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Influence of Trace Amount of Calponin on Smooth Muscle Myosin in Different States

Jing-Xian YANG, Xiao-Hua FENG, Ying ZHANG, and Yuan LIN*

Department of Pharmacology, Dalian Medical University, Dalian 116027, China

Abstract Calponin (CaP), a thin filament-associated protein, is thought to be involved in modulating smooth muscle contractile activity, but the role and mechanism keep unknown. In this study, trace amount of calponin (TAC) was found to obviously influence myosin in different states in Ca^{2+}-independent manner, suggesting a high efficient interaction between TAC and myosin. In this assay, the lowest ratio of CaP vs. myosin was 1:10,000, with the concentration of CaP 10,000-fold lower than that used previously. Myosin phosphorylation, myosin Mg^{2+}-ATPase activity and protein binding activity were detected to determine the effects of TAC on the myosin in different states. The amount of precipitated myosin that bound to TAC was used as the index to determine the interaction between myosin and TAC in binding assay. Our data showed that in the absence of actin, TAC significantly increased the precipitation of unphosphorylated myosin, Ca^{2+}-dependently or independently phosphorylated myosin by MLCK, and stimulated the Mg^{2+}-ATPase activities of these myosins slightly but significantly. However, no obvious change of precipitation of myosin phosphorylated by PKA was observed, indicating the relatively selective effect of TAC. In the presence of actin, the increase of myosin precipitations was abolished, and no obvious change of actin precipitations and actin-activated myosin Mg^{2+}-ATPase activities were observed implicating the high efficiency of TAC on myosin being present in the absence of actin. Although we can not give conclusive comments to our results, we propose that the high efficiency of TAC-myosin interaction is present when actin is dissociated from myosin, even if CaP/myosin ratio is very low; this high efficient interaction can be abolished by actin. However, why and how TAC can possess such a high efficiency to influence myosin and how the physiological significance of the high efficiency of TAC is in regulating the interaction between myosin and actin remain to be investigated.

Key words trace amount of calponin; protein binding; phosphorylation; myosin; Mg^{2+}-ATPase

The smooth muscle contraction and relaxation are primarily regulated by the reversible Ca^{2+}-calmodulin (CaM) dependent phosphorylation of myosin light chain catalyzed by myosin light chain kinase (MLCK) [1–5]. However, the detailed aspects of the regulation of the interaction between myosin and actin in smooth muscle contractile activity need further investigation [6–9]. Calponin (CaP), a thin filament-associated protein, was implicated to play certain role in the regulation of smooth muscle contractile activity, but what its role was and what mechanisms involved still remained under investigation. Tang *et al.* [10] and Rokolya *et al.* [11] reported that CaP might play an inhibitory role in the regulation of smooth muscle contractility through its interaction with F-actin and inhibiting the actin-activated Mg^{2+}-ATPase activity of phosphorylated myosin. Later on, Lin *et al.* [12] and Szymanski *et al.* [13] found that CaP also bound to myosin and activated Mg^{2+}-ATPase of both phosphorylated and unphosphorylated myosin in the absence of actin. Recently, a new phenomenon in our study attracted our attention: trace amount of CaP (TAC) with concentration of 10,000-fold lower than that in previous studies [12,14]

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Abbreviations: CaM, calmodulin; MLCK, myosin light chain kinase; CaP, calponin; TAC, trace amount of calponin; MLC_{20}-P, myosin light chain phosphorylation; PKA, protein kinase 3′:5′-cAMP-dependent; cAMP, adenosine 3′:5′-cyclic monophosphate; DTT, dithiothreitol; EGTA, ethylene glycol bis (2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; PMSF, phenylmethyl sulfonyl fluoride; CDPM, Ca^{2+}-CaM dependently phosphorylated myosin; CIPM, Ca^{2+}-CaM independently phosphorylated myosin

*Corresponding author: Tel/Fax, 86-411-84720652; E-mail, xiayang9999@sina.com & liny@dlmedu.edu.cn

could obviously influence smooth muscle myosin in different states in Ca^{2+}-independent manner.

In this assay, the lowest CaP/myosin ratio was 1/10,000 (mol/mol), and the myosins used in this assay included unphosphorylated myosin, phosphorylated myosin by MLCK in a Ca^{2+}-dependent way or Ca^{2+}-independent way, and phosphorylated myosin by PKA in a Ca^{2+}-independent way. Binding assay, myosin phosphorylation determination, and Mg^{2+}-ATPase measurement were used to explore the effects of TAC on the myosins of different states in the presence and absence of actin.

