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Calcium modulates conformational changes in F-actin induced by smooth muscle heavy meromyosin

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Abstract The effect of Ca^{2+} on conformational changes in rhodamine-phalloidin-labeled F-actin induced by binding of smooth muscle heavy meromyosin (HMM) with either phosphorylated or dephosphorylated regulatory light chains (LC_{20}) was studied by polarized fluorimetry. LC_{20} phosphorylation caused alterations in the F-actin structure typical of the force-producing (strong-binding) state, while dephosphorylation of the chains led to alterations typical of the formation of non-force-producing (weak-binding) state of the actomyosin complex. The presence of Ca^{2+} enhanced the effect of LC_{20} phosphorylation and weakened the effect of LC_{20} dephosphorylation. These data suggest that Ca^{2+} modulates actin-myosin interaction in smooth muscle by promoting formation of the strong-binding state.

Key words: Smooth muscle; Calcium regulation; Actin conformation; Strong binding; Weak binding; Fluorescence polarization

1. Introduction

In smooth muscle cells, Ca^{2+}-calmodulin-dependent phosphorylation of the 20-kDa light chains of myosin (LC_{20}) plays a central role in the regulation of contraction [1–3]. It has been suggested that the level of LC_{20} phosphorylation regulates the rate of inorganic phosphate release from the active site of actomyosin ATPase [4]. Therefore, according to the kinetic model proposed by Eisenberg and Greene [5], LC_{20} phosphorylation-dephosphorylation affects the transition from the weak-binding actomyosin complex to the strong-binding complex. Biochemical evidence suggests that in addition to its role in LC_{20} phosphorylation, which represents the main regulatory pathway for actin-myosin interaction, Ca^{2+} has a direct effect on actin-activated ATPase activity and thus may modulate the interaction between actin and myosin [6–8]. However, the molecular mechanisms of this modulation have remained unclear.

In this study, we used polarized fluorimetry, which demonstrates high sensitivity in evaluating conformational changes in the contractile proteins [9–15] to study the effect of LC_{20} phosphorylation and Ca^{2+} on conformational changes in actomyosin accompanying tension development in smooth muscle. It is shown that phosphorylated heavy meromyosin (pHMM) induces changes in actin typical of the force-producing (strong-binding) state and dephosphorylated HMM (dpHMM) induces changes in actin typical of the non-force-producing (weak-binding) state. Ca^{2+} strengthens the effect of phosphorylation and weakens the effect of dephosphorylation of myosin regulatory light chains. Thus, LC_{20} phosphorylation regulates the relative number of cross-bridges that are in the force-producing state in the fiber, and Ca^{2+} fine-tunes this regulation.

2. Materials and methods

2.1. Preparation of proteins

Smooth muscle myosin was extracted from chicken gizzards, phosphorylated using endogenous kinase, and purified by gel filtration on a Sepharose 4B-CL agarose column [16]. HMMs were prepared according to Kaminski and Chacko [8]. Phosphorylated myosin was concentrated and digested with α-chymotrypsin followed by chromatography on a Sepharose 6B-CL column. Dephosphorylated HMM was obtained by treating the phosphorylated HMM with phosphatase prepared as described [17]. After dephosphorylation, HMM was again chromatographed on a Sepharose 6B-CL column to remove the phosphatase and then concentrated [8]. The phosphorylation levels of the column-purified pHMM and dpHMM were found to be 98–100% and 0–2%, respectively, as determined using urea gel electrophoresis [18]. Myosin subfragment 1 (S1) modified by NEM (NEM-S1) and pPDM (pPDM-S1) was prepared from skeletal muscle according to Reisler [19] and Chalovich et al. [20], respectively. Protein concentration was determined as described [21].

