Volume 281, number 1,2, 51–54 FEBS 09549 April 1991

ADONIS 001457939100257A

Troponin I and caldesmon restrict alterations in actin structure occurring on binding of myosin subfragment 1

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Received 10 January 1991

The effect of troponin I and caldesmon on phalloidin-rhodamine- and 1,5-IAEDANS-labelled actin in skeletal muscle ghost fibers was investigated by polarized fluorescence. Both these proteins inhibited the structural alterations in the actin monomer and the increase of flexibility of actin filaments occurring on binding of myosin heads, and their effects were potentiated by tropomyosin. This immobilization of the actin filament through troponin I and caldesmon seems to originate from restriction of the relative motions of the two domains within the monomer.

Troponin I; Caldesmon; Actin dynamics; Actin-myosin interaction; Polarized fluorescence

1. INTRODUCTION

The molecular mechanism of muscle contraction involves ATP-dependent structural changes in the actomyosin complex during the crossbridge cycle, which are exploited for force production [1]. A variety of techniques, e.g. electron microscopy, X-ray diffraction, chemical crosslinking as well as fluorescence and EPR spectroscopy have been useful in detecting changes in the orientation of myosin crossbridges attached to actin filaments [2–10]. Physicochemical studies on actin, however, led to the conclusion that the actin filament does not play a merely passive role in the movement of myosin heads, but itself undergoes conformational changes upon interaction with myosin, providing an elastic element required in the mechanism of crossbridge action [11–16]. The elasticity of actin filaments is controlled by Ca^{2+} ions through actin-linked proteins, the troponin-tropomyosin complex [13,17] being the main regulatory system of striated muscle contraction [18,19] and the caldesmon-tropomyosin-calmodulin complex [20,21] a possible complement to myosin phosphorylation in the regulatory system of smooth muscle [22–24].

The functional properties of these two systems are similar: the properties of troponin I and troponin T in inhibiting actomyosin ATPase activity and in binding to tropomyosin, respectively, are both shown by caldesmon, while the role of troponin C, i.e. Ca^{2+}-dependent neutralization of the inhibitory effect, is taken over by calmodulin [22–24]. Both systems require the presence of tropomyosin for their functioning.

In this work we have used polarized microphotometry to study the effects of troponin I and caldesmon on the conformational changes in the two different regions of the F-actin monomer in skeletal muscle fibers upon binding of myosin heads. The results show that both troponin I and caldesmon prevent the increase in flexibility of the actin filament induced by myosin subfragment 1, probably by constraining the relative motions of the two domains in monomers.

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2. MATERIALS AND METHODS

2.1. *Preparation of proteins*

Caldesmon was prepared from chicken gizzard muscle according to the procedure of Bretscher [25].

Rabbit skeletal muscle actin and tropomyosin as well as smooth muscle tropomyosin were purified as previously described [26]. Rabbit skeletal muscle myosin subfragment 1 (S-1) was prepared as described by Weeds and Pope [27]. The troponin complex was extracted from rabbit skeletal muscle and troponin I purified from the complex by ion-exchange chromatography [28].

2.2. *Preparation of ghost fibers*

Ghost fibers were prepared from single glycerinated fibers of rabbit skeletal muscle by extraction of myosin and regulatory proteins as described previously [17]. Polarized fluorescence of either 1,5-IAEDANS or phalloidin-rhodamine bound to F-actin was measured before and after addition of skeletal muscle myosin S-1 to fibers containing tropomyosin and either caldesmon or troponin I. Incorporation of the proteins into the ghost fibers was performed as described earlier [29]. The degree of the incorporation of particular proteins was checked by SDS-PAGE. The molar ratios of caldesmon, troponin I, tropomyosin and S-1 bound to actin in ghost fibers determined by densitometric scans of the gels were approximately 1:20, 1:10, 1:6.5 and 1:3, respectively.

