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# Do thin filaments of smooth muscle contain calponin?

## A new method for the preparation

Wataru Nishida, Masahiro Abe, Katsuhito Takahashi and Kunio Hiwada

*The Second Department of Internal Medicine, Ehime Univerity School of Medicine, Onsen-gun, Ehime 791-02, Japan*

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A new method for the preparation of smooth muscle thin filaments which include calponin was established. We found that calponin readily separated from thin filaments in the presence of 10 mM ATP. By preventing thin filament extract from exposing to ATP, we obtained thin filaments which contained actin, tropomyosin, caldesmon and calponin in molar ratios of 7:0.9:0.6:0.7. We studied myosin Mg-ATPase activity by using the thin filaments in comparison with classical thin filaments prepared by the method of Marston and Smith, which contained the same amounts of caldesmon and tropomyosin as our thin filaments but lost almost all calponin. The presence of calponin reduced the $V_{max}$ value for thin filament-activated myosin Mg-ATPase activity by 33% without a significant change in $K_m$ value. These findings suggest that calponin inhibits myosin Mg-ATPase activity by modulation of a kinetic step as an integral component of smooth muscle thin filaments.

Thin filament; Calponin; Mg-ATPase activity; Smooth muscle; Chicken gizzard

## 1. INTRODUCTION

There are two contractile assemblies in the myofibrils. One is thick filament and the other is thin filament. It is generally accepted that the hydrolysis of ATP drives muscle contraction by sliding both filaments each other and this process is controlled by $Ca^{2+}$ concentration. The discovery of the phosphorylation of 20 kDa light chain of smooth muscle myosin by the $Ca^{2+}$-calmodulin complex and its enhancement of Mg-ATPase activity made clear the role of thick filament regulation in the initiation of $Ca^{2+}$-dependent contraction of smooth muscle [1–5]. Time-matched measurement of sarcoplasmic $Ca^{2+}$ concentration, myosin light chain phosphorylation, stress and tension development in smooth muscle, however, did not support the view that cross bridge phosphorylation is essential for the force maintaining interaction between thick and thin filaments [6–9].

It appears that an as yet unknown regulatory system (or systems) resides in the thin filaments. Caldesmon is proposed as one of the regulatory proteins in smooth muscle thin filaments [10–13]. Furthermore, Takahashi et al. found an additional actin-binding and tropomyosin-binding component in many vertebrate smooth muscles [14,15]. This novel protein, named calponin, is postulated to be a potential regulator of thin filaments on the basis of observations that it shows immunological cross-reactivity with troponin-T [16] and it binds to tropomyosin at a specific site for the $Ca^{2+}$-sensitive binding of troponin-T to tropomyosin [17]. Calponin existed in equimolar concentration to tropomyosin in the muscle extract [14,16]. These observations suggest that calponin is an integral component of smooth muscle thin filaments. Recently, Tsunekawa et al. reported that a free form of calponin was one of the most susceptible substrate for calpain I, a $C^{2+}$-dependent cysteine endopeptidase, in the smooth muscle contractile proteins. When calponin was a bound form in thin filaments, it became markedly resistant to calpain digestion [18]. This observation also suggests that calponin appears to be a constituent of smooth muscle thin filaments.

The thin filaments were first prepared from vertebrate smooth muscle by Sobieszek, Small and coworkers [1]. Then, the method developed by Marston and Smith [19] has been the most commonly used for the preparation of smooth muscle thin filaments. Calponin, however, is lost during the preparation of thin filaments by their method and others [20].

In this report, we established a new method for the preparation of thin filaments which contain calponin. We also showed that calponin had an inhibitory effect on thin filament-activated myosin Mg-ATPase activity.