2.2. Preparation and labeling of ghost fibers

Ghost fibers were prepared from single glycerinated fibers of rabbit psoas muscle by extraction of myosin and the regulatory proteins as described [12]. Actin filaments were labeled with rhodamine-phalloidin as described [22] by incubation of the fibers in a standard solution (10 mM KCl, 1 mM $MgCl_2$, 6,7 mM phosphate buffer, pH 7.0) containing 40 μM fluorescent dye for 20 min at room temperature. Unbound dye was removed by washing the fibers in the standard solution for 15 min at room temperature.

2.3. Binding of HMMs, NEM-S1 and pPDM-S1 to ghost fibers

To incorporate pHMM or NEM-S1 ghost fibers were immersed for 30 min in 20 mM KCl, 3 mM $MgCl_2$, 10 mM imidazole buffer (pH 7.0) and 0.5 mg/ml of the correspondent protein. Incorporation of dpHMM or pPDM-S1 was carried out in the same solution, containing 2 mg/ml of the correspondent protein, with incubation for 2 h. Ca/EGTA buffer was used to keep the concentration of free Ca^{2+} in the incubation mixture at 10^{-5} or 10^{-7} M [23]. After washing the fibers in the standard solution for 15 min at room temperature to remove unbound protein the fibers were monitored for composition by SDS-PAGE [24]. The molar ratios of HMM, NEM-S1 and pPDM-S1 bound to actin in ghost fibers were approximately 1:10 (S.D., ±3), 1:5 (S.D., ±2), and 1:5 (S.D., ±2), respectively, as determined by densitometric scanning of the gels (UltroScan XL, Pharmacia LKB).

2.4. Fluorescence polarization measurements

Polarized fluorescence of rhodamine-phalloidin bound to F-actin in ghost fibers was measured in the standard solution before and after addition of pHMM, dpHMM, NEM-S1 or pPDM-S1 at $pCa = 5$ and $pCa = 7$. Excitation of fluorescence probe was at 489 ± 5 nm and emission was recorded at 500–600 nm. Intensities of the components of

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Abbreviations: HMM, heavy meromyosin; S1, myosin subfragment 1

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polarized fluorescence were measured in parallel $_{\parallel}I_{\parallel}$, $_{\parallel}I_{\perp}$) and in perpendicular ($_{\perp}I_{\perp}$, $_{\perp}I_{\parallel}$) orientation of the fiber axis to the polarization plane of the exciting light. Data were analyzed as described [11,25,26]. The model used is based on the assumption that oscillators of fluorophores are helically arranged in the fiber, along the cone-shaped surface. The three ratios of fluorescence intensities, $_{\parallel}I_{\perp}/_{\parallel}I_{\parallel}$, $_{\perp}I_{\perp}/_{\parallel}I_{\parallel}$ and $_{\perp}I_{\parallel}/_{\parallel}I_{\parallel}$ were considered functions of angles Φ_A, Φ_E and $\Theta_{1/2}$, respectively, where Φ_A and Φ_E are angles between the fiber axis and the absorbance and emission dipoles, respectively, and $\Theta_{1/2}$ is the angle between the F-actin filament axis and the fiber axis. The changes in Φ_A and Φ_E are interpreted in terms of structural alterations of actin monomers in the region of fluorophore location and/or their twisting in filaments [11,14,27]. Φ_A changes were found to parallel corresponding Φ_E changes in all experiments; therefore, the values of Φ_A are not presented. $\Theta_{1/2}$ is not constant, and instead fluctuates and varies along each filament due to thermal bending motion. The increase in the value of $\Theta_{1/2}$ is considered to reflect increased flexibility of actin filaments in the fibers.

The significance of the differences observed was determined by Student's *t*-test.