2.3. *Fluorescence polarization measurements*

The polarized fluorescence from 1,5-IAEDANS bound to actin in ghost fibers was recorded at 480–600 nm after excitation at 365 ± 5 nm but that from phalloidin-rhodamine was recorded at 500–600 nm after excitation at 479 ± 5 nm. The intensities of the 4 components of polarized fluorescence were measured in the parallel ($I_{\parallel\parallel}$, $I_{\parallel}$) and in the perpendicular ($I_{\perp\perp}$, $I_{\perp}$) orientation of the fiber axis to the polarization plane of the exciting light. From these components we calculated the degree of fluorescence polarization ($P_{\parallel}$ and $P_{\perp}$), the angles of the emission dipoles of the fluorophore relative to the long axis of F-actin (Φ_E), the average angle between the F-actin axis and the fiber long axes ($\Theta_{1/2}$) and the number of randomly oriented fluorophores (N). The changes in Φ_E angle are interpreted in terms of structural alterations of actoin monomers in the vicinity of the fluorophore. The $\Theta_{1/2}$ angle shows the fluctuation of F-actin orientation about the direction of the fiber axis, reflecting filament flexibility. N measures the mobility of a C-terminal portion of actin polypeptide chain relative to the shaft of the actin filament; it was calculated on the basis of the $\Theta_{1/2}$ value estimated in parallel experiments with phalloidin-rhodamine labelled actin. The theoretical basis for these calculations is given in [12,30].

2.4. *Protein determination*

Protein concentrations were determined by measuring UV-light absorbance with the following absorption coefficients and M_r values: caldesmon, E_{278} = 0.38, 87 kDa [31]; G-actin, E_{290} = 0.63, 42 kDa [32], skeletal muscle tropomyosin E_{277} = 0.24, 68 kDa [33], smooth muscle tropomyosin, E_{277} = 0.19, 68 kDa [33], skeletal muscle S1, E_{280} = 0.75, 115 kDa [37]; troponin I, E_{280} = 0.50, 21 kDa [34].

3. RESULTS

Formation of rigor links between myosin heads and phalloidin-rhodamine- or 1,5-IAEDANS labelled actin in ghost fibers is accompanied by changes in orientation of these fluorophores relative to the long axis of the actin filament [20,29]: the angle of the emission dipole Φ_E of the phalloidin-rhodamine complex decreased, while that of 1,5-IAEDANS increased (Tables I and II). These effects were larger in the presence of tropomyosin. In contrast to tropomyosin, addition of troponin I or caldesmon caused a reduction in the effect of S-1 on actin conformation: in the presence of troponin I, the change in Φ_E for phalloidin-rhodamine on adding S-1 was 11% smaller than in its absence, and that for 1,5-IAEDANS was 25% smaller. In the presence of caldesmon, the corresponding changes in Φ_E were 77% and 46% smaller than in its absence. The effects of troponin I and caldesmon were not greatly affected by the presence of tropomyosin (Tables I and II). While the extent of the S-1-induced changes in the orientation of the 1,5-IAEDANS fluorophore was comparable in the presence of caldesmon and troponin I (Table II), the changes in orientation of the phalloidin-rhodamine complex upon binding of caldesmon were significantly higher than those induced by troponin I (Table I). Since 1,5-IAEDANS and phalloidin attach to different regions of the actin monomer we could determine the relative movement of these regions (segments) relative to the actin filament shaft from the difference ($\Delta\gamma$) between the angles of emission dipoles of the two fluorophores. This angle was significantly enhanced (by 34%) upon formation of

Table I

The effects of troponin I and caldesmon on the structural changes in phalloidin-rhodamine-labelled actin in ghost fibers induced by binding of myosin subfragment 1

