin subdomain-1 through its strong binding to the myosin head is in good agreement with previous data by Thomas et al. [28]. It was shown in particular by Prochniewicz et al. [29] that transition from weak to strong-binding structural state decreases the dynamic disorder in the actin molecule.

TM slightly increases the effect of S1 on the rotation of actin subdomain-1 toward the periphery of the thin filament (the values of Φ_E increased by 0.4°). In contrast, CD markedly inhibits the effect of TM, reversing the rotation of actin subdomain-1 (the values of Φ_E decreased by 1.6°). Both TM and CD increased the values of N by 20% and 24%, respectively (Fig. 1). Since the values of Φ_E and N for complexes of F-actin-S1, F-actin-CD, F-actin-TM-S1 and F-actin-TM-S1-CD differ, it may be suggested that actin conformation in these complexes varies at least in mobility and position of actin subdomain-1 in thin filaments.

Addition of nucleotides to F-actin-TM-S1 and F-actin-TM-S1-CD complexes changed the values of Φ_E and N (Fig. 1). In contrast, in the absence of S1 no dependence of the polarized fluorescence parameters on bound nucleotide was observed. Hence, the changes in the polarized fluorescence of the probe were caused by conformational changes in actin dependent on nucleotide-induced changes in the myosin head.

According to Fig. 1, the transition from A**·M**·ADP·Pi (in the presence of MgATP) to A·M state (in the absence of nucleotides) induced a multistep increase and decrease in Φ_E values (Fig. 1A) and N, respectively (Fig. 1B). Since the polarized fluorescence from AEDANS-actin was proved to be independent of S1 concentration in the fiber (see Materials and methods), the data showed that the transition from weakly to strongly bound states produced a multistep rotation of actin subdomain-1 toward the periphery of the thin filament and immobilization of subdomain-1 by its strong binding to S1. The most pronounced differences in the mobility and orientation of actin subdomain-1 were observed between the states A*·M*·ATP and A^·M^·ADP, i.e., at the stage at which force generation occurs [30,31]. Thus, the ATPase cycle was accompanied by multistep changes in smooth muscle actin conformational state (Fig. 1). Similar nucleotide-dependent changes in the direction of rotation and in mobility of subdomain-1 of skeletal actin in ghost fibers were observed recently [32].

It is widely believed that ionic and hydrophobic residues involved in actin binding are localized at one end of the myosin motor domain in the so-called "actin-binding" cleft, which is far from both the enzyme pocket and the converter domain. According to the model of the actomyosin interface [30,31], the motor domain binds to subdomain-1 of one actin monomer, with considerable contact with subdomain-2 of the adjacent actin molecule. As the enzyme pocket is connected to the apex of the cleft, actin binding causes the cleft to close, resulting in the opening of the enzyme pocket and acceleration of product release. We observed that the changes at myosin nucleotide-binding site were transmitted to actin, producing the nucleotide-dependent changes in the mobility and orientation of actin subdomain-1 in thin filaments (Fig. 1). The conformational changes in actin subdomain-1 observed in this work may be transmitted to the neighboring actin monomer, producing the earlier postulated cooperative changes [33] of thin filament rigidity [28] and changes in actin monomer orientation [16,26,34].

It is generally accepted that at initial stages of the ATPase cycle the myosin head is bound to one actin monomer and forms a weak-binding state. After a series of conformational changes, the myosin head additionally binds to one of the neighboring actin monomers, the area of the binding site being increased with each step of the ATPase cycle [30,31]. Our data do not contradict this suggestion. At transition from A**·M**·ADP·Pi to A·M state, a gradual rotation of the actin subdomain-1 away from the filament axis occurred (Fig. 1). Since the small domain of actin contains the strong myosin-binding site [30,31], it may be suggested that the increase in the strength of actin–myosin interaction is conditioned by the enhancement of the area of stereospecific and hydrophobic interaction between actin and myosin molecules. A change in configuration of the myosin-binding site leading to the enhancement of these interactions may occur upon rotation of the subdomain-1 away from the filament axis (Fig. 1A). If our suggestion is correct, then the increased rotation of subdomain-1 toward the periphery of the filament would correlate with the higher affinity of myosin to actin and with more pronounced movement of subdomain-1 to the periphery of the thin filament. If this were the case, the maximal movement of subdomain-1 should be expected at transition from A**·M**·ADP·Pi to A·M state. Such effect was indeed observed in our experiments (Fig. 1A).

