Influence of an actin-modulating protein from smooth muscle on actin–myosin interaction

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The actin-modulating protein from pig stomach smooth muscle (PSAM) which reduces the average filament length has two opposite effects on the interaction of actin with skeletal muscle myosin: (1) stimulation of both the Mg^{2+}-ATPase activity and superprecipitation at low KCl concentrations, and (2) inhibition of these two interrelated processes at an ionic strength close to physiological. Both stimulation and inhibition were Ca^{2+}-dependent, reflecting the requirement for Ca^{2+} for the interaction of the modulator with actin. With acto-subfragment-1, only inhibition of the actin-activated ATPase was observed. Possible implications of these effects for studies on the regulation of smooth muscle contraction are discussed.

Smooth muscle *Actin-modulating protein* *Actin–myosin interaction*

1. INTRODUCTION

It has been shown recently that vertebrate smooth muscle contains an actin-binding protein which affects the polymer state of actin in a Ca^{2+}-dependent manner. This actin modulator has been purified from pig stomach smooth muscle (PSAM = pig stomach actin modulator) [1]. In the presence of micromolar [Ca^{2+}] it binds to either G- or F-actin, nucleates polymerization, caps filament ends and severs F-actin, thereby reducing actin filament length in substoichiometric amounts. PSAM is similar to gelsolin from macrophages and other proteins of this type from non-muscle sources [2]. Corresponding proteins have also been detected in chicken gizzard [3] and aorta smooth muscle (unpublished) as contaminants of actin preparations indicating the actin modulators of this type may be generally present in vertebrate smooth muscle.

In the light of the still existing controversy on the mechanism of the Ca^{2+}-dependent regulation of vertebrate smooth muscle the presence of an actin-associated Ca^{2+}-sensitive protein in this muscle appears to be of special interest. Though the concept of myosin phosphorylation as the regulatory mechanism has now been widely accepted and is supported by the majority of published data (reviews [4,5]), several groups of workers raised the possibility of additional activation factors. In the search for actin-linked regulatory factors the activation of the Mg-ATPase of skeletal myosin by crude thin filament preparations from various smooth muscle types has recently been reinvestigated. Authors in [6] observed a Ca^{2+}-regulated activation of skeletal muscle myosin by thin filaments from pig aorta, whereas the activation by thin filaments from turkey gizzard, in agreement with earlier findings [7], was Ca^{2+}-insensitive. Authors in [8] have isolated a protein fraction from thin filament preparations of chicken gizzard muscle which activated skeletal actomyosin in a Ca^{2+}-independent manner, but a hybrid complex of the chicken gizzard thin filaments with skeletal muscle myosin was Ca^{2+}-sensitive. Similarly a Ca^{2+}-dependent activation of actomyosin reconstituted from chicken gizzard myosin and skeletal muscle actin by a protein fraction from

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actin-enriched pellets obtained from gizzard actomyosin has been reported [9]. As the modulation of actin filament length is likely to cause secondary effects on various actin-linked processes we have used PSAM as a tool to study the influence of actin filament length on these processes. Here, we show that depending on the ionic conditions and the molar ratio of PSAM and actin, PSAM may have both activating and inhibitory effects on the interaction of skeletal muscle myosin and actin. Our experiments show that PSAM modifies the Mg^{2+}-ATPase activity and the superprecipitation of actomyosin in a Ca^{2+}-dependent way. Since the modulator is effective at low molar ratios to actin and is likely to be present in thin filament preparations from smooth muscle, its effects may be relevant to the in vitro studies on the regulation of smooth muscle contraction.

2. MATERIALS AND METHODS

2.1. *Protein preparations*

All experiments were performed on actomyosin reconstituted from actin and myosin preparations from fast skeletal muscle of the rabbit. Actin was prepared according to [10] with an additional gel filtration on Sephadex G-150. Myosin and its subfragment-1 were obtained as in [11]. The actin modulator from pig stomach smooth muscle (PSAM) was prepared as in [1].

2.2. *Experimental procedures*

In all experiments G-actin was first mixed with PSAM and polymerized with 0.1 M KCl at actin concentrations of 1.3–1.8 mg/ml. Then myosin or subfragment-1 was added and the samples were diluted with other reagents to the required assay volumes. ATPase assays were carried out at 25°C. The reaction was initiated by adding (Mg)ATP (equimolar mixture of $MgCl_2$ and ATP adjusted to pH 7.0 with KOH) and terminated by addition of trichloroacetic acid at a final concentration of 5% (w/v). Inorganic phosphate was determined by the method [12].