Addition	S - 1	Φ_E(°)	$\Theta_{1/2}$	Addition	S - 1	Φ_E (°)	$\Theta_{1/2}$
None	-	39.9 ± 0.1	13.1 ± 1.0	None	-	39.9 ± 0.1	13.1 ± 1.0
None	+	39.0 ± 0.1	15.1 ± 1.0	None	+	39.0 ± 0.1	15.1 ± 1.0
TN-I	-	39.4 ± 0.1	15.2 ± 1.0	CD	-	39.3 ± 0.1	12.9 ± 1.0
TN-I	+	38.6 ± 0.1	15.9 ± 1.0	CD	+	39.1 ± 0.1	14.1 ± 1.0
TM_s	-	41.7 ± 0.2	10.9 ± 1.0	TM_g	-	39.8 ± 0.1	13.6 ± 1.0
TM_s	+	40.5 ± 0.1	15.0 ± 1.0	TM_g	+	38.6 ± 0.1	15.7 ± 1.0
TM_s-TN-I	-	40.9 ± 0.3	13.2 ± 1.0	TM_g-CD	-	39.3 ± 0.1	14.4 ± 1.0
TM_s-TN-I	+	39.7 ± 0.2	12.5 ± 1.0	TM_g-CD	+	39.6 ± 0.1	15.3 ± 1.0

The changes in Φ_E and $\Theta_{1/2}$ were calculated from fluorescence data obtained before and after incorporation of S-1 into ghost fibers containing either gizzard (TM_g) or skeletal muscle (TM_s) tropomyosin, according to [12]. TN-I, troponin I; CD, caldesmon. The average values and standard errors are for 60–80 measurements.

Table II

The effects of troponin I and caldesmon on the structural changes in 1,5-IAEDANS-labelled actin in ghost fibers induced by binding of myosin subfragment 1

Addition	S - 1	Φ_E(°)	N	Addition	S - 1	Φ_E (°)	N
None	-	52.9 ± 0.1	0.503 ± 0.006	None	-	52.9 ± 0.1	0.503 ± 0.006
None	+	55.7 ± 0.1	0.320 ± 0.012	None	+	55.7 ± 0.1	0.320 ± 0.012
TN-I	-	53.5 ± 0.1	0.449 ± 0.005	CD	-	52.8 ± 0.1	0.462 ± 0.008
TN-I	+	55.6 ± 0.1	0.359 ± 0.003	CD	+	54.3 ± 0.1	0.360 ± 0.008
TM_s	-	53.1 ± 0.1	0.457 ± 0.002	TM_g	-	52.6 ± 0.1	0.342 ± 0.009
TM_s	+	56.0 ± 0.1	0.363 ± 0.003	TM_g	+	56.4 ± 0.1	0.255 ± 0.010
TM_s-TN-I	-	53.4 ± 0.1	0.468 ± 0.003	TM_g-CD	-	54.2 ± 0.1	0.346 ± 0.009
TM_s-TN-I	+	53.9 ± 0.1	0.526 ± 0.003	TM_g-CD	+	55.7 ± 0.1	0.289 ± 0.009

The changes in the fluorescence parameters were calculated as described in the legend to Table I.