*Correspondence address:* W. Nishida, The Second Department of Internal Medicine, Ehime University School of Medicine, Onsen-gun, Ehime 791-02, Japan

*Abbreviations:* DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; Pipes, piperazine-N,N′-bis; ATP[γs], adenosine 5′-[γ-thio] triphosphate

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## 2. MATERIALS AND METHODS

### 2.1. *Preparation of smooth muscle thin filaments*

Washed myofibrils and thin filament extract of smooth muscle were prepared according to the method of Marston and Smith [19] with a slight modification. Smooth muscle of fresh chicken gizzard was cut away from the inner lining and the external connective tissue sheath. All further steps were carried out at 4°C. The muscle tissue was thoroughly fragmented by mincing twice with a meat grinder. The minced smooth muscle was then homogenized in 4 vols (volume/weight of starting minced muscles) of 10 mM imidazole/HCl buffer (pH 7.0) containing 50 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 1 mM $NaN_3$, 10 μg/ml of leupeptin, 1 mM trypsin inhibitor, 1 mM PMSF and 1 mM sodium tetrathionate dihydrate with home use blender for 30 s. The muscle residue was collected by centrifugation at 15 000 × *g* for 5 min. This washing step was repeated 5 times. Triton X-100 was added in the washing solution in case of the first three steps with decreasing its concentration from 1% to 0.5% (1, 1, 0.5%).

Thin filaments were extracted from washed myofibrils. The finally washed pellet was gently blended in 2.5 vols (v/w) of 20 mM Pipes buffer (pH 6.1) containing 80 mM KCl, 5 mM $MgCl_2$, 10 mM ATP, 1 mM EGTA, 1 mM DTT and protease inhibitors described above with blender for 3 s and was left quiet for 1 h. Then the suspension was centrifuged at 15 000 × *g* for 30 min and thin filament extract was collected in supernatant.

The extracted thin filaments were immediately dialysed against 100 vols (v/v) of 20 mM imidazole/HCl buffer (pH 7.0) containing 80 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT and 0.1 mM EGTA for 8 h at 4°C. The buffer was changed twice while dialysis for efficient elimination of ATP. After the dialysis, degenerated proteins were collected by the centrifugation at 30 000 × *g* and 30°C for 10 min. Finally, thin filaments were collected by high-speed sedimentation at 150 000 ± *g* and 30°C for 1 h. The precipitated pellet was resuspended in an appropriate buffer.

Marston and Smith's thin filaments were purified from chicken gizzard smooth muscle according to their original method [19].

### 2.2. *Preparation of reconstituted thin filaments*

Calponin was added to Marston and Smith's thin filaments to a final molar ratio of 5:1 (calponin:actin). They were incubated at 37°C for 30 min in 20 mM imidazole/HCl buffer (pH 7.0) containing 80 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT and 0.1 mM EGTA. Reconstituted thin filaments were collected by centrifugation at 150 000 × *g* and 30°C for 1 h. Densitometric analysis of SDS-polyacrylamide gel electrophoresis of the reconstituted thin filaments showed that actin, tropomyosin, caldesmon and calponin were present in molar ratios of 7:0.8:0.4:0.7.

### 2.3. *Preparations of other smooth muscle proteins*

Smooth muscle myosin was purified from chicken gizzard using the method of Persechini et al., [21] and it was thiophosphorylated according to the method of Heaslip and Chacko [22]. The extent of phosphorylation was confirmed by urea gel electrophoresis. Smooth muscle calponin was purified from chicken gizzard by the method described previously [23].

### 2.4. *Miscellaneous procedures*

SDS-polyacrylamide slab gel electrophoresis (12.5% w/v) was performed using the buffer system of Laemmli [24]. For the separation of β-tropomyosin from actin, gel electrophoresis was carried out in the presence of 2 mM $CaCl_2$ [25]. After the electrophoresis, the gel was stained with 0.25% Coomassie brilliant blue R-250 and was scanned using a microdensitometer. The molar ratios of each stained protein were estimated by the calibration with purified smooth muscle proteins. Molecular masses of actin, tropomyosin (α and β), calponin and caldesmon were taken to be 43 000, 35 000, 34 000 and 87 000, respectively. The molecular mass of caldesmon, 87 000 was based on the primary structure of caldesmon [26], although its estimated molecular mass from SDS gel electrophoresis was 120 000–150 000. Protein concentration was measured by the method of Bradford [27].