Superprecipitation was followed by recording changes in absorbance at 550 nm after addition of MgATP to the actomyosin suspension [13]. Samples were equilibrated at 25°C before starting the reaction and then the reaction was carried out at room temperature.

Concentration of myosin (dissolved in 0.5 M KCl) was calculated from ultraviolet absorbance with $A^{1\%}_{278}$ value of 4.5 cm^{-1}. Concentration of other proteins was detrmined with the biuret reagent [14].

Samples for electron microscopy were prepared by negative staining with 1% uranyl acetate on copper grids covered with a carbon-coated parlodion film. Before use the grids were rendered hydrophilic by irradiation with ultraviolet light. The samples were examined and photographed in a JEOL JEM 100B electron microscope operated at 80 kV.

3. RESULTS

Since the kinetics of ATP hydrolysis by actomyosin suspensions deviates from linearity [15], we first investigated the effect of PSAM on the time course of phosphate liberation. As shown in fig.1, in the presence of Ca^{2+} the hydrolysis rate at

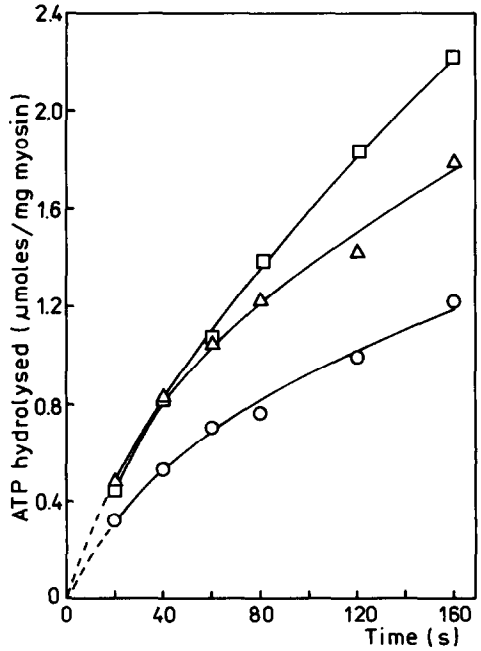

Fig.1. Effect of PSAM on the time course of actin-activated ATP hydrolysis by myosin. The reaction mixtures contained: 14 mM imidazole-HCl buffer (pH 7.0), 32 mM KCl, 0.1 mM $CaCl_2$, 0.55 mM MgATP, 0.165 mg/ml myosin, 0.108 mg/ml actin, and PSAM at a molar ratio to actin of 1:200 (△), 1:25 (□); (○) no PSAM.

low ionic strength (32 mM KCl) decreased with time over the early stage of the reaction regardless of whether actin had been polymerized in the presence or absence of the modulator. With actin polymerized in the presence of PSAM the ATPase rate, however, was already significantly higher at a modulator to actin molar ratio as low as 1:200, with an optimal activation at 1:25. No effect of PSAM was observed when 1 mM EGTA was substituted for $CaCl_2$ in the assay mixture (not shown). In the presence of Ca^{2+}, the modulator also increased, in a concentration-dependent manner, both the initial rate and final extent of superprecipitation (fig.2) the effect being maximal at a modulator to actin molar ratio of 1:25. On the other hand, under similar ionic conditions the actin activation of the Mg^{2+}-ATPase of myosin subfragment-1 was progressively decreased with increasing amounts of PSAM (table 1). A slight but significant decrease of activation was already observed at a PSAM to actin molar ratio of 1:400. At a 1:2 molar ratio the actin activation was practically zero, indicating that the tight 1:2 complex of PSAM and actin [1] is not able to activate myosin ATPase. At a slightly higher KCl concentration the relative decrease of actin activation by PSAM was substantially similar though the absolute ATPase rates were lower (table 1).