3. Results and discussion

In the present study rhodamine-phalloidin was used as an extrinsic fluorescent probe specifically attached to F-actin of the ghost fibers. Whereas phalloidin binding to F-actin increases the rigidity of thin filaments [28,29], it does not change the dynamic properties of the protein [30] and affects neither the tension development of glycerinated muscle fibers [30] nor the activity of actin-activated ATPase of myosin [31]. Ghost fibers are characterized by high anisotropy of polarized fluorescence emitted by the fluorophores, indicating a regular arrangement of rhodamine-phalloidin molecules and high rigidity of their bond with actin [32]. Thus, this fluorescent dye is appropriate for polarized fluorimetry experiments.

We used the complex of pPDM-S1 with F-actin as a model for the weak-binding state of actomyosin. Although pPDM-S1 itself may not be a good structural analogue of the S1-ATP or S1-ADP-P_i complexes [33], pPDM-S1 binds weakly to F-actin [20], and the orientation of pPDM-modified S1 relative to actin filament is the same as that of native S1 in the presence of ATP [34]. For consistency, a modified S1, NEM-S1, was used for the strong-binding conditions (for review, see [35]).

As shown in Table 1, the values of the angles Φ_E and $\Theta_{1/2}$ changed when pHMM, dpHMM, NEM-S1 or pPDM-S1 interacted with F-actin in the ghost fiber, consistent with the notion that F-actin interaction with each of the four proteins is accompanied by conformational changes in thin filaments. Both NEM-S1 and pHMM interaction with F-actin resulted in a decrease in Φ_E and an increase in $\Theta_{1/2}$. Consequently, the conformational state of actin in its complex with pHMM is similar to that in its complex with NEM-S1. As NEM-S1 complex with F-actin is a model of a force-producing (strong-binding) state of actomyosin (for review, see [35]), it is possible to conclude that pHMM forms strong binding with actin.

By contrast, both pPDM-S1 and dpHMM produced increased Φ_E and decreased $\Theta_{1/2}$ values (Table 1). Thus the conformational state of actin in its complex with dpHMM strongly resembles that in its complex with pPDM-S1. As pPDM-S1 binds to actin as if it formed weak bonds [20,35], dpHMM complex with actin is in a weak-binding rather than in a strong-binding state. This suggestion is supported by biochemical evidence. LC_{20} dephosphorylation was shown to decrease the affinity of HMM for actin [36,37]. Taken together, these data indicate that phosphorylation of LC_{20} shifts the character of actin-myosin interaction from weak binding to strong binding and reveal a mechanism by which LC_{20} phosphorylation drives smooth muscle contraction.

Smooth muscle contraction is accompanied by a change in the level of myosin regulatory light chain phosphorylation from dephosphorylation at relaxation to phosphorylation at activation. The initial rise in Ca^{2+} and LC_{20} phosphorylation induced by Ca^{2+}-calmodulin-dependent myosin light chain kinase is paralleled by rapid shortening and tension development. As the Ca^{2+} concentration decreases, the activity of myosin light chain phosphatase predominates over the kinase activity, and the extent of LC_{20} phosphorylation decreases. The decrease in phosphorylation correlates with the decrease in shortening velocity, while the level of force generated remains high [38,39]. This phenomenon is interpreted as the capacity of regulatory light chains to modify the velocity of the ATPase cycle through a change in the character of actin-myosin interaction. Phosphorylation of myosin LC_{20} is thought to accelerate P_i release from the complex AM-ADP-P_i, thus favoring strong binding of myosin heads to actin [4,40]. Conversely, dephosphorylation of LC_{20} inhibits P_i release, repressing the formation of the strong binding state (stage AM or AM-ADP in the ATP hydrolysis cycle). Our present data are in good agreement with this concept.

