FILAMIN INHIBITS ACTIN ACTIVATION OF HEAVY MEROMYOSIN ATPase

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1. Introduction

Filamin is a high molecular weight (240 000) actin-binding protein. It has been isolated from alveolar macrophages [1], leukemic leukocytes [2] and smooth muscle [3,4] and identified immunochemically in rat and mouse fibroblasts [3,5]. Although the protein is present in substantial amounts in some tissues (1–2% of the protein of macrophage and smooth muscle cells), little is known of its function in the cell. Stossel and Hartwig [1,6,7] have shown that when macrophage filamin and actin are mixed together at 25°C they form a gel. Shizuta et al. [4] also observed gel formation when chicken-gizzard filamin and actin were combined. Although little information is available on what effect filamin might have on actin function and what factors may regulate the interaction of filamin and actin, a known function of actin is activation of the Mg-ATPase activity of myosin or heavy meromyosin (HMM) (for review see ref. [8]). We have studied the effects of filamin on F-actin activation of HMM ATPase and myosin ATPase and have found that filamin markedly reduced actin activation of these activities.

2. Materials and methods

Actin was prepared from an acetone powder of rabbit skeletal muscle by the procedure of Spudich and Watt [9]. G-actin was converted into F-actin in a solution containing 2 mM Tris–HCl, pH 7.0, 60 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$ and 0.2 mM ATP. Human platelet actin and rabbit skeletal muscle myosin and HMM were a gift from Dr E. Korn, National Institutes of Health. Chicken-gizzard filamin was purified by the method of Shizuta et al. [4] except that Sepharose 4B equilibrated in 50 mM potassium phosphate (pH 7.5), 100 mM KCl, 1 mM EDTA, 1 mM DTT was used for the gel-filtration step. To prevent denaturation of filamin it was essential that during ammonium sulfate fractionation the pH be kept between pH 7.0–7.5 by addition of NH_4OH.

The precise conditions for ATPase assays are listed in the legends to the figures and tables. The general procedure was as follows: reaction tubes were prepared at 4°C containing 360 μl of 2 mM $MgCl_2$, 1 mM EGTA, 1 mM [γ-^{32}P]ATP (1–10 cpm/pmol), 10 mM MES [2-(*N*-morpholino)ethanesulfonic acid] (pH 7.0), and actin and filamin or appropriate buffer controls for the individual experiment. These components were pre-incubated for 5 min at 37°C, and the reaction started by addition of 40 μl of HMM or myosin followed by vigorous mixing. Aliquots (100 μl) were taken at various times and the reaction stopped by the addition of 1 ml cold 10% trichloroacetic acid. The $^{32}P_i$ formed was measured as described by Pollard and Korn [10]. In all experiments the recovery of $^{32}P_i$ was greater than 95% and was unaffected by filamin. Background $^{32}P_i$ in the assay varied from lot to lot of [γ-^{32}P]ATP but was less than 2%.

Under the conditions utilized, actin-activated HMM ATPase activity was linear for at least 30 min at 37°C. The rate of reaction was directly proportional to the HMM concentration over the range 10–100 μg/ml and ATPase activity was activated in a linear manner by F-actin in the concentration range of 10–300 μg/ml. Both F-actin and filamin had negligible ATPase activities (less than 1 nmol/min/mg).

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3. Results

3.1. *Effect of filamin on actin activation of HMM ATPase*

Increasing concentrations of filamin caused a marked decline in the F-actin activated ATPase activity of skeletal muscle HMM (fig.1). The ATPase activity of HMM was 30 nmol/min/mg and filamin had no effect on this activity; however, F-actin (100 μg/ml) increased the HMM ATPase 30-fold to 820 nmol/min/mg. Concentrations of filamin below 2 μg/ml (10^{-8} M) had little effect on the actin-activated HMM ATPase, whereas filamin at 2–10 μg/ml produced a small but consistent stimulation of actin activated HMM ATPase. When filamin at 10 μg/ml or greater was used, actin-activated HMM ATPase was progressively reduced until a final value of 100 nmol/min/mg was reached. At three different actin concentrations this final ATPase activity was approximately the same and was greater than the activity of HMM alone (fig.1).

The effect of filamin on actin activation of HMM was independent of the sequence of addition of the protein components to the reaction mixture. Further-