Materials and Methods

Materials

Protein kinase 3′:5′-cAMP-dependent (PKA), adenosine 3′:5′-cyclic monophosphate (cAMP), ATP, calmodulin (CaM), dithiothreitol (DTT) and phenylmethyl sulfonyl fluoride (PMSF) were purchased from Sigma. Ethylene glycol bis (2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) was from Wako. Scp70H centrifuge was made by Hitachi and UV-120-02 spectrophotometer was the product of Shimadzu.

Purification of CaP, myosin, MLCK and actin

CaP [15], myosin [16–19] and MLCK [20–22] were prepared from fresh chicken gizzard smooth muscle as described previously. Actin was purified from acetone powder of chicken breast muscle as previously described [23].

Myosin light chain phosphorylation

Ca^{2+}-CaM dependent or independent myosin light chain phosphorylation (MLC_{20}-P) catalyzed by MLCK was carried out according to the method described previously [5,24,25]. For MLC_{20}-P catalyzed by PKA, the assay condition was as described in reference [26] with slight modification in that the final concentration of PKA was 12.9 μg/ml and cAMP was 2 μM respectively. The myosin phosphorylated by MLCK or by PKA was used in the binding assays.

Binding assay

The amount of the precipitation of myosin was used as the index to determine the interaction between myosin and TAC in our assay. CaP from 0.0001 μM to 10 μM was mixed with 1 μM myosin respectively in the absence or the presence of actin. Myosins used in this assay were unphosphorylated myosin, Ca^{2+}-CaM dependently phosphorylated myosin (CDPM) by MLCK, Ca^{2+}-CaM independently phosphorylated myosin (CIPM) by MLCK, and CIPM by PKA respectively. As control assay, 0.0001 μM to 10 μM CaP was mixed with 1 μM actin respectively in the absence of myosin. The mixtures of CaP with myosin, CaP with myosin/actin, and CaP with actin were incubated respectively at 25 °C for 10 min in buffer (20 mM Tris-HCl, pH 7.4, 1 mM DTT, 5 mM $MgCl_2$, 60 mM KCl, 2 mM EGTA and 0.5 mM ATP), and then centrifuged in a rotor (Beckman) at 4 °C and 140,000 *g* for 25 min. The precipitate and supernatant were separately subjected to sodium dodecyl sulfate gel electrophoresis. Myosin that bound to CaP was recovered from precipitate by centrifugation [12]. The amounts of myosin in supernatant and in pellet were quantified by densitometry using Scoin image software, a densitometry software from Scoin Co., Ltd. [5,27].

Measurement of myosin Mg^{2+}-ATPase activity

0.00004 μM to 0.4 μM CaP was mixed with 0.4 μM myosin respectively. Myosins used include unphosphorylated myosin, CDPM by MLCK, CIPM by MLCK and CIPM by PKA respectively in the absence or the presence of actin. The measuring of Mg^{2+}-ATPase activity of myosin was carried out in a mixture comprising 60 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 2 mM EGTA, 0.5 mM ATP and 20 mM Tris-HCl (pH 7.5) at 25 °C for 10 min by using the malachite green method [27,28].

Other procedures

Protein concentrations were determined with the method of Bradford [29] using bovine serum albumin as a standard. For calculation of the molarity of the proteins, the molecular weight of each protein used was as following: myosin, 440 kD; MLCK, 108 kD; actin, 42 kD; CaP, 34 kD; CaM, 17 kD. The data were expressed as $\bar{x} \pm s$ and Student's *t*-test was used to evaluate the statistical significance.

