# Disassembly and reconstitution of the $Ca^{2+}$-sensitive thin filaments of vascular smooth muscle

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The $Ca^{2+}$-sensitive thin filaments of aorta smooth muscle have been, disassembled into their constituent proteins, actin, tropomyosin and a 120-kDa protein. The 120-kDa protein bound to aorta actin-tropomyosin and inhibited its ability to activate myosin MgATPase. This inhibition correlated with the binding of one 120-kDa protein molecule per 29 actin monomers. Upon the addition of calmodulin to the actin-tropomyosin-120-kDa protein complex, the inhibition was relieved in $10^{-4}$ M $Ca^{2+}$ but not $10^{-9}$ M $Ca^{2+}$. The full release of inhibition was not accompanied by a full release of 120-kDa protein binding to actin-tropomyosin. A fully active, $Ca^{2+}$-sensitive aorta thin filament has thus been reconstituted from just four components: actin, tropomyosin, 120-kDa protein and calmodulin.

*Smooth muscle*    *$Ca^{2+}$ regulation*    *Calmodulin*    *Caldesmon*    *Actin*    *Tropomyosin*

## 1. INTRODUCTION

In smooth muscle the activation of myosin by actin, and hence contraction, is controlled by changes in the cytoplasmic $Ca^{2+}$ concentration in the range $10^{-7}$–$10^{-5}$ M [1]. There is widespread agreement that the phosphorylation of the 20-kDa light chain of myosin by the $Ca^{2+}$–calmodulin-dependent myosin light chain kinase is a key event in this activation [1–3]. However, various studies using intact [4,5] and chemically skinned [6,7] smooth muscle, and isolated contractile proteins [8–12] indicate that some other $Ca^{2+}$-dependent mechanism is involved in the regulation of contractile activity. We have previously purified thin filaments from aorta smooth muscle which activate skeletal muscle myosin MgATPase in a $Ca^{2+}$-dependent manner [8,9]. We can now disassemble these thin filaments and separate their three major components: actin, tropomyosin and a 120-kDa protein. These three components can be reconstituted along with calmodulin to give a thin filament which activates myosin MgATPase in a $Ca^{2+}$-dependent manner like the native thin filaments.

## 2. METHODS

Our starting material was $Ca^{2+}$-regulated thin filaments from sheep aorta, prepared according to our recent procedure [9]. Their main protein components were actin, tropomyosin and the 120-kDa protein (see fig.1A). Sheep aorta actin and the 120-kDa protein were purified from thin filaments as follows. The thin filament pellets obtained from $100-120$ g artery were homogenised in 35 ml cold 0.8 M KCl, 2 $\mu$g pepstatin/ml + inhibitor solution (2 mM dithiothreitol, 2 $\mu$g chymostatin/ml; 2 $\mu$g leupeptin/ml). The actin was sedimented at $407\,000 \times g$ for 90 min leaving the tropomyosin, 120-kDa protein, and myosin in the supernatant. The supernatant was made to 10 mM citric acid-NaOH (pH 5.5) from 1 M stock and the pH adjusted with 1 M HCl to pH 2.9–3.0. At this pH, the tropomyosin precipitated together with any remaining actin and myosin whilst most of the 120-kDa protein remained in solution. After centrifugation ($47\,800 \times g$ for 10 min) the pH of the supernatant was adjusted to 7.0 and it was then dialysed overnight against 500 ml ATPase buffer (60 mM KCl, 5 mM $MgCl_2$, 10 mM $NaN_3$, 10 mM

Fig.1. Polyacrylamide (4–30% gradient)/0.1% SDS/40 mM Tris/20 mM sodium acetate/2 mM EDTA (pH 7.4) gel electrophoresis of sheep aorta thin filament proteins. Gels were stained with 0.25% Coomassie blue and scanned by an LKB Ultrascan densitometer. (i) Native thin filament preparation [9], identified bands and band area relative to actin are: (1) myosin heavy chain, 0.05; (2) 120-kDa protein, 0.10; (3) actin, 1; (4) tropomyosin, 0.40. (ii) Purified F-actin. (iii) Purified tropomyosin. (iv) Purified 120-kDa protein.

