Vol. 130, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1985 Pages 286-292

THE EFFECT OF LEIOTONIN FRACTION ON STABLY PHOSPHORYLATED SMOOTH MUSCLE MYOSIN

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Received May 28, 1985

This study was designed to determine the effect of leiotonin on the actin-activation once the myosin is stably phosphorylated. Gizzard myosin was stably phosphorylated by ATP-γ-S using the gizzard light chain kinase. Addition of leiotonin preparation to phosphorylated myosin reconstituted with actin and tropomyosin did not alter the ATPase activity. Furthermore, leiotonin did not confer the calcium sensitivity of the ATPase activity. These experiments show that the actin-activated ATPase activity of stably phosphorylated gizzard myosin is not altered by leiotonin.

Phosphorylation of the 20,000 dalton light chain of the myosin by a calcium-dependent kinase is associated with an increase in actin-activated ATP hydrolysis by myosins isolated from smooth muscles (1,2,3). The experiments correlating phosphorylation and actin-activation were carried out either by adding the calcium dependent kinase preparation to myosin (1,3) or by using pure myosin phosphorylated to varying degrees prior to purification (4). The proponents of the phosphorylation theory claim that phosphorylation of the myosin light chain by

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Abbreviations: EDTA, 1,2-bis [(2-discarbosymethyl)aminoethoxy] ethane; DDT, dithiothreitol; DOD, dodecyl; TCA, trichloroacetic acid

0006-291X/85 $1.50

the calcium dependent kinase regulates the actin-activated ATP hydrolysis.

Ebashi and his collaborators also showed that the ATPase activity of actomyosin from smooth muscle was activated by the addition of a factor isolated from smooth muscle (5,6). This activator, termed leiotonin, differs from the kinase. It has not been purified in a homogenous state, however, and is often contaminated by kinase (7,8,9).

The purpose of this study was to determine the effect of leiotonin on the actin-activation of phosphorylated myosin and to determine whether or not the leiotonin confers calcium sensitivity once the myosin is stably phosphorylated.

MATERIALS AND METHODS

The thiophosphorylated myosin was prepared by two procedures: 1) The 35-50% ammonium sulfate fraction prepared according to Chacko and Rosenfeld (10) was dialyzed against 0.06 M KCl 20 mM imidazole-HCl (pH 7.0), and 1 mM DTT was incubated with 0.5 mM ATP-γ-S, 2 mM $MgCl_2$ and 0.01 mM $CaCl_2$ at 23°C for 10 minutes. The pH of the sample was lowered to 6.5 and centrifuged. The pellet containing myosin was dissolved in 0.5M KCl buffer and subjected to further purification by ultra centrifugation in the presence of 10 mM Mg-ATP. The myosin present in the supernatant was precipitated with 10 mM $MgCl_2$ (11). 2) Myosin prepared according to Ebashi (11) was incubated with 1 mM DTT, 0.5 mM ATP-γ-S, 0.01 mM $CaCl_2$, 2 mM $MgCl_2$ in the presence of leiotonin fractions for 10 min at 23°C prior to actin-activated ATPase assays. The myosin became fully phosphorylated by the kinase contaminating the leiotonin preparations used in this series of experiments.

For preparation of leiotonin, the muscle mince, from which myosin had been extracted, was diluted by 3 volumes of 1 mM $NaHCO_3$ and left standing for two hours. After centrifugation (38,000x g for 15 min), the residue was suspended in 20 mM KCl contaning 1 mM $NaHCO_3$, and centrifuged after 1 hours. An equal volume of 1.2 M NaCl was added to the residue and left for three hours with occasional stirring. After centrifugation (38,000x g for 15 min), the supernatant was subjected to the inverse ammonium sulfate fractionation as described previously (12). The fraction precipitated at ammonium sulfate concentrations between 1.4 M and 1.8 M was used as the leiotonin preparation. This fraction still contained some kinase activity, but no further purification to reduce the kinase activity was made in order to facilitate the phosphorylation of myosin (see above).

The degree of phosphorylation of myosin was determined after urea gel electrophoresis (13)a and scanning densitometry as published (10). The ATPasse assays were carried out as

previously described (4). A Ca/EGTA buffer (14) was used for some experiments. The protein concentrations used, unless indicated otherwise, were: myosin, Ø.14 mg/ml; smooth muscle actin, Ø.2Ø mg/ml; tropomyosin, Ø.Ø57 mg/ml. Samples were incubated at 37°C and aliquots were removed at zero time and at two additional times to ascertain linearity of phosphate release. P_i was measured according to either Youngburg and Youngburg (15) or Martin and Doty (16).