Results

TAC enhancing the precipitations of myosin in different states with the absence of actin

To observe the effects of TAC on myosin in different states with the absence of actin, we mixed 0.0001 μM to 10 μM CaP with 1 μM myosin in different states at 25 °C for 10 min, and then got them centrifuged. The results (Fig. 1) showed that CaP in the range of 0.0001 μM to 10

μM significantly increased the unphosphorylated myosin (A), CDPM (B) and CIPM (C) by MLCK. Even when 0.0001 μM CaP was applied, the precipitation increased by 64.52 % for unphosphorylated myosin, 6.13 % for CDPM by MLCK and 50.21% for CIPM by MLCK, compared with the corresponding control respectively [Fig. 1(E), a–c, *P<0.05, **P<0.01 vs. control]. Moreover, the myosin precipitation increased with the increasing of CaP concentration. However, CaP of 0.0001–10 μM had no influence on the precipitation of myosin phosphorylated by PKA in the absence or the presence of CaP [Fig. 1(D); Fig. 1(E), d, $^{\#}P$>0.05 vs. control]. These results indicated that TAC significantly increased the precipitation of myosin unphosphorylated and phosphorylated by MLCK in the absence of actin, even CaP/myosin ratio was very low, but it could not increase the precipitation of myosin phospho-

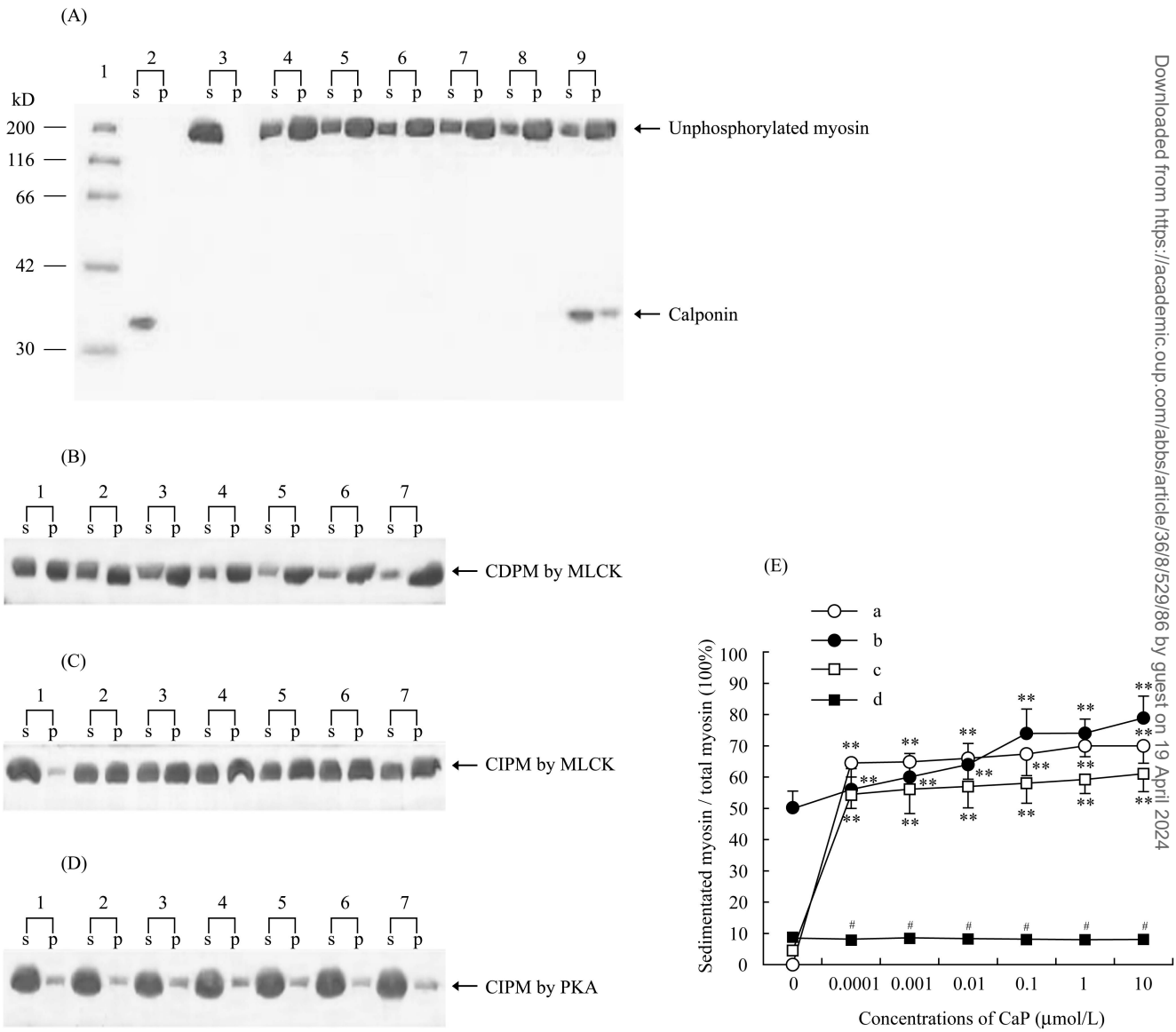

Fig. 1 The influence of TAC on the precipitation of myosins in the absence of actin