The fluctuations of the polarized fluorescence parameters of pure actin filaments caused by addition or removal of Ca^{2+} from the washing solution were not significant (Table 1). Similarly, no difference was found between the changes of the parameters resulting from NEM-S1 or pPDM-S1 binding to F-actin at p$Ca = 5$ and the corresponding changes observed at

Table 1
Alterations of rhodamine-phalloidin-labeled F-actin in ghost fibers induced by binding of pHMM, dpHMM, NEM-S1 and pPDM-S1 in the absence and presence of Ca^{2+}

Conditions		Φ_E (°)	$\Theta_{1/2}$ (°)
F-actin	p$Ca = 7$	54.4 ± 0.1	13.1 ± 0.1
	p$Ca = 5$	54.3 ± 0.1	13.2 ± 0.1
F-actin + pPDM-S1	p$Ca = 7$	54.6 ± 0.1	12.6 ± 0.1
F-actin + NEM-S1	p$Ca = 7$	53.1 ± 0.1	14.4 ± 0.1
F-actin + dpHMM	p$Ca = 7$	55.8 ± 0.1	12.4 ± 0.2
	p$Ca = 5$	54.9 ± 01	13.1 ± 0.2
F-actin + pHMM	p$Ca = 7$	53.8 ± 0.1	13.9 ± 0.2
	p$Ca = 5$	52.3 ± 0.1	15.9 ± 0.2

Φ_E, the angle of the emission dipole of the dye relative to the F-actin axis. $\Theta_{1/2}$, the angle between the F-actin axis and the fiber long axis. Data are the mean (± S.E.M.) of at least 75 measurements made on 15 ghost fibers for each experimental condition.

p$Ca = 7$ (for these experiments, only the values of Φ_E and $\Theta_{1/2}$ calculated for p$Ca = 7$ are shown in Table 1).

In contrast, the increase in Φ_E and the decrease in $\Theta_{1/2}$ resulting from dpHMM binding to F-actin were smaller in the presence of Ca^{2+} than in its absence. The decrease in the value of the angle Φ_E and the increase in the value of the angle $\Theta_{1/2}$ caused by F-actin interaction with pHMM were larger in the presence of Ca^{2+} than in its absence. These data are in a good agreement with previous biochemical evidence indicating that at least in some tissues, Ca^{2+} acts to increase Mg^{2+}-ATPase activity of smooth muscle actomyosin [6–8]. Together, these observations strongly support the notion that Ca^{2+} modulates the interaction of myosin with actin, in addition to its effect on phosphorylation.

The presence of Ca^{2+} enhances the transition of actin subunits to the state typical of strong-binding actomyosin complex, inhibiting the effect of dpHMM and intensifying the effect of pHMM on actin conformation. Thus, actin-myosin interaction is modulated by Ca^{2+} in a way that might enable some dephosphorylated cross-bridges to form the strong-binding complex with F-actin in the presence of Ca^{2+} and prevent some phosphorylated cross-bridges from forming the same complex in the absence of Ca^{2+}. The physiological role of this modulation by Ca^{2+} might be as follows. At activation, when Ca^{2+} concentrations increase faster than the level of LC_{20} phosphorylation, the presence of Ca^{2+} enables formation of the strong-binding complex even by some cross-bridges that are still unphosphorylated. This mechanism facilitates the triggering of muscle contraction by LC_{20} phosphorylation. At maximal activation, Ca^{2+} levels begin to decrease, preventing further changing of the actin filament state by phosphorylated cross-bridges and inhibiting the formation of strong binding between the phosphorylated cross-bridges and actin. This process conserves ATP and contributes to the high economy of force production in smooth muscle. At relaxation, low Ca^{2+} concentrations would decrease ATP hydrolysis, thereby facilitating the fiber relaxation.

Thus, our data indicate that both phosphorylation of myosin regulatory light chains and Ca^{2+} can change the character of the interaction of myosin heads with F-actin in muscle fibers. Phosphorylation of myosin LC_{20} causes the formation of the force-producing, strong-binding state, while dephosphorylation of the light chains leads to the non-force-producing, weak-binding state of the actomyosin complex. Ca^{2+} favors the transition of actin monomers to the state typical of strong binding and thereby strengthens the effect of phosphorylation and weakens the effect of dephosphorylation of myosin regulatory light chains.

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