Tris-Mes, pH 7.0) + inhibitor solution. The 120-kDa protein was further purified and concentrated by ammonium sulphate fractionation (40–50%). The 120-kDa protein was typically obtained in a yield of 7–12 mg/g thin filament protein and with a purity of 70–80% as judged by SDS gel electrophoresis (see fig.1). The principal impurities were protein bands of 80–110 kDa. These were probably degraded fragments of the 120-kDa protein since they were not present in native thin filaments and the 120-kDa protein has been found to be highly susceptible to proteolytic degradation [9]. Aorta tropomyosin and skeletal muscle myosin were prepared by standard methods [9] and bovine brain calmodulin was purified by phenyl-Sepharose affinity chromatography [13].

## 3. RESULTS

### 3.1. 120-kDa protein inhibits actin-tropomyosin cofactor activity

The vascular smooth muscle actin-tropomyosin complex can activate skeletal muscle myosin MgATPase activity at least 50-fold [9]. Low concentrations of the purified 120-kDa protein were potent inhibitors of this activation as shown in fig.2. A maximum of 95% inhibition was observed. The 120-kDa protein inhibited actin-tropomyosin activation of myosin MgATPase activity under all conditions tested (ionic strength 0.095–0.165; 25–37°C) and was independent of the $Ca^{2+}$ concentration (range $10^{-9}$–$10^{-4}$ M, fig.2). In contrast, the 120-kDa protein did not in-
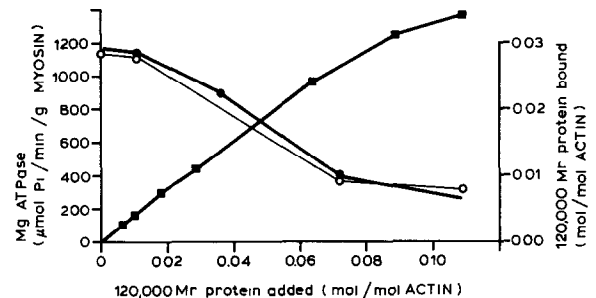


Fig.2. Binding of the 120-kDa protein to sheep aorta actin tropomyosin at $10^{-4}$ M $Ca^{2+}$ (■) and inhibition of skeletal myosin/aorta actin-tropomyosin MgATPase activity by the 120-kDa protein at $10^{-9}$ M $Ca^{2+}$ (●) and $10^{-4}$ M $Ca^{2+}$ (○). Conditions: 37°C, 70 mM KCl, 5 mM $MgCl_2$, 10 mM $NaN_3$, 10 mM Tris-Mes (pH 7.0), 0.1 mM dithiothreitol, 0.5 mg/ml (12 $\mu$M) aorta actin, 0.12 mg/ml aorta tropomyosin and 0–0.16 mg/ml (1.3 $\mu$M) 120-kDa protein. For binding measurements, 120-kDa protein was covalently labelled with iodo[$^{14}$C]acetamide [18]. Aorta actin, aorta tropomyosin and $^{14}$C-labelled 120-kDa protein were mixed in a volume of 0.25 ml. Actin-120-kDa protein complexes were separated from free 120-kDa protein by high-speed sedimentation (1.5 h at 50000 × g) [18]. Aliquots were taken for assay of radioactivity before (= total 120-kDa protein) and after sedimentation (= unbound 120 kDa protein). Difference = bound 120 kDa protein. ATPase measurements were made with the addition of 0.12 mg/ml skeletal muscle myosin. The reaction was started by adding MgATP to 2 mM and terminated after 10 min by quenching in an equal volume of 5% trichloroacetic acid. Phosphate released was assayed by the method of Taussky and Schorr [17].

hibit activation by pure smooth muscle actin filaments under comparable conditions.