### 2.5. *Measurement of myosin Mg-ATPase activity*

Myosin used in this experiment was thiophosphorylated by ATP-[γS] to eliminate the possibility of dephosphorylation by contaminant phosphatase. We confirmed that there was no significant change in the extent of phosphorylation of myosin throughout the experimental procedures.

Myosin Mg-ATPase activity was measured at 37°C in 20 mM imidazole/HCl buffer (pH 7.0) containing 90 mM KCl, 4 mM $MgCL_2$, 1 mM DTT, 0.2 mM $CaCl_2$, 1 μM thiophosphorylated myosin and 0–70 μ thin filaments (in terms of actin monomer). Each sample was preincubated at 37°C for 5 min. The reaction was initiated by addition of ATP (final concentration, 2 mM) and terminated after 10 min by trichloroacetic acid (final concentration, 7%). Released inorganic phosphate was measured by the method of Fiske and SubbaRow [28]. Control samples containing the thin filaments alone were run at the same time for a correction of background activity of the thin filaments. To get a net ATPase activity, myosin Mg-ATPase activity in the absence of thin filaments was also subtracted from each measurement made in the presence of thin filaments.

## 3. RESULTS

### 3.1. *Thin filaments from chicken gizzard smooth muscle*

Fig. 1 shows SDS-polyacrylamide gel electrophoresis (12.5%) of two kinds of thin filaments extracted from chicken gizzard smooth muscle. Whole homogenate (Fig. 1, lane 1), washed myofibrils (Fig. 1, lane 2) and thin filament extract (Fig. 1, lane 3) contained calponin. But there was only a trace of calponin in thin filaments prepared by the method of Marston and Smith (Fig. 1, lane 5). They contained actin, tropomyosin, caldesmon and calponin in molar ratios of 7:0.8:0.5:0.01. In contrast, thin filaments prepared by our method contained substantial amounts of calponin (Fig. 1, lane 4) and the molar ratios were 7:0.9:0.6:0.7 (the same order described above). The molar ratios of major proteins in reconstituted thin filaments (7:0.8:0.4:0.7) were very close to our thin filaments.

The yields of thin filaments prepared by our method and by Marston and Smith's method were 4–6 mg and 2–3 mg per gram of minced smooth muscle tissue, respectively.

### 3.2. *Effect of thin filaments on myosin Mg-ATPase activity*

Both thin filaments activated myosin Mg-ATPase activity in a dose-dependent manner. Fig. 2 shows a representative experiment. The data were fitted to the Michaelis-Menten equation. $V_{max}$ value for our thin filaments was 162 ± 18 nmol $P_i$/min/mg of myosin ($n = 4$) and that for Marston and Smith's thin filaments was 242 ± 26 nmol $P_i$/min/mg of myosin ($n = 4$). $K_m$ value for our thin filaments was 4.2 ± 0.9 μM ($n = 4$) and that for Marston and Smith's thin filaments was 4.0 ± 0.5 μM ($n = 4$). Apparent binding constant ($K_{ATPase}$) values [29], reciprocal number of $K_m$, for our filaments and for Marston and Smith's thin filaments

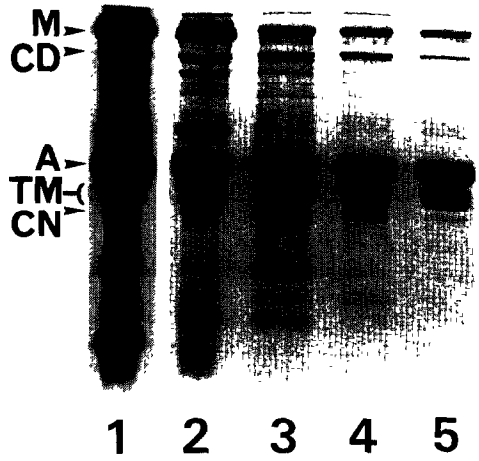


Fig. 1. SDS-polyacrylamide gel electrophoresis (12.5%). The gel included 2 mM $CaCl_2$ for the separation of $\beta$-tropomyosin from actin. (Lane 1) whole homogenate; (lane 2) washed myofibrils; (land 3) thin filament extract; (land 4) thin filaments prepared by our method; (lane 5) thin filaments prepared by Marston and Smith's method. M, myosin; CD, caldesmon; A, actin; TM, tropomyosin ($\alpha$ and $\beta$); CN, calponin.