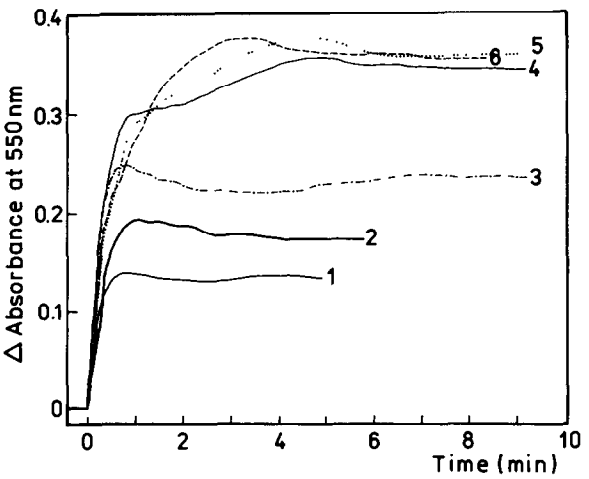

Fig.2. Superprecipitation of actomyosin in the presence of PSAM at various molar ratios to actin: (curve 1) no PSAM, (curve 2) 1:413, (curve 3) 1:200, (curve 4) 1:50, (curve 5) 1:25, and (curve 6) 1:12.5. The abscissa shows the time after addition of MgATP, the ordinate the increase in absorption at 550 nm.

Table 1

Effect of PSAM on actin-activated Mg^{2+}-ATPase activity of myosin subfragment 1

Modulator to actin molar ratio	ATPase activity (μmol $P_i \cdot$ mg S-1 $^{-1} \cdot min^{-1}$	
	17 mM KCl	54 mM KCl
0	0.885	0.504
1:400	0.862	0.495
1:200	0.854	0.484
1:100	0.813	0.442
1: 50	0.761	0.382
1: 25	0.690	0.337
1: 12.5	0.575	0.275
1: 6.25	0.467	0.22
1: 2	0.039	0.03

Conditions: 0.23 mg/ml subfragment 1, 0.29 mg/ml actin, PSAM at molar ratios to actin as indicated, 10 mM imidazole-HCl buffer (pH 7.0), 0.1 mM $CaCl_2$, 2 mM MgATP, 25°C. ATPase activity of subfragment 1 alone was subtracted from the activities measured in the presence of actin

The stimulatory effect of PSAM on the actin-activated ATPase of filamentous myosin diminished with increasing ionic strength and changed into an inhibition when ionic strength approached the physiological level. As shown in fig.3, in 54 mM KCl the ATPase rate in the presence of the modulator was still enhanced relative to the control sample but in 100 and 120 mM KCl the actin activation of ATP hydrolysis decreased with increasing modulator to actin ratio.

Corresponding differences in the effects of PSAM on the superprecipitation of actomyosin in 54 and 100 mM KCl are illustrated in fig.4. Under the conditions used here there was still no detectable clear phase at 100 mM KCl in the absence of PSAM. With actin polymerized in the presence of the modulator at a ratio of 1:200 the increase in turbidity was preceded by a clear phase and its rate was considerably reduced. A further increase of the amount of PSAM (1:25) produced a long-lasting clear phase. Generally, for the whole range of KCl concentrations studied, the final extent of turbidity increase was larger in the presence of PSAM than with actin alone.

The superprecipitating actomyosin gel formed a more uniform, translucent, and stable suspension when actin had been polymerized in the presence

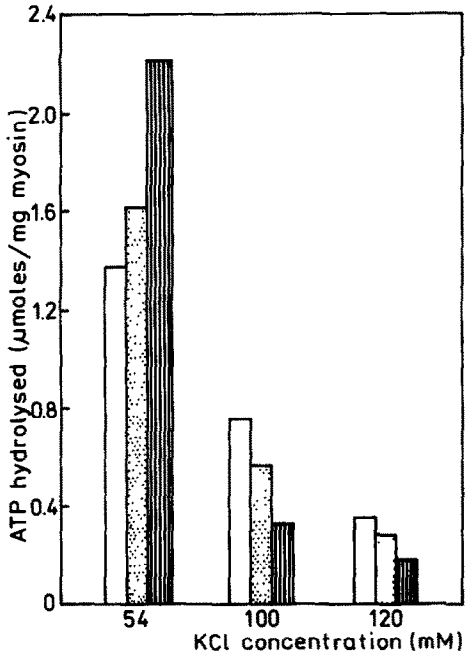

Fig.3. Comparison of the effects of PSAM on actin-activated Mg^{2+}-ATPase of myosin at various concentrations of KCl. Conditions: 10 mM imidazole-HCl buffer (pH 7.0), 0.1 mM $CaCl_2$, KCl at concentrations indicated in the figure, 0.52 mM MgATP, 0.165 mg/ml myosin, 0.105 mg/ml actin. The molar ratio of PSAM to actin was 1:200 (dotted columns), 1:25 (striped columns), or no PSAM (clear columns). The ordinate shows ATP hydrolysed within the first 160 s of the reaction.

