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Unphosphorylated calponin enhances the binding force of unphosphorylated myosin to actin

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ABSTRACT

Background: Smooth muscle has the distinctive ability to maintain force for long periods of time and at low energy costs. While it is generally agreed that this property, called the latch-state, is due to the dephosphorylation of myosin while attached to actin, dephosphorylated-detached myosin can also attach to actin and may contribute to force maintenance. Thus, we investigated the role of calponin in regulating and enhancing the binding force of unphosphorylated tonic muscle myosin to actin.
Methods: To measure the effect of calponin on the binding of unphosphorylated myosin to actin, we used the laser trap assay to quantify the average force of unbinding (F_{unb}) in the absence and presence of calponin or phosphorylated calponin.
Results: F_{unb} from F-actin alone (0.12 ± 0.01 pN; mean ± SE) was significantly increased in the presence of calponin (0.20 ± 0.02 pN). This enhancement was lost when calponin was phosphorylated (0.12 ± 0.01 pN). To further verify that this enhancement of F_{unb} was due to the cross-linking of actin to myosin by calponin, we repeated the measurements at high ionic strength. Indeed, the F_{unb} obtained at a [KCl] of 25 mM (0.21 ± 0.02 pN; mean ± SE) was significantly decreased at a [KCl] of 150 mM, (0.13 ± 0.01 pN).
Conclusions: This study provides direct molecular level-evidence that calponin enhances the binding force of unphosphorylated myosin to actin by cross-linking them and that this is reversed upon calponin phosphorylation. Thus, calponin might play an important role in the latch-state.
General significance: This study suggests a new mechanism that likely contributes to the latch-state, a fundamental and important property of smooth muscle that remains unresolved.

1. Introduction

Tonic smooth muscle is well known to maintain force for long periods of time at low energy levels. It is generally agreed that this property of smooth muscle, called the latch-state, is due to the dephosphorylation of myosin molecules while attached to actin filaments [1]. However, evidence is accumulating to suggest that calponin, a 35 kDa [2] actin binding protein [3,4] may also play a role in smooth muscle force maintenance, presumably through its effects on unphosphorylated myosin [5–7].

Calponin has an inhibitory effect on the ATPase activity of smooth muscle myosin [8] and on the velocity (v_{max}) of actin propulsion in the in vitro motility assay [3,9]. However, contrary to caldesmon, the inhibitory effect of calponin is more of an all-or-none mechanism [10], i.e. the filaments are either moving or they are stopped. At the cellular level, it has also been shown that calponin is necessary to inhibit the slow cycling of unphosphorylated myosin, stopping the shortening and force production of resting smooth muscle [5]. The inhibitory action of calponin on the actomyosin ATPase rate is due to its binding to actin which can be suppressed by calponin phosphorylation [8]. However, calponin also binds to unphosphorylated myosin [7] so it presumably cross-links unphosphorylated myosin to actin. This interaction between calponin

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and unphosphorylated myosin is dependent on ionic strength and it gets weaker at high [NaCl] [7].

We previously demonstrated, at the single molecule level, that unphosphorylated myosin purified from both tonic and phasic smooth muscles can bind to unregulated actin filaments with a binding force of approximately $1/10^{th}$ of the force generated by phosphorylated myosin [11]. Unphosphorylated, and presumably dephosphorylated detached myosin, could therefore participate in force maintenance during the latch state. The actin regulatory proteins could possibly potentiate this binding force. Thus, in the current study, we investigated the role of calponin in the enhancement of this binding force as well as its regulation by phosphorylation.

2. Materials and methods

2.1. Proteins

Myosin was purified from pig stomach fundus following a previously published protocol [12]. For the protocols requiring myosin activation, myosin was thiophosphorylated [13]. Actin was purified from chicken pectoralis acetone powder [14] and fluorescently labeled by incubation with tetramethylrhodamine isothiocyanate (TRITC)–phalloidin (P1951, Sigma-Aldrich Canada) [15]. Turkey gizzard calponin was purified as a by-product of caldesmon purification [16]. For the protocols requiring calponin activation, calponin was phosphorylated using Ca^{2+}/calmodulin-dependent protein kinase II and MgATP (1 mol of Pi/mol of calponin) [8]. After 10 min, Ca^{2+} chelation was performed by adding 10 mM EGTA to avoid contamination of the next assays with Ca^{2+}.

2.2. Buffers

Myosin buffer (300 mM KCl, 25 mM imidazole, 1 mM EGTA, 4 mM $MgCl_2$, and 30 mM DTT; pH adjusted to 7.4); actin buffer (25 mM KCl, 25 mM imidazole, 1 mM EGTA, 4 mM $MgCl_2$, and 30 mM DTT, with an oxygen scavenger system consisting of 0.25 mg/ml glucose oxidase, 0.045 mg/ml catalase, and 5.75 mg/ml glucose; pH adjusted to 7.4); and assay buffers: the in vitro motility assay buffer consisted of actin buffer to which methylcellulose (0.5%) was added, to favor binding of myosin to actin, and MgATP (2 mM). The laser trap assay buffer consisted of actin buffer to which methylcellulose (0.3%) and MgATP (200 μM) were added.