were $2.4 \pm 0.5 \times 10^5\ M^{-1}$ and $2.5 \pm 0.2 \times 10^5\ M^{-1}$. Since our thin filaments and those of Marston and Smith contain the same amounts of caldesmon and tropomyosin in relation to actin monomer, the difference in the kinetic parameters between the two thin filaments must be due to the presence of calponin in our thin filaments. This was confirmed by the reconstitutive experiment which showed that the kinetic parameters of Marston and Smith's thin filaments supplemented by calponin in molar ratio of actin to calponin 7:0.7 were very similar to those of our native thin filaments ($V_{max}$ $171 \pm 19$ nmol $P_i$/min/mg of myosin, $K_m$ $3.8 \pm 1.1$ $\mu$M and $2.6 \pm 0.6 \times 10^5\ M^{-1}$ ($n = 4$)).

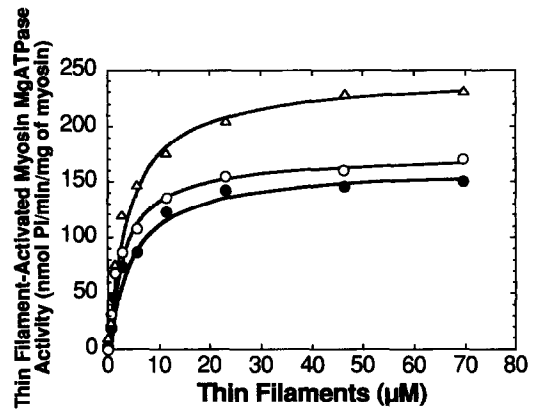


Fig. 2. Effects of 3 kinds of thin filaments on myosin Mg-ATPase activity. Conditions: 37°C, 20 mM imidazole-HCl (pH 7.0), 90 mM KCl, 4 mM $MgCl_2$, 1 mM DTT, 0.2 mM $CaCl_2$, 1 $\mu$M thiophosphorylated myosin and 0–70 $\mu$M thin filaments. Marston and Smith's thin filaments (△), thin filaments including calponin prepared by our method (●) and Marston and Smith's thin filaments supplemented by calponin (○).

## 4. DISCUSSION

Using our preparative procedures for smooth muscle thin filaments, we demonstrated that thin filaments consisting of actin, tropomyosin, caldesmon and calponin could be purified from chicken gizzard smooth muscle. Lehman has reported that calponin does not appear to be a constituent of smooth muscle thin filaments [20]. We found that this discrepancy was due to the presence or absence of ATP in the preparative procedures. A high concentration of ATP readily liberated calponin from thin filaments. We obtained successful preparations of thin filaments which contain substantial amounts of calponin by preventing thin filament extract from exposing to ATP in further steps. The amount of calponin in the thin filaments prepared by our procedures was 70 times greater than that in the widely used thin filaments prepared by the method of Marston and Smith [19]. The present result and our previous findings [14–18] indicate that calponin is an integral component of smooth muscle thin filaments and it may serve as a regulator for smooth muscle contraction. The presence of calponin in thin filaments caused 33% reduction in $V_{max}$ for the myosin Mg-ATPase activity by native thin filaments as well as reconstituted thin filaments in comparison with thin filaments which are devoid of calponin. The reduction of $V_{max}$ was achieved without a significant change in $K_m$ and $K_{ATPase}$ value. These results suggest that, like troponin-tropomyosin system in striated muscle, the inhibitory effect of calponin on Mg-ATPase activity of smooth muscle myosin is not due to a change in the affinity of myosin to thin filaments, but to a change in other steps such as phosphate and ADP release in the kinetic cycle.

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