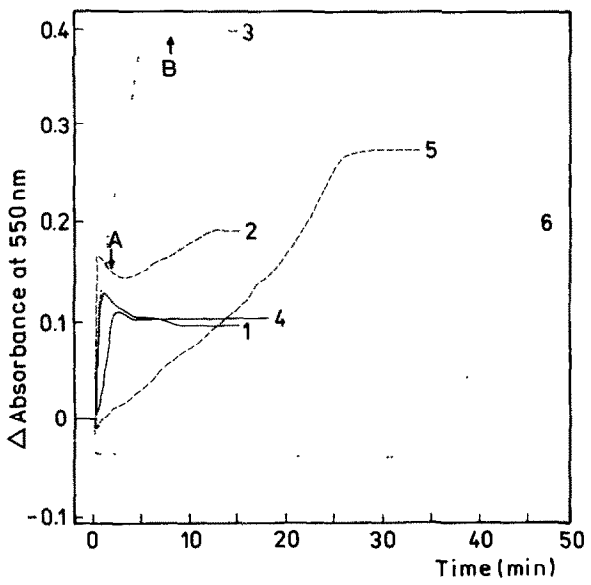

Fig.4. The influence of PSAM on the superprecipitation of actomyosin in 54 and 100 mM KCl. Conditions were the same as in fig.3, with 54 (curves 1-3) or 100 mM KCl (curves 4-6), and in the absence (——) or presence of PSAM at a molar ratio to actin of 1:200 (– – –) or 1:25 (· · ·). At times indicated by arrows samples were taken for examination in the electron microscope.

of PSAM. In order to obtain more information on the effect of PSAM on the structural organization of actomyosin gel, samples of actomyosin with and without the modulator were examined by electron microscopy at various stages of superprecipitation. Fig.5a shows a fragment of a dense aggregate of myosin and actin filaments passing into a looser filament network. The appearance is typical of superprecipitating actomyosin with or without actin modulator, except that with increasing modulator to actin ratio the average length of actin filaments and the size of the dense aggregates diminished (fig.5b). Acto myosin with actin polymerized in the presence of PSAM additionally showed numerous small bundles of short actin filaments (fig.6). Within some of these aggregates it was possible to recognize myosin filaments by their larger diameter and lack of the beaded appearance characteristic of actin filaments (fig.6, arrows). One can therefore suppose that the bundles contain a core of one or more myosin filaments obscured by the surrounding actin filaments. These numerous small aggregates, seen both before and after splitting of the added ATP, seem to be responsible for the turbidity increase above the level observed in the absence of the modulator.

At elevated KCl concentrations, the structural organization of actomyosin during the clear phase induced by PSAM (not shown) was not substantially different from what was earlier observed for actomyosin reconstituted from pure actin and myosin undergoing clearing at high KCl and ATP concentrations [16], except the length of actin filaments. The same holds true for a characteristic lateral association of myosin and actin filaments which we observed during the early stages of slow turbidity increase subsequent to clearing which has been reported for actomyosin reconstituted from pure actin and myosin [17] and leads to an extensive filament bundling after splitting of ATP.