CaP at 0.0001 μM to 10 μM were incubated with 1 μM myosin in the absence of actin. S, supernatant; P, pellet. In (A): 1, molecular weight standard; 2, 10 μM CaP; 3, 1 μM unphosphorylated myosin; 4–9, 1 μM unphosphorylated myosin + 0.0001, 0.001, 0.01, 0.1, 1 or 10 μM CaP, respectively. In (B–D): 1, 1 μM CDPM by MLCK for (B); 1 μM CIPM by MLCK (C), and 1 μM CIPM by PKA for (D), respectively; 2–7, 1 μM corresponding myosin + 0.0001, 0.001, 0.01, 0.1, 1 or 10 μM CaP, respectively. (E) The amounts of the myosins in supernatant and in pellet are quantified by densitometric scanning, and the percentages of sedimented myosins (sedimented myosin vs. total myosin) are plotted against the concentration of the CaP. a, unphosphorylated myosin; b, CDPM by MLCK; c, CIPM by MLCK; d, CIPM by PKA. Data were represented as $\bar{x} \pm s$, n=6. *P<0.05, **P<0.01, $^{\#}P$>0.05 vs. corresponding control without CaP.

rylated by PKA, which suggested that the effect of TAC on myosin had high efficiency and selectivity.

TAC showing no significant influence on actin precipitation in the absence of myosin

To determine whether TAC influenced actin as it affected myosin, we centrifuged 1 μM actin with 0.0001 μM to 10 μM CaP respectively in the absence of myosin. It was shown in Fig. 2(A,B) that no obvious change of actin precipitation was observed in the presence of CaP from 0.0001 μM to 0.1 μM ($^{\#}P>0.05$). When the CaP concentration increased to higher than 1 μM, the amount of precipitated actin increased significantly ($*P<0.05$, $**P<0.01$). These results showed that TAC with CaP/actin ratio within 10^{-5}–0.1 could not influence actin obviously as it influenced myosin.

The influence of TAC on the precipitation of myosins was abolished by actin

In order to make clear whether the effect of TAC on myosin was affected by actin, we mixed 0.0001 μM to 10 μM CaP with both 1 μM actin and 1 μM myosin, and then got them centrifuged. The results showed that, in the presence of actin, CaP from 0.0001 μM to 0.1 μM could not obviously influence the precipitation of 1 μM myosin unphosphorylated [Fig. 3(A)] and phosphorylated by PKA [Fig. 3(B)], and no significant statistical change was observed [Fig. 3(C), $^{\#}P>0.05$ vs. control]. When the