Smooth muscle actin was prepared from acetone-washed fibers from gizzard according to the method of Ebashi (17). An alcohol/ether powder (18) of the residue after myosin extraction was used for extracting the tropomyosin. The tropomyosin was extracted and purified as described by Eisenberg & Kielley (19).

Protein concentrations were determined according to Lowry (2Ø) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The leiotonin preparations were tested for their activator effect by measuring their ability to produce superprecipitation of unphosphorylated myosin in the presence of Ca^{2+}, gizzard actin and tropomyosin (6). The preparations which produced the maximum turbidity were used for determining the effect of leiotonin on thiophosphorylated myosin.

Most of the preparations of thiophosphorylated myosins were fully phosphorylated at the beginning of the ATPase assay and they remained phosphorylated during the course of the ATPase assay (Figure 1) since thiophosphorylated protein is a poor substrate for phosphatases (21). The K^+-EDTA and Ca^{2+}-activated ATPase activities measured in high salt (Ø.5 M KCl) and Mg-ATPase activity and actin-activated Mg-ATPase activities of phosphorylated and thiophosphorylated myosins are not significantly different (Table 1). Thus the active sites of these enzymes appear to be unaltered. Calcium has not direct effect on the actin-activation at Mg^{2+} concentration higher than 4 mM (22, 23).

The actin-activated ATPase activity was measured in the presence and absence of Ca^{2+}. The actin-activated ATPase activities of thiophosphorylated myosins in the presence of leiotonins are not remarkably different from ATPase activities in

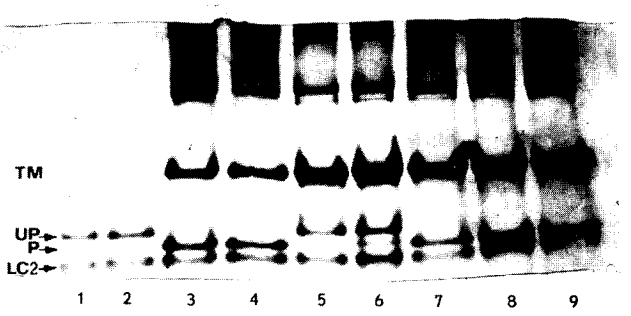

Figure 1. Urea gel electrophoresis of myosin. 1 & 2, myosin alone; 3-9 are myosin reconstituted with actin, tropomyosin and leiotonin. UP and P respectively indicate unphosphorylated and phosphorylated 20,000 dalton light chain (LC) of myosin. LC_2 stands for 16,000 dalton second light chain of myosin and TM stands for tropomyosin. 1 & 2, myosin removed at 0 time (well 1) and 10 min (well 2) from the ATPase assay mixture in the presence of calcium. It remained unphosphorylated under the conditions of the ATPase assay. 3 & 4, incubaed at 25°C in the presence of 0.01 mM $CaCl_2$, 6 mM $MgCl_2$ and 0.5 mM ATP-γ-S for 10 minutes prior to the ATPase assay. the ATPase assay was initiated by adding 2 mM ATP incubation at 37°C. Samples were removed at 0 time, 10 min and 20 min for measuring the ATP hydrolysis in the presence of Ca^{2+} and leiotonin. The supernatant obtained after TCA precipitation was used for inorganic phosphate analysis (19) and the precipitated protein was used for urea gel electrophoresis. The 20,000 dalton light chain is completely phosphorylated at 0 time (well 3) and 10 min (well 4). 5 & 6, same as 3 & 4, except that 2 mM EGTA was added instead of $CaCl_2$. The 20,000 dalton light chain was completely unphosphorylated at 0 time (well 5), but at 10 min (well 6) it shows 10% phosphorylation, presumably due to the presence of trace activity of Ca^{2+} insensitive kinase. 7 & 9, same as 3 & 4, except that after preincubation in Ca^{2+} and ATP-γ-S for 10 min to achieve stable phosphorylation, 2 mM EGTA was added prior to ATPase assay. Hence, the actin-activitated ATPase activity was measured in the absence of free Ca^{2+} ions but in the presence of leiotonin. The 20,000 dalton light chain was fully phosphorylated at 0 time (well 7) aand it remained phosphorylated during thc ATPase assay (well 8, 10 min and well 9, 20 min).

the absence of leiotonin (compare activities in Table 1 and Table 2) indicating that the leiotonin did no additionally stimulate the actin-activated ATP hydrolysis. Furthermore, removal of free calcium by the addition of EGTA had no effect on the actin activation in the presence of leiotonin (Table 2). Therefore, the leiotonin preparation did not confer calcium sensitivity to actin-activated ATP hydrolysis once the myosin was stably phosphorylated.