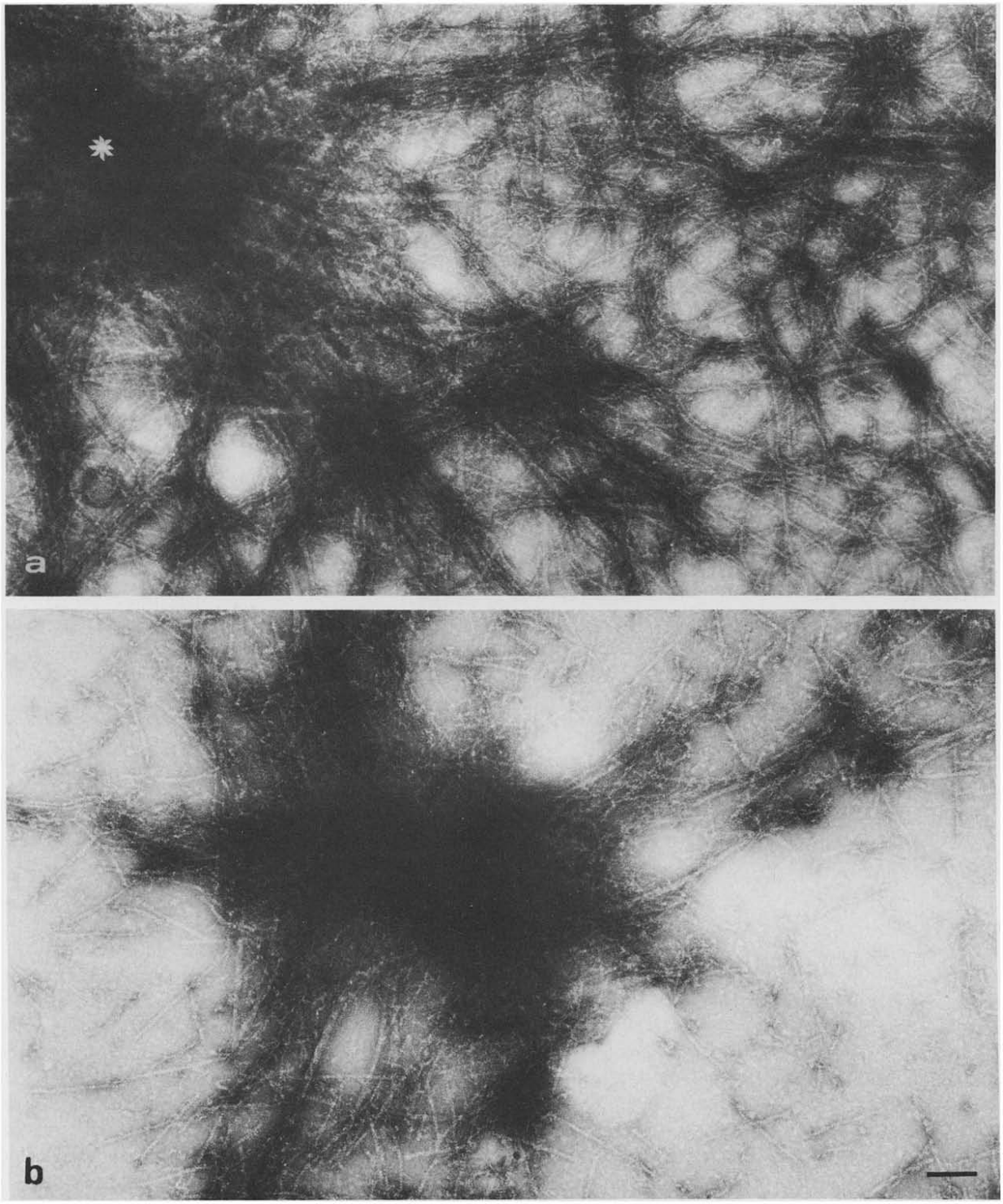

Fig.5. Electron micrographs of aggregates formed during superprecipitation of actomyosin reconstituted from skeletal muscle myosin and actin to which PSAM had been added a molar ratio of (a) 1:200 or (b) 1:25. The conditions of superprecipitation were the same as in fig.4, the concentration of KCl was 54 mM. Samples were negatively stained (a) 2 min and (b) 8 min after addition of ATP (points A and B in fig.4, respectively). The asterisk marks a peripheral region of a large aggregate of randomly distributed myosin and actin filaments. Bar, 0.1 μm.