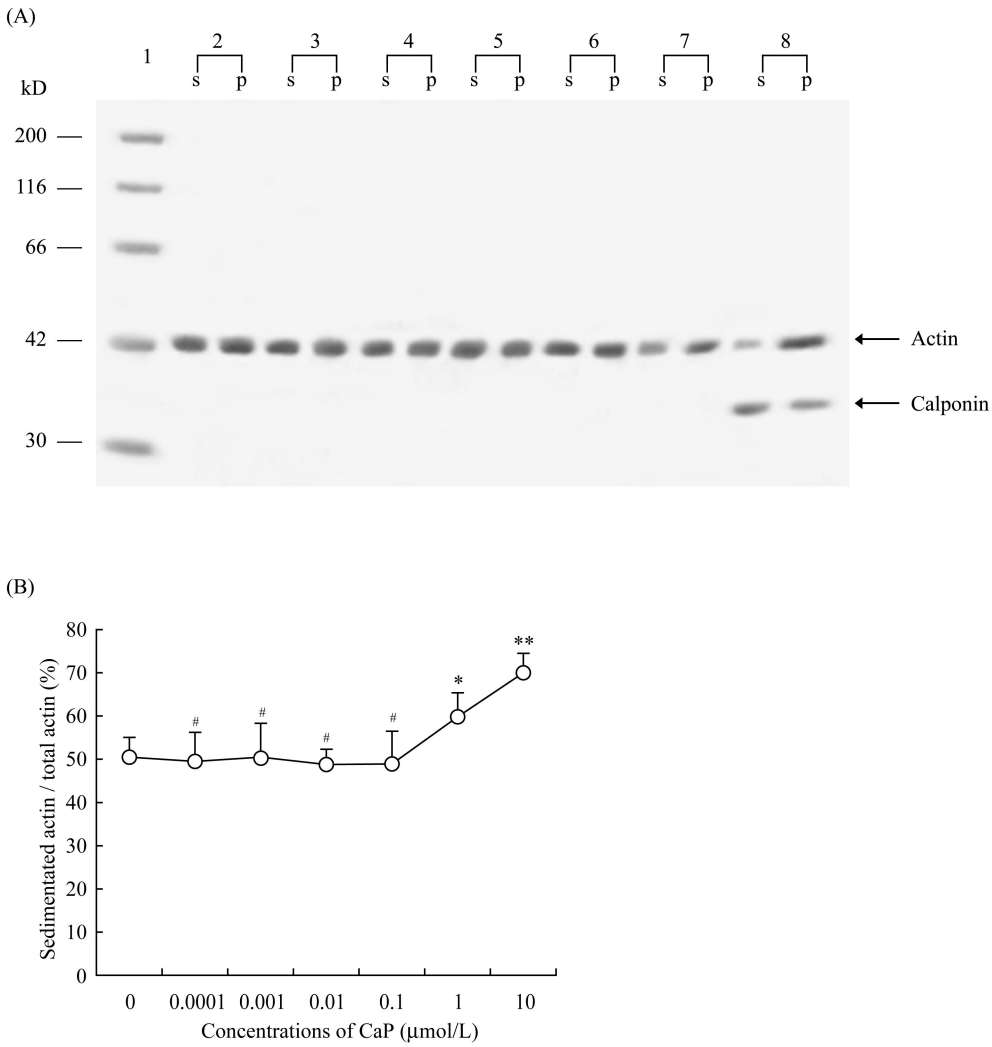

Fig. 2 The influence of TAC on actin precipitation in the absence of myosin

0.0001 μM to10 μM CaP were incubated with 1 μM actin in the absence of myosin. In (A): 1, molecular weight standard marker; 2, 1 μM actin; 3–8, 1 μM actin mixed with CaP 0.0001, 0.001, 0.01, 0.1, 1 and 10 μM respectively. (B) The amounts of actin in supernatant and in pellet are quantified by densitometric scanning, and the percentages of sedimented actin (sedimented actin vs. total actin) are plotted against the concentration of the CaP. Data were represented as $\bar{x} \pm s$, $n=6$. $*P<0.05$, $**P<0.01$, $^{\#}P>0.05$ vs. control without CaP.