Addition of caldesmon modified the S1-induced conformational state of actin, markedly inhibiting the formation of strong and weak forms of S1 binding to actin. This was indicated by changes in the structural state of actin subdomain-1 revealed at simulating various intermediate stages of the ATPase cycle in the presence of

caldesmon (Fig. 1). For example, at simulation of strong-binding states A·M and A^·M^·ADP (in the absence of nucleotide and presence of MgADP), the mobility and spatial arrangement of actin subdomain-1 turned out to be close to that observed for A^·M^·ADP and A′·M′·ADP states, respectively, in the absence of caldesmon, i.e., at formation of the weaker binding state. Caldesmon-inhibited rotation of actin subdomain-1 to the periphery of the thin filament during transition to the strong-binding states, which could be one of the reasons for the drop in actomyosin ATPase activity [35]. At simulation of weak-binding states (in the presence of MgATPγS and MgATP), the subdomain-1 rotated toward the center of the thin filament, showing that A′·M′·ADP and A*·M*·ATP states, i.e., the stronger binding states, emerged instead of A*·M*·ATP and A**·M**·ADP·Pi states, respectively. Inhibition of weak actin-to-myosin-binding state and hampering of the actomyosin ATPase activity [11,12] are also consistent with the observation that caldesmon overlaps the weak-binding sites of myosin on actin filaments [1]. Hence, binding of caldesmon altered the mobility and inhibited the rotation of actin subdomain-1 during the transition from AM**·ADP·Pi to AM state, resulting in inhibition of both strong and weak-binding intermediate states.

Since the strong myosin-binding site is located in the small domain of actin [30,31], it can be suggested that the attenuated actin–myosin interaction can result from the decrease of the area of stereospecific and hydrophobic interaction due to inhibition of actin subdomain-1 rotation away from the center of the thin filament (Fig. 1). It is possible that the decrease of this area may result in inhibition of both strong and weak-binding intermediate states, which is likely to underlie at least in part the mechanism of the suppression of ATPase activity [3–5] induced by caldesmon. Inasmuch as it was found that caldesmon, shifting on thin filament [36] inhibits the formation of the strong- and weak-binding states by "freezing" tropomyosin in blocked position, resulting in inhibition of tropomyosin strands movement on the thin filament [9], it may be suggested that rotation of actin subdomain-1 to the periphery of the thin filament (Fig. 1) occurs due to "freezing" of myosin-dependent tropomyosin movements and transition of the thin filaments to the "OFF" state.

It has been found recently that skeletal muscle regulatory protein, troponin (in the absence of Ca^{2+}) inhibited the formation of strong-binding states essential for force generation by shifting tropomyosin strands toward the periphery of the thin filament and restricting myosin-dependent movements of actin subdomain-1 toward the thin filament periphery. It seems that troponin may alter the pattern of the binding of tropomyosin α- and β-bands with F-actin in the process of rolling [32]. It is possible that caldesmon, similarly to troponin, alters rotation of actin subdomain-1 from the center to the thin filament periphery (Fig. 1) due to myosin-dependent changes in the pattern of the binding of tropomyosin α- and β-bands with F-actin during the ATPase cycle.

It has been shown recently [37] that the C-terminal fragment of caldesmon interacts with actin through multiple actin contact points located in two clusters. Binding of calmodulin stabilizes an extended structure of caldesmon C-terminus and maintains the separation of the two clusters which prevents interaction of caldesmon with actin. On the other hand, caldesmon phosphorylation leads to dissociation of one of the two clusters, while the other one remains bound to actin. Further insight into the modes of caldesmon binding to actin has been achieved in the paper by Foster et al. [38]. The results obtained by these authors were consistent with phosphorylation-dependent conformational change in which the C-terminal segment of caldesmon moves away from F-actin. The "staple-like" connectivity between the helical strands may help to explain stabilization of actin filaments [38] and is in good agreement with our previous results [15]. The results presented here for the first time demonstrate directly the rotation of actin subdomain-1 during the ATP hydrolysis cycle. These results show that actin filament does not remain passive; fine movements of actin protomers seem to be concerted with conformational changes in caldesmon itself, thus facilitating the transition of the whole filament into the off-state and inhibition of the ATPase cycle by caldesmon.

Acknowledgments

We thank Svetlana S. Khaimina for technical assistance. This work was supported by the Russian Fund for Fundamental Research (08-04-00960a), program "Leading Scientific Schools of Russia" (NSH-1961.2008.4) and the statutory funding of the Nencki Institute. The authors wish to express their gratitude to late Professor Renata Dąbrowska who stood at the origins of these studies.

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