A tight-binding interaction between smooth-muscle native thin filaments and heavy meromyosin in the presence of MgATP

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The binding of the Ca^{2+} -regulated native thin filaments from vascular smooth muscle to vascular smooth-muscle heavy meromyosin was measured in the presence of 3 mM-MgATP. At 25 °C and 1/0.25 binding had an affinity of 1×10^{-6} – $0.3 \times 10^{-6} \text{ M}^{-1}$ with a stoichiometry of one molecule bound to one actin monomer. The K_m for the activation of heavy-meromyosin ATPase was 20–50 μM . Thin filament–heavy meromyosin binding was not altered by Ca^{2+} (pCa 9–4) or the extent of myosin phosphorylation. With skeletal-muscle heavy meromyosin affinity was $0.023 \times 10^6 \text{ M}^{-1}$ in parallel with activation of the ATPase (K_m 54 μM). It is concluded that tight binding is specific to smooth-muscle proteins and that it is not related to the ATPase activation site.

INTRODUCTION

Smooth-muscle contraction is regulated by the intracellular Ca^{2+} concentration, which acts on Ca^{2+} -dependent switches associated with the myosin filaments (via myosin phosphorylation) and the actin-based thin filaments. It is possible to isolate from vascular and other smooth muscles native thin filaments that retain an intact Ca^{2+} -regulatory mechanism [1,2]. These filaments are made up of actin, tropomyosin and caldesmon in the molar proportions 1:0.2:0.04 [1,2] plus a Ca^{2+} -sensitizing protein [3].

Caldesmon is the central component of the Ca^{2+} -regulatory system [4,5]. The properties of purified caldesmon in synthetic models of thin filaments have been thoroughly studied [5,6]. Caldesmon acts by binding to actin or actin–tropomyosin and inhibiting activation of myosin MgATPase activity. In certain cases inhibition is found to be associated with a large increase in the binding affinity of myosin– or heavy meromyosin (HMM)–(products) complex for actin–caldesmon. A 40-fold increase in affinity was reported for the skeletal-muscle actin/gizzard muscle HMM system when gizzard caldesmon was added [7,8]. This ‘tight’ binding does not appear to be a general phenomenon: with skeletal-muscle actin/skeletal-muscle HMM only a small increase in binding affinity due to gizzard caldesmon was reported, and with skeletal-muscle myosin subfragment 1 (S-1) a decrease was found [8]. In our experience vascular smooth-muscle (vsm) caldesmon inhibited vsm actin activation of vsm thiophosphorylated HMM (HMM[SP]) but did not alter binding [9].

The general conclusion from this work is that the inhibitory action of caldesmon can be accounted for by a decrease in the rate-limiting step of actomyosin MgATPase, namely product release [9], whereas the binding interaction between HMM–(products) complex and actin–caldesmon does not correlate with inhibition. It has therefore been proposed that the tight binding could be at a separate binding site [8–10].

Much of the diversity in reported binding results may

arise from the different sources and modes of preparation of the proteins used in synthetic thin-filament experiments. In the intact smooth muscle myosin interacts with native thin filaments, and we know that these do not always behave like the synthetic model systems [6,11]. In particular calmodulin, which can confer Ca^{2+} -sensitivity on MgATPase inhibition by caldesmon [5,6] and on binding of actin–caldesmon to myosin [7,8,10], is a very poor analogue of the Ca^{2+} -sensitizing component of native thin filaments [3,6].

If tight binding is physiologically relevant it ought to occur in native thin filaments. I have therefore investigated the relationship between myosin MgATPase activation by native vsm thin filaments and its binding to the filament under different regulatory conditions. Does tight binding occur in the more physiological native thin filament system? How is it regulated and can we obtain any clues to its function?

METHODS

Native thin filaments were prepared from sheep aorta as described previously [1]. Crude aorta myosin was prepared as described by Sellers *et al.* [12]. It was thiophosphorylated as described by Heaslip & Chacko [13]. HMM was prepared as described by Sellers *et al.* [12] and purified by chromatography on Sepharose CL-4B. S-1 was prepared from column-purified aorta myosin by papain digestion as described previously [14]. Skeletal-muscle HMM was prepared as described by Weeds & Taylor [15]. Immediately before use HMM and S-1 were centrifuged for 15 min in a Beckman Airfuge to remove sedimentable material.