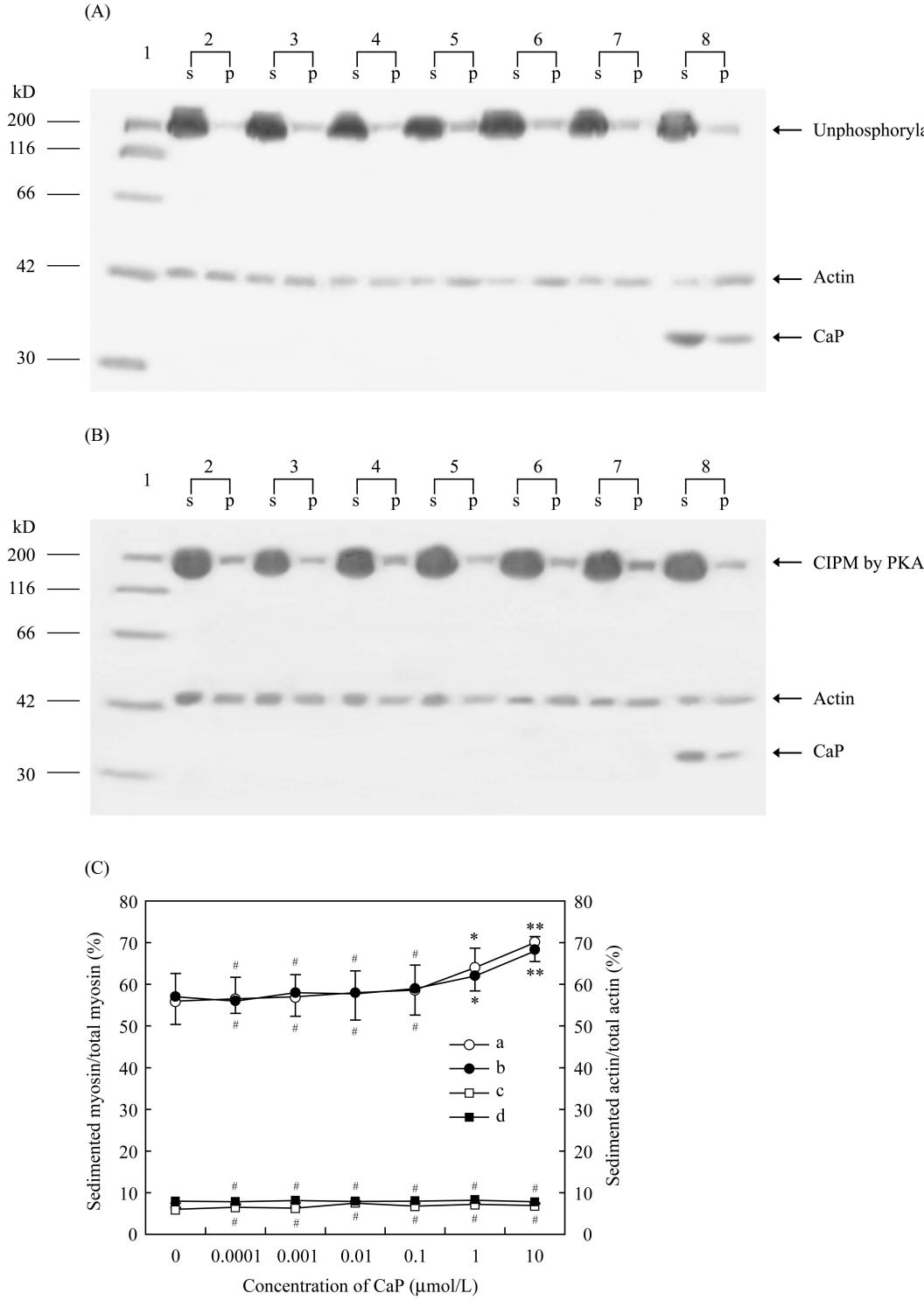

Fig. 3 The influence of TAC on the precipitations of myosin and actin in the presence of both myosin and actin

0.0001 μM to 10 μM CaP was incubated with 1 μM myosin and 1 μM actin. In (A,B): 1, molecular weight standards; 2, 1 μM actin + 1 μM unphosphorylated myosin (A) or CIPM by PKA (B), respectively; 3–8, 1 μM actin + 0.0001, 0.001, 0.01, 0.1, 1 or 10 μM CaP + 1 μM unphosphorylated myosin (A) or CIPM by PKA (B), respectively. (C) The percentages of sedimented actin (sedimented actin vs. total actin) and sedimented myosin (sedimented myosin vs. total myosin) quantified by densitometric scanning are plotted against the concentration of the CaP. a, sedimented actin over total actin in the mixture comprising unphosphorylated myosin; b, sedimented actin over total actin in the mixture comprising CIPM by PKA; c, sedimented unphosphorylated myosin over total unphosphorylated myosin in the presence of actin; d, sedimented CIPM by PKA over total CIPM by PKA in the presence of actin. Data were represented as $\bar{x} \pm s$, n=6. **P*<0.05, ***P*<0.01, #*P*>0.05 vs. corresponding control without CaP.

concentration of CaP was over 1 μM, it increased actin precipitations significantly (*P<0.05, **P<0.01) rather than myosin precipitations. The influence of TAC on the precipitations of both CDPM and CIPM by MLCK in the presence of actin was the same as that of the myosin unphosphorylated or phosphorylated by PKA (data not shown). These results showed that the highly efficient TAC-myosin interaction was abolished by actin, implicating this high efficiency of TAC on myosin only appeared when actin was dissociated from myosin.