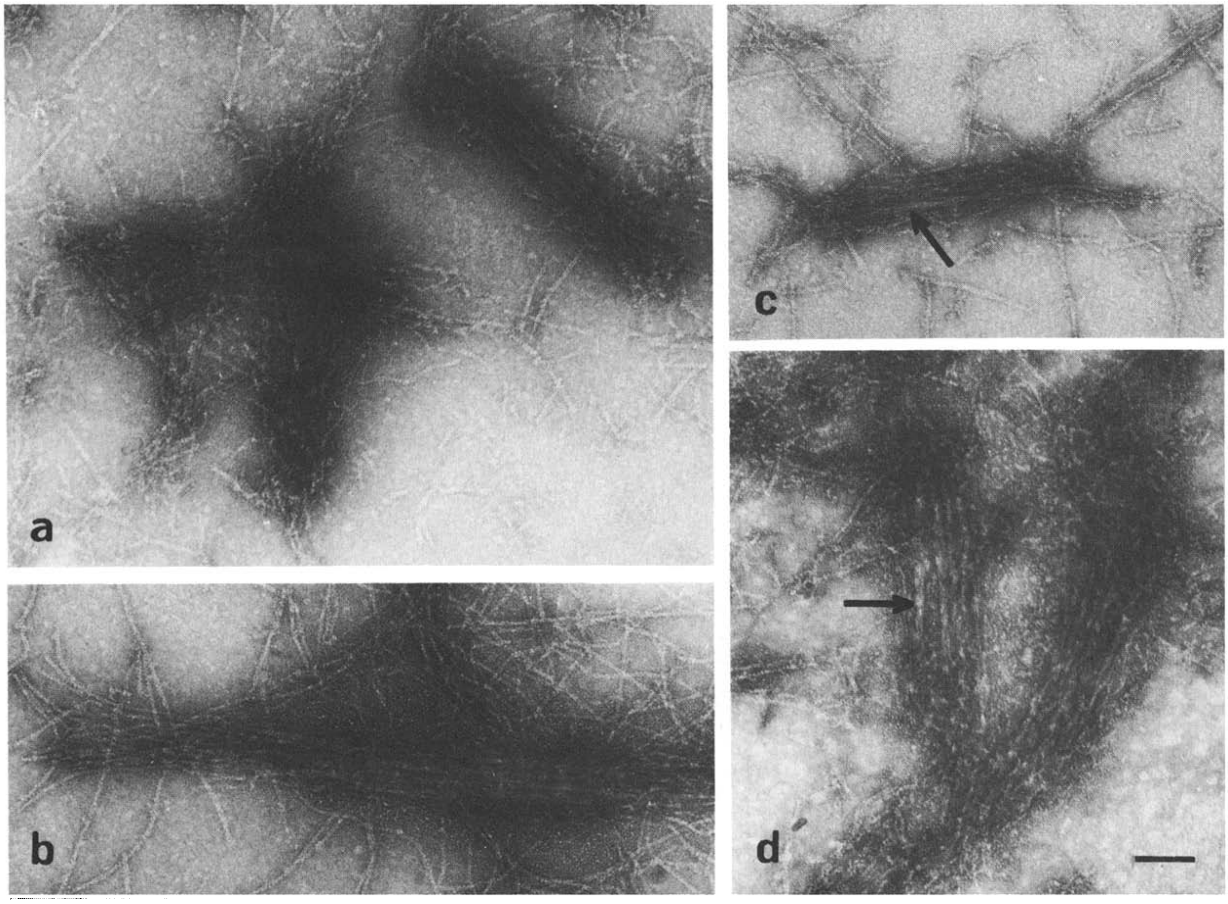

Fig.6. Electron micrographs showing bundles of parallel-aligned myosin and short actin filaments in superprecipitated actomyosin reconstituted from myosin and actin complexed with PSAM. The modulator to actin molar ratios were (a,b) 1:200 or (c,d) 1:25, 54 mM KCl, other conditions as in figs.3,4. Samples in (a,b) were stained 2 min after addition of ATP (point A in fig.4, ATP hydrolysed in 39%), those in (c,d) 8 min after addition of ATP (point B in fig.4, ATP fully hydrolysed). Arrows point to myosin filaments. Bar, 0.1 μm.

4. DISCUSSION

As we have shown, there are two opposite effects of the actin modulator from pig stomach smooth muscle on the interaction between skeletal muscle myosin and actin: (1) stimulation of the Mg^{2+}-ATPase activity and superprecipitation of actomyosin at low KCl concentrations, and (2) inhibition of these two interrelated activities at KCl concentrations yielding an ionic strength close to physiological. With acto-subfragment-1, only inhibition was observed. Both effects were Ca^{2+}-dependent, reflecting the requirement for Ca^{2+} for the interaction of the modulator with actin [1].