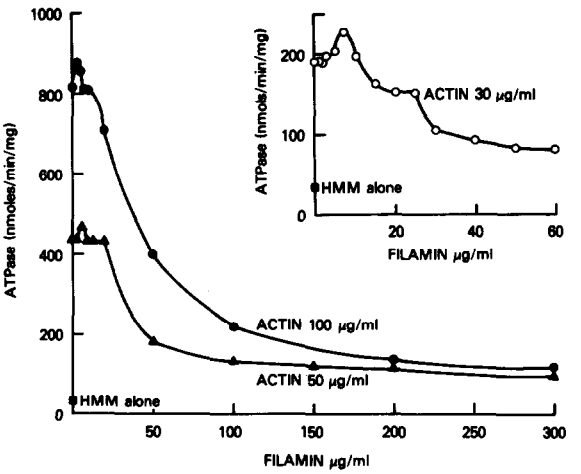

Fig.1. Effect of filamin on actin-activated HMM ATPase. Assays were performed at 37°C with HMM 60 μg/ml and rabbit skeletal muscle F-actin: (•) 100 μg/ml, (▲) 50 μg/ml, (○) 30 μg/ml. The reaction mixture contained 25 mM MES pH 7.0, 1 mM $MgCl_2$, 2 mM EGTA, 1 mM ATP, 16 mM KCl, 5 mM potassium phosphate. Note that the inset from a separate but comparable experiment has an expanded scale. ATPase measurements were performed as described in Materials and methods. (■) Indicates HMM ATPase in the absence of actin or filamin.

Table 1
Effects of ATP and $MgCl_2$ on filamin inhibition of actin activation of HMM ATPase

Components	ATPase activity (nmol/min/mg)					
	ATP (mM)			$MgCl_2$ (mM)		
	0.1	0.5	1.0	0.1	1.0	5.0
	(nmoles/min/mg)					
HMM	57	28	76	37	48	52
HMM + filamin	ND[a]	ND	82	ND	48	ND
HMM + actin	550	658	830	557	604	668
HMM + actin + filamin	145	193	206	95	111	151

[a] ND, not determined

Assays were performed at 37°C with HMM 84 μg/ml, rabbit skeletal muscle F-actin 100 μg/ml, chicken-gizzard filamin 200 μg/ml in a reaction mixture containing 25 mM MES, pH 7.0, 2 mM EGTA, 16 mM KCl, 5 mM potassium phosphate. Incubations with 0.1 mM ATP were up to 1 min, 0.5 M ATP up to 5 min and 1 mM ATP up to 10 min. When ATP concentration was varied $MgCl_2$ was 1 mM. When $MgCl_2$ concentration was varied ATP was 1 mM. Values represent the mean of triplicate determinations.

more all ATPase measurements were linear for at least 25 min but from 25–60 min there was an approximate 40% decrease in the magnitude of the filamin inhibition. To avoid the complications of prolonged incubations, all experiments were performed for less than 20 min.

3.2. *Effect of $MgCl_2$, ATP, KCl and cytochalasin B on filamin's inhibitory activity*

As shown in table 1, varying the ATP concentration from 0.1–1.0 mM or the $MgCl_2$ concentration from 0.1–5.0 mM had no effect on filamin inhibition of actin activation of HMM ATPase. Removal of free Ca^{2+} by EDTA or addition of 0.1 mM Ca^{2+} also had no effect. Increasing the ionic strength by addition of KCl reduced the actin-activated HMM ATPase activity but did not relieve the inhibition by filamin (fig.2). The inhibitory effect of filamin was not affected by cytochalasin B, at 10^{-4}–10^{-6} M and 23°C (table 2).

Heating of filamin at 60°C for 5 min abolished its inhibitory capacity. Freezing of filamin solutions did not reduce the ability to inhibit actin activation of HMM.

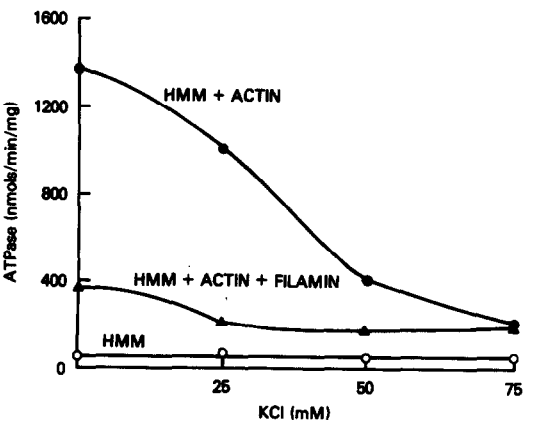

Fig.2. Effect of KCl on filamin inhibition of actin activation of HMM ATPase. Assays were performed at 37°C with HMM 85 μg/ml, rabbit skeletal muscle F-actin 100 μg/ml, chick-gizzard filamin 100 μg/ml, 10 mM MES, pH 7.0, 1 mM EGTA, 1 mM $MgCl_2$ 1 mM ATP, 4 mM KCl, 2 mM potassium phosphate. Abscissa indicates KCl added to reaction mix. Values represent the mean of triplicate determinations. (○) HMM, (●) HMM + actin, (▲) HMM + actin + filamin.