### 3.2. The 120-kDa protein binds to actin, actin-tropomyosin and calmodulin

The 120-kDa protein binding to actin and actin-tropomyosin was measured by sedimentation of the actin−120-kDa protein complex using the radioactivity labelled 120-kDa protein (see fig.2). When binding was measured over a concentration range of 0.01 to 14 $\mu$M 120-kDa protein (12 $\mu$M actin-tropomyosin) the binding curve was not simple. At low concentrations, high affinity binding to actin-tropomyosin was observed ($K$ = 2 $\times$ $10^7$ $M^{-1}$, 60 mM KCl, 25°C) saturating at one 120-kDa molecule bound per 20−30 actins. Further weaker binding ($K$ = 3 $\times$ $10^6$ $M^{-1}$, 60 mM KCl, 25°C) saturated at around one 120-kDa protein per 4−7 actins. Comparison of binding and ATPase data (fig.2) shows that the inhibitory action of the 120-kDa protein correlated (correlation coefficient = 0.994) with binding of one molecule per 29 actins. The binding constant for the high affinity sites depended on ionic strength (5-fold decrease between $I$ = 0.95 and $I$ = 0.155) but was not very temperature-dependent (less than 2-fold decrease between 25°C and 37°C). The 120-kDa protein also bound to pure smooth muscle actin filaments with a similar stoichiometry, but a 4−7-fold reduction in affinity compared with actin-tropomyosin.

The 120-kDa protein bound to bovine brain $Ca^{2+}$−calmodulin coupled to CNBr Sepharose with an affinity of about $10^6$ $M^{-1}$. This binding did not occur in the absence of $Ca^{2+}$.

### 3.3. Inhibition is reversed by $Ca^{2+}$−calmodulin

When the 120-kDa protein was tested with skeletal muscle myosin and actin and aorta tropomyosin, its inhibition of MgATPase could be readily reversed by the addition of $Ca^{2+}$−calmodulin [20]. To demonstrate a similar effect of $Ca^{2+}$−calmodulin upon the system reconstituted from aorta actin, tropomyosin and the 120-kDa protein, the assay conditions had to be adjusted to favour the hydrophobic interaction of the 120-kDa protein with $Ca^{2+}$−calmodulin at the expense of its interaction with actin-tropomyosin. At 37°C and 70 mM KCl, the addition of calmodulin to the actin-tropomyosin−120-kDa protein complex produced a relief of inhibition in $10^{-4}$ M $Ca^{2+}$ but had
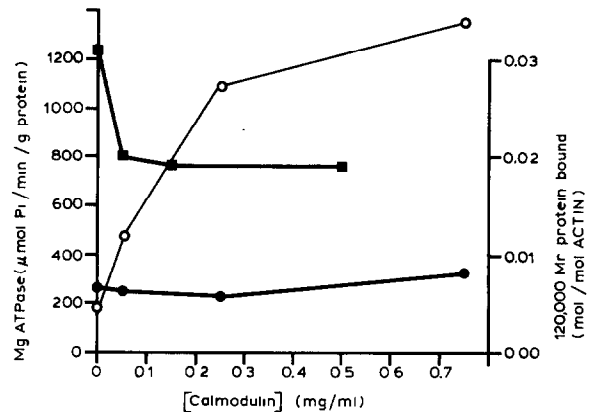


Fig.3. Release of inhibition due to 120-kDa protein by $Ca^{2+}$ and brain calmodulin. Conditions as in fig.2; 0.5 mg/ml (12 $\mu$M) aorta actin, 0.12 mg/ml aorta tropomyosin, 0.15 mg/ml (1.2 $\mu$M) 120-kDa protein, 0−0.75 mg/ml calmodulin and 0.12 mg/ml skeletal muscle myosin (ATPase measurements only). (O) MgATPase activity at $10^{-4}$ M $Ca^{2+}$. (●) MgATPase activity at $10^{-9}$ M $Ca^{2+}$. (■) 120-kDa protein binding at $10^{-4}$ M $Ca^{2+}$.

no effect in $10^{-9}$ M $Ca^{2+}$ (fig.3). Measurements of 120-kDa protein binding to actin-tropomyosin under the same conditions showed that $Ca^{2+}$−calmodulin caused a decrease in binding of up to 40%, when the relief of inhibition was complete (fig.3).