The stimulatory effect of the modulator on the insoluble actomyosin system under conditions favouring superprecipitation seems to be a direct consequence of its effect on actin filament length. A characteristic feature of superprecipitation is the formation of dense, disordered aggregates of myosin and actin filaments as a result of entanglement and concentration of myosin filaments in microregions of the original filament network in the process of sliding of myosin and actin filaments past each other [16,18,19]. It has been shown that a large fraction of actin is excluded from these aggregates even when myosin is in excess over actin [15,17,20]. This, as well as buckling

the filaments trapped in the aggregates, lowers the concentration of actin available for interaction with myosin and thus diminishes the rate of actin-activated hydrolysis of ATP.

These effects are most likely overcome by the shortening of actin filaments in the presence of PSAM. In fact, a significant influence of PSAM on the organization of superprecipitating actomyosin gel was observed already at the low modulator to actin molar ratio of 1:200: in addition to large, unordered aggregates, another type of aggregate appears in which actin and myosin filaments are aligned in parallel (fig.6). The latter type is predominant at a high modulator to actin ratio (1:25). It is therefore concluded that shortening of actin filaments provides better conditions for ordered association of myosin and actin filaments and facilitates the interaction of myosin heads with actin. On the other hand, the inhibition of actomyosin ATPase at higher KCl concentrations may also be directly related to the effect of the modulator on the length of actin filaments. Assuming that the interaction of myosin and actin in solution involved some kind of sliding of myosin past actin filaments, the progressive inhibition with increasing the modulator to actin ratio is explainable in terms of shortening the time during which any particular myosin filament interacts with a given actin filament as actin filament length is decreased. Additionally, it is well known that G-actin is incapable of activating significantly the myosin ATPase. This unequivalence to actin subunits within the polymer has been attritubed to either the lack of a polymerization-induced conformational transition that may be necessary for activation of myosin ATPase, or a requirement of two adjacent subunits for effective interaction with the site on myosin [21–25]. Thus, increasing the relative number of polymer ends might result in diminishing the activation of myosin ATPase if we assume that the conformation of monomers at polymer ends is closer to that of free monomers rather than of the internal subunits within the polymer.

Both the stimulatory and inhibitory effects of the modulator are not specific for this protein. Essentially similar effects of β-actinin, an actin-capping protein from skeletal muscle, on the ATPase activity of actomyosin have been reported [26–28]. Amplification of actomyosin ATPase by myelin basic protein at certain ranges of its ratios to actin, coinciding with extensive fragmentation of actin filaments, has recently been observed (Barylko and Dobrowolski, personal communication). A considerable inhibition of actin activation of heavy meromyosin Mg^{2+}-ATPase by fragmin, a Ca^{2+}-dependent actin-capping protein from *Physarum polycephalum,* has been observed by one of us [29]. Taken together, these observations suggest a nonspecific and indirect effect of actin-capping and severing proteins on actin-myosin interaction via a shortening of actin filaments.

An actin-capping activity has been detected in extracts from various types of vertebrate smooth muscle including chicken gizzard [3] and bovine aorta (unpublished). Protein fractions exhibiting this activity contain a polypeptide with a chain mass of 85–90 kDa, similar to that of PSAM and may nucleate actin polymerization with corresponding reduction of actin filament length in a Ca^{2+}- dependent manner.

Even though the activity of PSAM [1], and possibly that of the corresponding proteins from other types of smooth muscle, is regulated by changes in free Ca^{2+} concentration in the physiological range, it seems unlikely that the modulator plays a role in the regulation of smooth muscle contraction. First of all, the dissociation of the modulator from its actin complex upon lowering free Ca^{2+} concentration is much too slow to account for the changes in the state of muscle. Moreover, the Ca^{2+}-induced severing of actin filaments would decrease rather than increase tension developed by ordered arrays of myosin and actin filaments in muscle, as has been observed with artificial *Physarum* actomyosin threads in the presence of fragmin [32]. The effects of PSAM on the interaction between myosin and actin, however, may have some implications for in vitro studies on the regulation of smooth muscle contraction. In view of its strong affinity to actin, the possible presence of the modulator in natural actomyosin or thin filament preparations from smooth muscle should be taken into consideration even when the preparation procedure includes washing with EDTA solution. Contamination with the modulator in amounts hardly detectable by SDS–polyacrylamide gel electrophoresis – depending on assay conditions – may either amplify or decrease the extent of activation of myosin

ATPase by actin in a Ca^{2+}- dependent manner. As we have shown, the net effect of the modulator should depend not only on the ionic strength of the medium, but also on other factors determining the affinity between myosin and actin in the presence of ATP.

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