3.3. *Effect of filamin on activation of myosin by skeletal muscle and platelet F-actin*

Activation of skeletal muscle myosin by skeletal muscle actin was sensitive to inhibition by filamin in a manner analogous to HMM. Filamin at 250 μg/ml produced complete inhibition of actin activation. Filamin was also effective in inhibiting the activation

Table 2
Lack of effect of cytochalasin B on filamin inhibition of actin activation of HMM ATPase

Components	ATPase activity (nmol/min/mg)			
	Control	Cytochalasin B		
		10^{-4} M	10^{-5} M	10^{-6} M
HMM	13	12	ND[a]	ND
HMM + filamin	14	15	ND	ND
HMM + actin	222	236	ND	ND
HMM + actin + filamin	89	112	116	108

[a] ND, not determined

Cytochalasin B, rabbit skeletal muscle F-actin 100 μg/ml and chicken-gizzard filamin 100 μg/ml were added to tubes containing 10 mM MES, pH 7.0, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM ATP, 6 mM KCl, 2 mM potassium phosphate. Tubes were incubated 5 min at 23°C and the reaction initiated by addition of HMM 90 μg/ml. All tubes contained 1% dimethylsulfoxide. Values represent the mean of duplicate determinations.

of skeletal muscle myosin with human-platelet actin, a non-muscle actin. Filamin at 25°C and 250 μg/ml markedly reduced the activation of skeletal muscle myosin by human-platelet actin.

4. Discussion

The major point developed by these studies is that filamin leads to a marked reduction in the ability of actin to activate the ATPase of myosin. The mechanism of inhibition is probably not due to a change in the catalytic properties of the myosin ATPase since in the absence of actin, filamin has no effect on HMM or myosin ATPase activity. Furthermore ATPase measurements were carried out under conditions where neither ATP nor Mg^{2+} were limiting and the ATPase activity was proportional to the F-actin concentration. It is therefore probable that the reduction in actin activation of myosin ATPase results from filamin reducing the concentration of free F-actin available for activating the myosin ATPase.

How could filamin decrease the amount of actin available?

(i) Filamin could bind to actin and prevent actin's interaction with HMM or the actin–filamin complex could interact with HMM but not activate it.

(ii) Actin and filamin could combine to form a gel which would restrict the ability of HMM to interact with actin.

Considering the first possibility, our data demonstrates that substantial inhibition of actin activation of HMM occurs at molar ratios of actin:filamin of 25:1 or less (fig.1). An inhibition of 50% occurs at ratios of 10:1 and maximal inhibition occurs at actin to filamin molar ratios of 3:1. If each filamin subunit were capable of blocking 3–5 actin subunits, this could account for the observed decrease in actin activation. This value is not unreasonable if one considers the relative molecular weight of actin (43 000) and filamin (240 000).

However, considering the second possibility, actin and filamin when combined form a gel and gelation could restrict the diffusion of HMM to the actin. Although this possibility cannot be ruled out, it has been reported that several proteins isolated from the cytoplasm of *Acanthamoeba* are much more effective than chicken-gizzard filamin in promoting actin gel formation and much less effective in causing inhibition of actin activation of HMM ATPase [11]. Also, Stossel and Hartwig [6] have reported that macrophage filamin is very effective in promoting actin-gel formation but has no effect on actomyosin ATPase. Significant differences between macrophage and gizzard filamins is suggested by the failure by cytochalasin B to effect gizzard filamin inhibition of actin activation of HMM. Cytochalasin B has been reported to block the interaction of macrophage filamin and actin [7]. In summary our data and that of others supports the possibility that binding of filamin by actin directly reduces its ability to activate HMM.

The physiological consequences of the inhibitory activity of filamin upon actin activation of myosin are not defined by these studies. The data do suggest that actin complexed with filamin, presumably in vivo in the form of microfilament bundles [3,4] may not be competent for activating myosin. If so, then factors that regulate the interaction of filamin and actin may also regulate the activity of the contractile apparatus of the cell. It has recently been observed that chicken-gizzard filamin is a phosphoprotein that can be phosphorylated by cyclic AMP-dependent protein kinase [4,5]. Studies are now in progress to determine if phosphorylation of filamin modifies its ability to inhibit actin activation of myosin.

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References

[1] Hartwig, J. H. and Stossel, T. P. (1975) J. Biol. Chem. 250, 5696–5705.
[2] Boxer, L. A. and Stossel, T. P. (1976) J. Clin. Invest. 57, 964–976.
[3] Wang, K., Ash, J. F. and Singer, S. J. (1975) Proc. Natl. Acad. Sci. USA 72, 4483–4486.
[4] Shizuta, Y., Shizuta, H., Gallo, M., Davies, P., Pastan, I. and Lewis, M. S. (1976) J. Biol. Chem. 251, 6562–6567.
[5] Davies, P., Shizuta, Y., Olden, K., Gallo, M. and Pastan, I. (1977) Biochem. Biophys. Res. Commun. 74, 300–307.

[6] Stossel, T. P. and Hartwig, J. H. (1976) J. Cell Biol. 68, 602–619.
[7] Hartwig, J. H. and Stossel, T. P. (1976) J. Cell Biol. 71, 295–303.
[8] Mannherz, H. G. and Goody, R. S. (1976) Ann. Rev. Biochem. 45, 427–466.
[9] Spudich, J. A. and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871.
[10] Pollard, T. D. and Korn, E. D. (1973) J. Biol. Chem. 248, 4682–4690.
[11] Maruta, H. and Korn, E. D. (1977) J. Biol. Chem. 252, 399–402.