2.3. In vitro motility assay

The velocity (v_{max}) of actin filament propulsion by myosin was measured in the in vitro motility assay as previously described [11] with minor changes. Briefly, a flow-through chamber (20 μl) was constructed from a nitrocellulose-coated coverslip and a glass microscope slide [15]. Non-functional myosin molecules were removed by ultracentrifugation (Optima ultracentrifuge L-90K and 42.2 Ti rotor, Beckman Coulter, Fullerton, CA) of myosin (500 μg/ml) with equimolar filamentous actin and 1 mM MgATP in myosin buffer. Myosin was then perfused in the flow-through chamber at a concentration of 125 μg/ml and allowed to randomly attach to the nitrocellulose for 2 min. The following solutions were then perfused sequentially in the flow-through chamber (all in actin buffer): BSA (0.5 mg/ml), unlabeled G-actin (1.33 μM) to bind to any remaining non-functional myosin and followed by MgATP (1 mM) to remove the unlabeled actin from the functional heads. Then two washes of actin buffer were followed by TRITC labeled actin (0.03 μM), with or without calponin (0.3 μM), incubated for 1 min, and finally, motility buffer. All molecular mechanics measurements were performed at 30 °C. Motility was then assessed using an inverted microscope (IX70, Olympus, Melville, NY) equipped with a high numerical aperture objective (×100 magnification Ach 1.25 numerical aperture, Olympus, Melville, NY) and rhodamine epifluorescence. An image intensified video camera (KP-E500 CCD Camera, Hitachi Kokusai Electric, Woodbury, NY, 720 × 480 resolution, 68.6 μm × 45.7 μm real frame size, 29.94 frame/s, 8 bit grayscale) was used to visualize and record the actin filament movement on computer (Custom Built by Norbec Communication, Montreal, QC) using a frame grabber (Pinnacle Studio AV/DV V.9 PCI Card) and image capturing software (AMCap software V9.20) at 29.94 Hz. v_{max} was determined from the total path described by the filaments divided by the elapsed time using our automated version of the National Institutes of Health tracking software (NIH macro in Scion Image 4.02, Scion) coded in Matlab (R2009b). Only the filaments present for at least 20% of the recorded video time (50 s) and describing a path of at least 3 μm were considered. To calculate the percentage of stopped filaments, a threshold of 0.1 μm/s was set below which, the filaments were considered immobile and moving only due to Brownian motion. This threshold was estimated by analyzing the frame-to-frame velocity of actin filaments in the absence of ATP [17]. Finally, filaments that were not moving continuously were eliminated from this analysis [10].

2.4. Laser trap assay

Our single beam laser trap assay was built around the Laser Tweezers Workstation (Cell Robotics, Albuquerque, NM) and the motility assay described above and as previously reported [11]. Briefly, pedestals were created by spraying 4.5 μm polystyrene microspheres (Polybead, Polysciences, Warrington, PA) on the coverslips before coating with nitrocellulose. 3 μm polystyrene microspheres (Polybead, Poly-sciences, Warrington, PA) coated by 30 min incubation at room temperature with N-ethylmaleimide-modified (NEM) skeletal myosin [11] were used for trapping. The perfusion of proteins and solutions in the flow-through chamber followed the same sequence as for the motility assay except that the myosin was unphosphorylated and at a concentration of 16.7 μg/ml, TRITC-labeled actin was mixed with microspheres (13×10^3 microspheres/μl) in a laser trap assay buffer, and there were no unlabeled G-actin and MgATP steps. A diode pumped Nd:YAG solid-state laser (TEM_{00}, 1.5 W, 1064 nm) was used to create the trap. To perform the assay, a microsphere visualized in bright field by a charge coupled device (CCD) camera (XC-75, Sony Corporation of America, New York, NY) was captured in the laser trap, and its position was recorded on a computer as described above. An actin filament, visualized by fluorescence imaging (described above for the motility assay) was attached to the microsphere and brought in contact with unphosphorylated myosin molecules randomly adhered to a pedestal (Fig. 1A). Contact between myosin and actin was allowed for approximately 10 s. During that time, the microsphere baseline position in the trap was recorded. The pedestal was then moved away from the trap at a slow and constant velocity of 0.5 μm/s. The microsphere initially followed the pedestal (Fig. 1B) until the force exerted on it by the trap became greater than that exerted by the myosin molecules on the actin filament. At this point, the microsphere sprang back to its unloaded baseline position in the center of the trap (Fig. 1C). The total unbinding force (Total F_{unb}) of the myosin molecules was calculated as follows:

$$\text{Total } F_{unb} = k * \Delta d \quad (1)$$

where k is the trap stiffness and Δd is the maximal displacement of the trapped microsphere from its baseline position. k was calibrated using the Stokes force (F_f) approach, as previously reported [11]. Briefly, a viscous drag was applied to a trapped microsphere by moving it at a constant velocity (v) in 0.3% methylcellulose while measurements of Δd were performed. According to Stokes' law, the frictional force exerted on spherical objects with very small Reynolds numbers is calculated as follows:

$$F_f = 6\ \pi\ \eta\ r\ \nu \quad (2)$$

where η is the dynamic viscosity and r is the microsphere radius. The viscosity of 0.3% methylcellulose was measured with a viscometer (DV-I at