TAC stimulating the Mg^{2+}-ATPase activity of myosins in the absence of actin

We examined how TAC in 0.00004 μM to 0.4 μM modulated the Mg^{2+}-ATPase activity of 0.4 μM myosin of different states in the absence of actin. The results (Fig. 4) showed that even 0.00004 μM CaP could slightly but significantly stimulate the Mg^{2+}-ATPase activity to 1.13-fold of unphosphorylated myosin (a), 1.089-fold of CDPM by MLCK (b), 1.095-fold of CIPM by MLCK (c) and 1.192-fold of CIPM by PKA (d) as compared to the corresponding controls without CaP. With the increase of CaP concentration to 0.4 μM, the maximal Mg^{2+}-ATPase activities of the myosins reached 1.19-fold (a), 1.35-fold (b), 1.38-fold (c) and 1.68-fold (d) respectively. It must be noted that the extent of stimulation was not large, but statistically significant (*P<0.05, **P<0.01). This indicated that in the absence of actin, TAC stimulated the Mg^{2+}-ATPase activities of the four states of myosin slightly but significantly. The result also showed that the stimulation of myosin Mg^{2+}-ATPase activity might not be directly correlated with the increase of myosin precipitation, since TAC enhanced the Mg^{2+}-ATPase activity of CIPM by PKA but didn't influenced its precipitation.

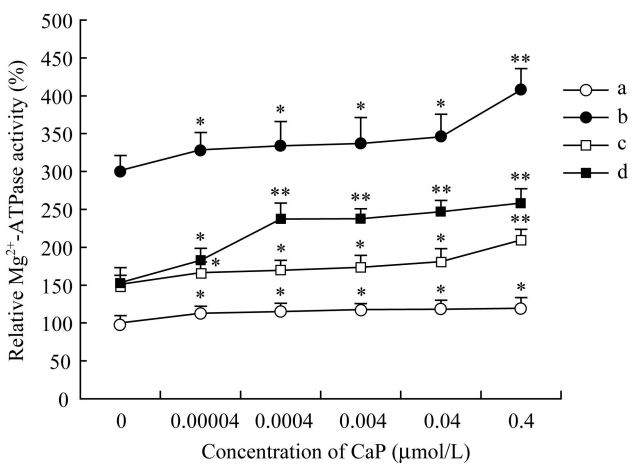

Fig. 4 The effects of TAC on Mg^{2+}-ATPase activity of myosins in the absence of actin

The relative Mg^{2+}-ATPase activity of myosins in the absence of actin is plotted against the concentration of CaP. The Mg^{2+}-ATPase activity of unphosphorylated myosin without CaP is calculated as 100% and the others are relative value comparing to it. a, unphosphorylated myosin; b, CDPM by MLCK; c, CIPM by MLCK; d, CIPM by PKA. Data were represented as $\bar{x} \pm s$, n=6. *P<0.05, **P<0.01 vs. corresponding control without CaP.

TAC showing no influence on actin-activated myosin Mg^{2+}-ATPase activity

To observe the effect of TAC on the myosins Mg^{2+}-ATPase activities in the presence of actin, we mixed 0.4 μM actin with 0.4 μM myosin in different states and measured the Mg^{2+}-ATPase activity of myosins, then added 0.00004 μM to 0.4 μM CaP into the mixture and measured the Mg^{2+}-ATPase activity again. The results (Fig. 5) showed that in the absence of TAC, actin significantly enhanced the Mg^{2+}-ATPase activity to 1.28-fold for unphosphorylated myosin (a), 1.38-fold for CDPM by MLCK (b), 1.71-fold for CIPM by MLCK (c) and 2.57-fold for CIPM by PKA (d) respectively (*P<0.05, **P<0.01). After adding TAC to the mixtures (CaP/myosin ratio from 1/10,000 to 1/1), no obvious change of actin-activated Mg^{2+}-ATPase activities of the four states of myosin was observed ($^{\#}P$>0.05). This result indicated that TAC could not in-