Measurements of MgATP hydrolysis and binding were made in 5 mM-Pipes/KOH buffer, pH 7.1, containing 2.5 mM-MgCl₂, 1 mM-dithiothreitol, 0.3 mg of serum albumin/ml, 1.5–3 μM -HMM[SP] and 0–150 μM thin filaments (concentration of actin monomers is given, based on a 62% actin content in native thin filaments [1]) at 25 °C. Samples (200 μl) were made up for each assay

point and were split into two equal portions. One half was used to measure MgATPase activity. MgATP was added to 3 mM, and after 10 min 0.5 ml of 5% (w/v) trichloroacetic acid was added to terminate the reaction. The P_i released was assayed [16]. Control samples containing thin filaments only were also run, and these values were subtracted to obtain the ATPase activity. The other half of the sample was used for binding measurements; MgATP was added to a final concentration of 3 mM, and the mixture was immediately centrifuged for 20 min in a Beckman Airfuge. The thin filaments and any bound HMM were pelleted. Samples (40 μ l) of the supernatant after sedimentation were taken and the quantity of HMM present was assayed by measuring ATPase activity in the presence of EDTA (25 min incubation at 28 °C) as described by Sellers *et al.* [17]. Aorta HMM[SP] ATPase activity in the presence of EDTA was 7–11 mol of P_i /s per mol of HMM[SP] (heads). To measure the stoichiometry of binding, measurements were made with a constant 4 μ M thin filaments and 0–16 μ M-HMM[SP]. In control experiments no HMM was sedimented in the absence of thin filaments, at least 97% was sedimented with an excess of thin filaments in the absence of ATP

Fig. 1. Comparison of activation of vsm HMM[SP] MgATPase activity by vsm thin filaments with HMM[SP] binding to the thin filaments

Measurements were made in 5 mM-Pipes/KOH buffer, pH 7.1, containing 2.5 mM-MgCl₂, 1 mM-dithiothreitol, 0.3 mg of bovine serum albumin, 3 mM-MgATP, 1.5 μ M vsm HMM[SP] (heads) and 0–100 μ M vsm thin filaments (actin monomer concentration) at 25 °C. ●, ATPase activation in the presence of 0.1 mM-Ca²⁺; ○, ATPase activation in the presence of 1 mM-EGTA; △, HMM[SP] bound to thin filaments in the presence of 0.1 mM-Ca²⁺; ▽, HMM[SP] bound to thin filaments in the presence of 1 mM-EGTA. Activation is plotted as turnover rate {mol of P_i /min per mol of HMM[SP] (heads)} and as percentage of V_{max} . The continuous line is a best fit of the data in the presence of 0.1 mM-Ca²⁺ to the Michaelis–Menten equation. The derived kinetic parameters are K_m 26 ± 5.9 μ M and V_{max} 30 ± 3.8 mol of P_i /min per mol. Binding is plotted as percentage of HMM[SP] that was sedimented with the thin filaments. The broken line is a best fit of the pooled Ca²⁺ and EGTA data to an equation of simple binding. The dissociation constant (K_d) was 2.1 ± 0.6 μ M and the maximum binding (B_{max}) was 88 ± 5%.

and an average 86 ± 15% (n = 15) of HMM was sedimented in the presence of MgATP and an excess of thin filaments. The maximum amount of MgATP hydrolysed during sedimentation under the most unfavourable circumstances was 16%. Some binding experiments were done with HMM radioactively labelled by thiophosphorylation in [γ -³⁵S]thio]ATP and assaying radioactivity in the supernatant after centrifugation; this technique produced identical results.

RESULTS

Native vsm thin filaments activated vsm HMM[SP] Mg²⁺-dependent ATPase activity in the presence of 0.1 mM-Ca²⁺. At 25 °C activation followed Michaelis–Menten kinetics with a V_{max} of 20–30 mol of P_i /min per mol of HMM[SP] (heads) and a K_m of 32 ± 13 μ M (n = 4). These values are similar to the activation due to pure vsm actin under these conditions (V_{max} 40 mol of P_i /min per mol of HMM[SP] (heads) and K_m 47 μ M; see [9]). In the absence of Ca²⁺ (1 mM-EGTA) activation was substantially inhibited. The Ca²⁺-sensitivity was comparable with that previously reported for vsm thin filaments activating vsm myosin[SP] [2,4,18].