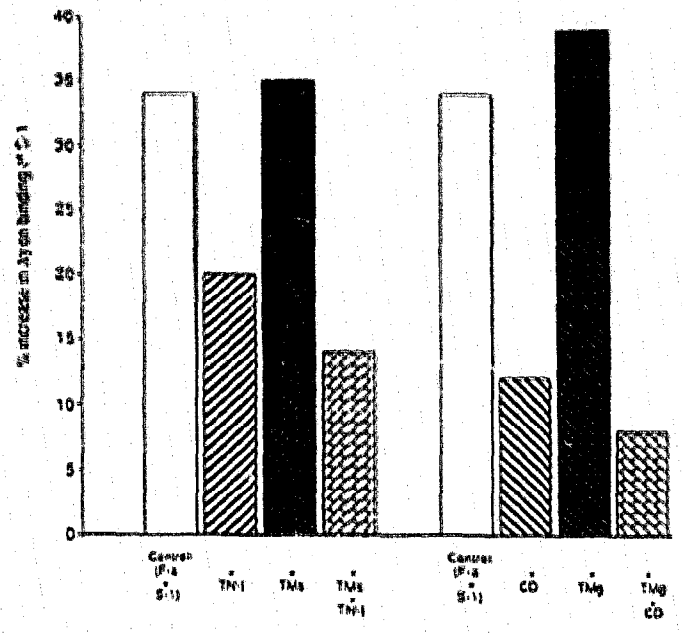

Fig. 1. The effect of troponin I and caldesmon on the changes in the relative orientation of 1,5-IAEDANS and phalloidin-rhodamine fluorophores attached to actin induced by binding of myosin subfragment 1. F-a. F-actin; other abbreviations as in legend to the Table I.
$\Delta\gamma = \Phi_{E\ (IAEDANS)} - \Phi_{E\ (phalloidin\text{-}rhodamine)}$

rigor complexes between actin and S-1; this enhancement was markedly reduced in the presence of either troponin I (20%) or caldesmon (12%) (Fig. 1). In the presence of tropomyosin it dropped to 14% and 8%, respectively. This could mean that interaction of actin with myosin heads involves rotational motions of segments in the actin monomer that are inhibited by troponin I and caldesmon. In other words, these two latter proteins immobilize actin filaments by restricting the relative motions of segments in the monomer to which fluorophores are attached. Measurements of the angle between the actin filaments and the fiber long axes ($\Theta_{1/2}$) in the case of phalloidin-rhodamine label, and of the number of randomly oriented fluorophores (N) in the case of the 1,5-IAEDANS label, show that upon interaction of actin with myosin heads the flexibility of actin filaments containing tropomyosin increases ($\Theta_{1/2}$ increases) while the mobility of the C-terminal part of the actin polypeptide chain decreases (N lower). In the presence of troponin I or caldesmon the flexibility of actin filaments markedly decreased, whereas the mobility of the C-terminus increased. The effect of troponin I here was greater than that of caldesmon (compare Tables I and II).

4. DISCUSSION

1,5-IAEDANS and phalloidin-conjugated rhodamine are fluorescent probes which, when attached to actin in skeletal muscle ghost fibers, monitor alterations in actin structure and dynamics that occur upon binding of myosin heads [20,29]. The different changes in the orientation of these two fluorophores suggest that formation of rigor links with S-1 affects their respective regions of attachment to actin monomers in distinct ways. It is known that phalloidin binds in the cleft between the two domains of actin monomer [35,36], while 1,5-IAEDANS associates with the C-terminal Cys-374 [37] located in the smaller domain [38]. Since the difference between angles of emission dipoles of these two fluorophores relative to the long axis of the actin filament increased significantly, one can suppose that during interaction of actin with myosin some relative movement of actin domains (or their segments) takes place. Such movement of actin domains was also suggested by Borovikov and Kakol [39] who studied conformational changes of actin in ghost fibers accompanying transformation of actomyosin complex from the weak-binding to the strong-binding state, as well as by Miki [40] who measured energy transfer between various points of reconstituted actin filaments in the presence and absence of Ca^{2+}. The interdomain motions in actin monomers seem to be fundamental to the generation of force since immobilization of actin filaments by crosslinking inhibited development of isometric tension upon interaction with myosin [41] and sliding movement of F-actin on heavy meromyosin that was fixed onto a glass surface [42]. The results of this work show that both troponin I and caldesmon, in the presence of tropomyosin, restrict domain motions and in this way immobilize actin filaments. This immobilization may be related either to the weakening of the actin-myosin interaction, as was shown in the case of caldesmon [29], or to the slowing of one of the kinetic steps of ATP hydrolysis, as suggested for troponin I [43], both resulting in the inhibition of actomyosin ATPase activity.

In conclusion, this study indicates that alterations in the intramonomer dynamics of actin may contribute to the mechanism of actin-linked regulation of muscle contraction.

Acknowledgements: The authors thank Drs. W. Kabsch and J. Wray for critical reading of the manuscript.

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