TABLE I. ATPase activities of phosphorylated and thiophosphorylated chicken gizzard myosin

ATPase assay*	ATPase activity† (μmole Pi liberated/mg myosin/min)	
	Phosphorylated myosin††	Thiophosphorylated myosin††
Myosin ATPase		
+ EDTA	1.263 ± 0.073	1.333 ± 0.091
+ Ca^{2+}	0.728 ± 0.052	0.727 ± 0.039
+ Mg^{2+}	0.068 ± 0.009	0.043 ± 0.010
Actin-activated ATPase (0.02 M KCl)		
+ Actin and tropomyosin	0.134 ± 0.017	0.143 ± 0.028
- Actin and tropomyosin	0.022 ± 0.003	0.033 ± 0.010

* ATP hydrolysis was measured according to the method of Martin and Doty (20). Myosin ATPase activities were determined at 37°C in 0.5 M KCl, 30 mM imidazole (pH 7.5), 2 mM ATP, 20 μg ml myosin, plus EDTA (2 mM), $CaCl_2$ (10 mM) or $MgCl_2$ (5 mM) as indicated. Actin-activated myosin ATPase activities were measured at 37°C in 20 mM imidazole (pH 7.0), 6 mM $MgCl_2$ and $CaCl_2$ (pCa 4) using 0.14 mg/ml myosin, 0.20 mg/ml chcken gizzard actin (1:16 molar ratio, myosin:actin) and 0.057 mg/ml chicken or turkey gizzard tropomyosin (6:1 molar ratio actin:tropomyosin) as indicated above.

† Values indicated represent mean ± standard error of 4-6 preparations

†† Myosins used were 85-100% phosphorylated.

The experiments reported in this paper were aimed at determining the role of leiotonin on myosin which is already "turned-on" by phosphorylation.

TABLE II: Effect of leiotonin on actin-activited ATP hydrolysis by thiophosphorylated myosin

Prep #		Actin activated ATPase activity (μmole Pi/mg/min)		Percent phosphorylation	
		+ $CaCl_2$ (pCa 5)	- $CaCl_2$ (pCa 8)	+ $CaCl_2$ (pCa 5)	- $CaCl_2$ (pCa 8)
1	M	0.015	0.017		
	M+A+Tm+Ln	0.117	0.098	100	100
2	M	0.018			
	M+A+Tm+Ln	0.057	0.057	100	100
3	M	0.018			
	$M+A+Tm+Ln_{(1)}$	0.123	0.102	100	100
	$M+A+Tm+Ln_{(2)}$	0.101	0.103	100	100
4	M	0.015			
	$M+A+Tm+Ln_{(1)}$	0.090	0.121	95	95
	$M+A+Tm+Ln_{(2)}$	0.122	0.118	95	95

Conditions of the assays: Same as in Table 1. 1 & 2 in Prep# 3 and 4 represent different leiotonin preparations. Inorganic phoshate was determined by Youngburg & Youngburg (19).

Thiophosphorylated myosin (M) was reconstituted with actin (A), tropomyosin (Tm), and leiotonin preparation (Ln) and the ATPase activities were assayed either in the presence of Ca^{2+} or in its absence. The ATPase assay was started by adding ATP. The level of phosphorylation was determined from TCA precipitated proteins removed at 0 time, 10 min and 20 min (see Fig. 1). The degree of phosphorylation remained the same during the ATPase assay.

It is important to point out that the data reported in this paper do not rule out the possibility that the actomyosin ATPase activity is regulated by an activator which acts through the thin filament. However, the stably phosphorylated myosin is fully activated by actin and tropomyosin in the absence of additional activator. The effect of additional regulatory factors on the actomyosin ATPase of myosin under conditions which favor cyclic phosphorylation and dephosphorylation of myosin is still not clear. Further studies are necessary to understand the role of phosphorylation and/or activators which work through the thin filament in the regultion of actomyosin ATPase activity and smooth muscle contraction under conditions which are close to the physiological conditions.

ACKNOWLEDGEMENTS

The authors wish to thank Professor Y. Nonomura and Professor M. Endo for valuable discussions during the course of this work. We also thank Mrs. J. Flinn and Ms. Debbie Rupp for typing this manuscript and Miss H. Nakasone and Mr. G. Thomas for excellent technical assistance.

This investigation was supported in part by a fellowship from the Fogarty International Center of the National Institute of Health (1 FO6-TW0076) and research grant HL 22264 from the National Heart Lung and Blood Institute.

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