When HMM[SP] binding was measured simultaneously with ATPase measurements it was found that substantial binding occurred at low concentrations of thin filaments. In the example shown in Fig. 1 binding reached nearly 80% of total HMM[SP] with 5 μ M thin filaments. The binding data were fitted to a simple binding equation. In 11 experiments the mean dissociation constant was 2.7 ± 1.2 μ M, which was 10–20-fold lower than the apparent K_m of the ATPase activation. This tight binding was observed both in the presence and in the absence of Ca²⁺ [(K_d in Ca²⁺)/(K_d in EGTA) 1.06 ± 0.7; n = 5]. Tight binding was also observed when

Fig. 2. Titration of vsm HMM[SP] binding to vsm thin filaments

Conditions were as for Fig. 1. Thin filament concentration was a constant 4 μ M and HMM[SP] was added in the range 0–15 μ M. Binding is expressed as HMM[SP] (heads) bound per actin monomer in the thin filaments (assuming M_r 165 000 per HMM head, M_r 42 000 per actin monomer and 62% actin content of thin filaments). The continuous line is drawn freehand through the experimental points. The broken line indicates the titration curve that would have been followed if binding of 2.1 HMM heads per actin monomer was infinitely tight.

Fig. 3. Comparison of activation of skeletal-muscle HMM Mg-ATPase activity by vsm thin filaments with HMM binding to thin filaments

Conditions were as for Fig. 1. ●, Activation of HMM ATPase in the presence of 0.1 mM- Ca^{2+} ; ○, activation of HMM ATPase in the presence of 1 mM-EGTA; △, binding of HMM in the presence of 0.1 mM- Ca^{2+} . Data are presented as in Fig. 1. The continuous line represents a best fit of the ATPase activation in the presence of 0.1 mM- Ca^{2+} to the Michaelis-Menten equation. Derived parameters are K_m $54 \pm 14 \mu\text{M}$ and $V_{\max}$ 500 ± 64 mol of P_i /min per mol of HMM (heads). The HMM-binding data gave dissociation constant (K_d) $44 \pm 6 \mu\text{M}$ and maximum binding ($B_{\max}$) $96 \pm 7\%$.

the HMM was not phosphorylated (K_d $3.6 \pm 1.2 \mu\text{M}$). In comparable experiments with HMM[SP] and pure vsm actin the dissociation constant of the HMM-(products) complex was 1–1.6 times the K_m of the MgATPase [9]. Increasing [KCl] from 0 to 100 mM at $10 \mu\text{M}$ thin filament concentration caused a 25% decrease in binding, indicating that K_d increased less than 2-fold.

The stoichiometry of tight binding was determined by measuring binding with a constant concentration of thin filaments and increasing concentrations of HMM[SP] (Fig. 2). Binding reached a plateau with two HMM[SP] heads (i.e. one molecule) bound per actin monomer. The dissociation constant derived from the titration curve was approx. $1.5 \mu\text{M}$.

Smooth-muscle S-1 activated vsm thin filaments in Ca^{2+} similarly to vsm HMM[SP] but was not as Ca^{2+} -sensitive (maximum 3-fold Ca^{2+} activation observed). In the binding assay S-1 bound to vsm thin filaments with a K_d ($18.3 \mu\text{M}$ in the presence of Ca^{2+} , $16.6 \mu\text{M}$ in the presence of EGTA) just one-third of the K_m ($55 \pm 9 \mu\text{M}$ in the presence of Ca^{2+}). Skeletal-muscle HMM activated vsm thin filaments with a $V_{\max}$ of 500 mol of P_i /min per mol of HMM (heads) and a K_m of $54 \mu\text{M}$ (Fig. 3) but exhibited very little Ca^{2+} -sensitivity (maximum 1.5-fold Ca^{2+} activation observed; four preparations assayed). In the binding assay the binding of skeletal-muscle HMM closely paralleled the activation of the ATPase (Fig. 3); there was no tight binding.

DISCUSSION

In the presence of 3 mM-MgATP, vsm HMM, both phosphorylated and unphosphorylated, binds tightly to

vsm thin filaments. The dissociation constant is 2–3 μM and the stoichiometry is one HMM molecule (two active sites) per actin monomer (Figs. 1 and 2).

Tight binding of smooth-muscle HMM-(products) complex was originally reported with synthetic filaments made of purified actin + caldesmon [7]. In common with the synthetic filaments, tight binding to native thin filaments depends on the nature of the myosin; it is essentially specific to two-headed smooth-muscle fragments but it is independent of extent of myosin phosphorylation (Figs. 1 and 3) [7,8].