### 4. DISCUSSION

Thin filaments of vascular smooth muscle contain three major protein components: actin, tropomyosin and the 120-kDa protein. We isolated all these components (fig.1) and found that the 120-kDa protein is a potent inhibitor of actin-tropomyosin cofactor activity (fig.2). With the further addition of $Ca^{2+}$−calmodulin we reconstituted a fully $Ca^{2+}$-regulated synthetic thin filament (fig.3). Thus, 120-kDa protein plays a key role in regulation.

The stoichiometry of the actin−120-kDa protein interaction in particular, is worthy of note. $Ca^{2+}$-sensitive native aorta thin filaments contain one 120-kDa protein per 28 actins (see fig.1) [9] and full inhibition of actin-tropomyosin by pure 120-kDa protein is correlated with one 120-kDa protein bound per 29 actins (see fig.2). This ratio

is considerably higher than the 7:1 molar ratio of actin:troponin in striated muscle thin filaments [14], the only other documented system of actin-based regulation. Investigation of how 120-kDa protein works at these low ratios will be of great interest.

The calmodulin requirement in these reconstitution experiments is also of interest. Under the conditions of our assay (fig.2), the concentration of calmodulin required for full relief of inhibition in $10^{-4}$ M $Ca^{2+}$ is greater than the total tissue content of calmodulin in bovine aorta (0.15 mg/g) [19]. Furthermore, native aorta thin filaments have a low complement of calmodulin-like protein and yet are $Ca^{2+}$-sensitive [8,9]. There are two possible ways of accounting for this discrepancy. First, it is apparent that the calmodulin requirement of the reconstituted regulatory system is highly dependent upon the conditions (temperature, ionic strength) used for measurement. Second, it is possible that the native thin filaments contain a different $Ca^{2+}$-binding protein which is more potent than the brain calmodulin used in this system.

Our results suggest a hypothesis for the regulation of smooth muscle thin filaments by $Ca^{2+}$. Vascular smooth muscle thin filaments have 4 components: actin, tropomyosin, 120-kDa protein and a calcium-binding protein. The actin, tropomyosin and 120-kDa protein are present in approximate molar ratios of 1:1/7:1/28. At resting levels of $Ca^{2+}$, the actin-tropomyosin is unable to activate myosin due to the inhibitory effect of the bound 120-kDa protein. At high $Ca^{2+}$ concentrations, a $Ca^{2+}$-calmodulin-binding protein complex interacts with the 120-kDa protein, thereby altering its binding to actin-tropomyosin and releasing its inhibitory effect upon the activation of myosin.

The regulatory properties of our 120-kDa protein are very similar to those reported for the calmodulin-binding protein, caldesmon [10,23]. We have found that the 120-kDa protein and caldesmon are indistinguishable as judged by the SDS-gel electrophoresis [22], the cleavage pattern using V8 protease and antibody cross reaction (in preparation). Our hypothesis for regulation by the 120-kDa protein differs from that proposed by Sobue et al. [10], namely, a mutually exclusive binding of caldesmon, either to $Ca^{2+}$-calmodulin or actin-tropomyosin ('flip-flop' mechanism),

whilst we find a complete neutralisation of the inhibitory effect of the 120-kDa protein by $Ca^{2+}$-calmodulin associated with only partial release of the 120-kDa protein from actin-tropomyosin (fig.3). However, the results of Ngai et al. [15] with gizzard caldesmon are in agreement with our observations (fig.3).

Caldesmon promotes bundling of actin filaments [23,24] and so does the 120-kDa protein. (C. Moody, unpublished). We do not think bundling is responsible for the inhibitory effect of the 120-kDa protein since it requires much larger 120-kDa protein:actin ratios than inhibition and unlike inhibition, occurs equally well in the presence or absence of tropomyosin (C. Moody, unpublished).

The observations reported here add further weight to the contention that the contractile activity of vascular smooth muscle is controlled by separate $Ca^{2+}$-dependent mechanisms associated with both the myosin and actin filaments [1,8–10,16].

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