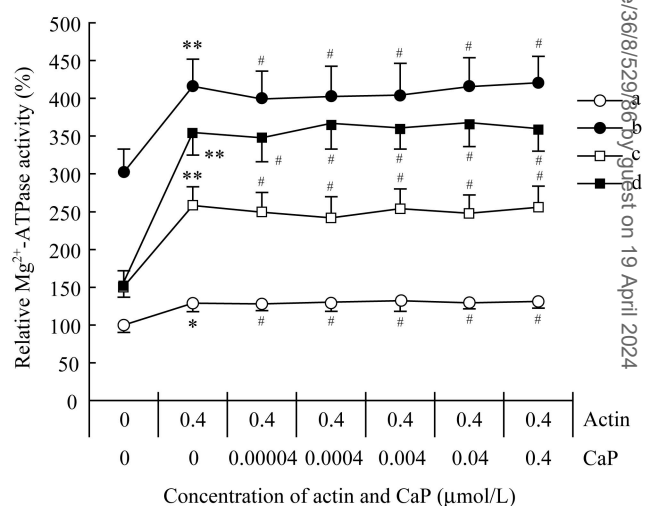

Fig. 5 The effect of TAC on actin-activated myosin Mg^{2+}-ATPase activity

The relative Mg^{2+}-ATPase activity of myosins activated by actin is plotted against the concentrations of actin and CaP. The Mg^{2+}-ATPase activity of unphosphorylated myosin without CaP and actin is calculated as 100%, and the others are relative value comparing to it. a, unphosphorylated myosin; b, CDPM by MLCK; c, CIPM by MLCK; d, CIPM by PKA. Data were represented as $\bar{x} \pm s$, n=6. *P<0.05, **P<0.01 vs. corresponding control without actin and CaP; $^{\#}P$>0.05 vs. corresponding control with actin but without CaP.

fluence actin-activated myosin Mg^{2+}-ATPase activity.

Discussion

In this study, we used TAC to examine its effects on the myosin in different states. Our results showed that in the absence of actin, TAC significantly increased the precipitations of unphosphorylated myosin, CDPM and CIPM by MLCK, and stimulated the Mg^{2+}-ATPase activities of these myosins slightly but significantly. The lowest CaP/myosin ratio in the assay was 1/10,000 (mol/mol), i.e., 10,000-fold lower than the lowest CaP/myosin ratio used in previous studies in our and other laboratories (1/1, mol/mol) [12,14,30]. Our results suggest that the high efficiency of TAC-myosin interaction exists when actin is dissociated from myosin, even if CaP/myosin ratio is very low. We hypothesize the possible mechanism involved in the increasing of myosin precipitation is the change of myosin's spatial configuration induced by TAC, which made the myosins easy to be polymeric. However, whether this proposal is true and how this high efficient interaction regulates the function of myosin need further investigation.

The high efficiency of the interaction between TAC and myosin was confirmed by the control assay that albumin, a non-myosin-binding protein, even in the same concentration as myosin (mol) did not affect myosin precipitations in any states tested for TAC (data not shown).

We also used PKA which phosphorylated MLC_{20} in a Ca^{2+}-CaM independent way [26] as a reference to reveal the characterization of the effects of TAC on myosin in different states. The results showed that TAC significantly enhanced its Mg^{2+}-ATPase activity of myosin phosphorylated by PKA, but did not increase its precipitation, suggesting that the enhancement of myosin Mg^{2+}-ATPase activity might not be directly correlated with the increase of myosin precipitation. The phenomenon that TAC increased the precipitations of myosin unphosphorylated and phosphorylated by MLCK but did not increase the precipitation of myosin phosphorylated by PKA in the absence of actin suggested the relative selective effect of TAC.

In the absence of myosin, TAC could not influence actin precipitation obviously in the range of CaP/actin ratio from 1/10,000 to 1/10; In the presence of both myosin and actin (CaP:myosin:actin being 1:10,000:10,000 to 1:10:10), the increase of myosin precipitation was abolished, and no obvious change of actin precipitations and actin-activated myosin Mg^{2+}-ATPase activities were observed. These results also support the high efficiency of TAC on myosin when actin is dissociated from myosin.

To explain the physiological significance of the high efficiency of TAC, we hypothesize that TAC is involved in the regulation of the interaction between myosin and actin in the three interrelated states. (a) Initial Ca^{2+}-dependent fast contraction state (normally cycling cross-bridge characterized by strong interaction between CDPM and actin [1–4]); in this state, the high efficient interaction between TAC-CDPM is abolished by actin, and no obvious interaction between TAC-actin was observed. (b) Ca^{2+}-independent sustained tension keeping state (slowly cycling "latch" cross-bridge characterized by weak interaction between CIPM and actin [5]); the effect produced from TAC-CIPM interaction is abolished by actin and no obvious TAC-actin interaction was observed. (c) Ca^{2+}-independent relaxation state (myosin and actin in dissociation state); the high efficiency of TAC is present in this state via interaction with myosin and is to keep smooth muscle not over relaxed. However, whether the hypothesis is true needs further study.

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Edited by
Zu-Xun GONG