Native vsm thin filaments are Ca^{2+} -regulated, but tight binding does not correlate with Ca^{2+} control of ATPase activation (Fig. 1). This result is substantially different from results reported with synthetic filaments (skeletal-muscle actin + caldesmon), where not only caldesmon inhibition but also tight binding can be abolished by Ca^{2+} and calmodulin [7,8,10]. It should be noted that this is a highly artificial system that can only Ca^{2+} -regulate actin + caldesmon under a restricted set of conditions [5,6], whereas native thin filaments are Ca^{2+} -regulated under virtually all conditions [1,3,6,11]. The discrepancies probably arise because native vsm thin filaments have a Ca^{2+} -sensitizing protein that is different from calmodulin [3].

The observations with native vsm thin filaments can only be explained by the hypothesis that tight binding does not involve the same contact sites on the thin filament as the site where myosin attaches to actin to activate MgATP hydrolysis and generate tension [8,9]. K_d is 10–20-fold less than the K_m for activation of MgATPase activity, and no plausible kinetic model can generate these two values from a single mechanism [8,9,19]. Furthermore, tight binding of HMM-(products) complex by thin filaments is not related to changes in the ATPase activity due to Ca^{2+} (acting on the thin filaments) (Fig. 1) or myosin phosphorylation. Finally, tight binding is only observed with smooth-muscle myosin and its subfragments (Figs. 1 and 3), yet both smooth-muscle and skeletal-muscle myosin MgATPase can be activated by vsm thin filaments in a Ca^{2+} -dependent manner [1,2,4,11].

In synthetic thin-filament models caldesmon is essential for tight binding, and thus it is possible that a direct binding of smooth-muscle myosin to caldesmon is involved. In support of this, several groups have shown that smooth-muscle HMM can bind to caldesmon affinity columns [8,10] or that caldesmon can bind to smooth-muscle myosin affinity columns [20]. There is also evidence for a myosin-caldesmon association in vsm thin filaments. Anti-caldesmon antibody can abolish Ca^{2+} regulation in native thin filaments activating skeletal-muscle myosin, but is blocked from doing so when smooth-muscle myosin is present [4].

This does not exclude the possibility that other thin-filament proteins could be involved in tight binding. It has been observed that the anticalmodulin drug calmidazolium [21] can abolish Ca^{2+} activation of the thin filament-skeletal-muscle myosin ATPase activity and yet has no effect on Ca^{2+} -activated thin filament-smooth-muscle myosin ATPase activity. This suggests that the Ca^{2+} -binding protein may also be involved in molecular contacts with smooth-muscle myosin either directly or indirectly via caldesmon. Furthermore, we find that the stoichiometry of tight binding of HMM-(products) complex to thin filaments is one molecule to one actin

monomer (i.e. up to 28 HMM molecules per caldesmon molecule). This observation implies either many interaction sites on every caldesmon or a co-operative conformational change that may be triggered by myosin binding to caldesmon but that also involves sites on tropomyosin and actin. The structural arrangement of actin, tropomyosin and caldesmon in the native thin filament is consistent with such a mechanism [22].

Since tight binding is a phenomenon unique to smooth-muscle contractile proteins it is interesting to make a correlation with another property unique to smooth muscles, namely the ability to maintain tonic contractures with very little ATP consumption, known as the latch state. During the latch state extent of myosin phosphorylation, Ca^{2+} concentration and shortening velocity of intact smooth muscles are close to their values in a relaxed muscle, and yet isometric tension is maintained [23–25]. Could the tight bonds formed between thick and thin filaments be responsible for latch?

The properties of tight bonds described in this paper are certainly compatible with the hypothesis. Since tight bonds are independent of Ca^{2+} concentration and extent of myosin phosphorylation, they would be expected to exist in smooth muscles under the conditions of latch. The affinity of HMM[SP]-(products) complex for thin filaments is similar to the affinity of skeletal-muscle HMM- $[\alpha\beta\text{-imido}]\text{ATP}$ complex for actin, and it has been shown that this complex is capable of sustaining tension over many hours [26]. One major problem remains for this hypothesis: in the vsm thin filament preparation that we have used tight bonds were always found, independent of Ca^{2+} concentration and myosin phosphorylation. For tight bonds to be involved in latch their formation must be controlled to allow the muscle to relax. There is some evidence that latch is controlled separately from contractility [25,27]; one can only speculate that this separate regulatory pathway is absent from my preparation.

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