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EFFECT OF PHOSPHORYLATION ON THE ACTIN-ACTIVATED ATPASE ACTIVITY OF MYOSIN

A. Persechini, U. Mrwa[1] and D. J. Hartshorne

Muscle Biology Group, Dept. of Nutrition and Food Science
University of Arizona, Tucson, AZ. 85721

[1]Universität Heidelberg, II. Physiologisches Institut
Heidelberg, D-6900, West Germany

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SUMMARY

The purpose of this study was to test the hypothesis that the phosphorylation of myosin is solely responsible for the activation of the Mg^{2+}-ATPase activity of gizzard actomyosin. Using a washed natural actomyosin and a reconstituted actomyosin it was shown that phosphorylation alone caused only a slight activation of ATPase activity. Full activity was obtained only when proteins in addition to the myosin light chain kinase were added. It is evident from these results that: 1) there is no simple relationship between the extent of myosin phosphorylation and the specific Mg^{2+}-ATPase activity of actomyosin and 2) in order for full activation by actin of the Mg^{2+}-ATPase activity of phosphorylated myosin additional factors are required.

INTRODUCTION

The most widely accepted mechanism to account for the regulation of smooth muscle actomyosin is based on the phosphorylation and dephosphorylation of the myosin molecule. A considerable body of evidence has been accumulated which is supportive of this theory (1) and the logical conclusion is that activation of ATPase activity or the contraction of smooth muscle is accompanied by the phosphorylation of myosin. However, this is not accepted universally and Ebashi and his colleagues (2,3) maintain that the phosphorylation of myosin is not involved in the regulatory mechanism of smooth muscle, but rather this is due to a system termed leiotonin. It was also pointed out recently that the phosphorylation theory is not compatible with all phases of the contractile cycle in smooth muscle (4,5) and it was concluded that a simple quantitative relationship between myosin phosphorylation and isometric tension development does not exist (4,6). These experiments raise doubts about the relative importance

Abbreviation: MLCK, myosin light chain kinase.

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of myosin phosphorylation and the possibility of an alternative, or complimentary, mechanism must be considered. In this context it is interesting that a dual regulatory system has been proposed (7) for aorta actomyosin.

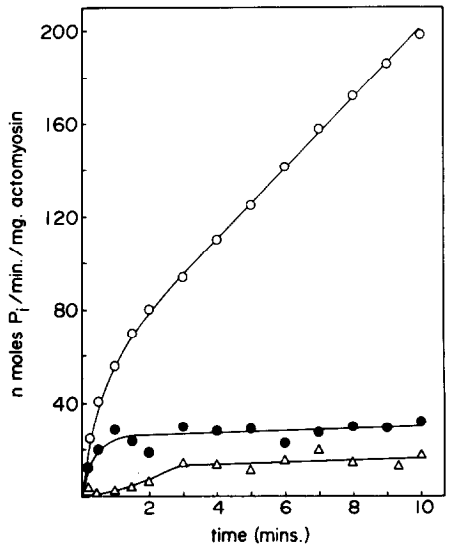
We decided, therefore, to challenge the phosphorylation hypothesis and to question the significance of myosin phosphorylation. In earlier studies which attempted to establish this point the protein constituents were not adequately characterized and the results are not conclusive. While it is likely that some of our proteins are not homogeneous we feel that the basic assay system is defined sufficiently in order to test the simplest hypothesis, i.e. that myosin phosphorylation is the only event that is required for full activation of the Mg^{2+}-ATPase activity of actomyosin.

METHODS

Frozen chicken gizzards (Pel-Freeze) were used throughout. For the preparation of myosin and actomyosin the washing procedures prior to extraction were as described previously (8) except that 0.2 mM dithiothreitol was included in all solutions. The extraction of actomyosin was achieved by homogenizing briefly the washed gizzard residue with 2 volumes (based on the original weight of gizzard) of 40 mM imidazole (pH 7.2), 4 mM EDTA, 5 mM ATP and 0.5 mM dithiothreitol. The supernatant obtained after centrifugation at 14,300 g for 20 mins. was used for the preparation of myosin and actomyosin. The latter was precipitated by the addition of $MgCl_2$ to 25 to 30 mM (9) and was collected after 12 to 18 hours by centrifugation at 14,300 g for 15 mins. The precipitate was suspended in 60 mM KCl, 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.2 mM dithiothreitol and dialyzed against this solvent to yield the Ca^{2}-sensitive natural actomyosin. This was precipitated three times at 25% ammonium sulfate saturation (10) to yield the washed actomyosin. Tropomyosin, MLCK and several minor components were removed by this treatment. The actin content was also reduced from a molar ration of actin:myosin of about 4:1 in the natural actomyosin to 2:1 in the washed actomyosin.

For the preparation of myosin the ATP-EDTA-imidazole extract was adjusted to 0.15 M $MgCl_2$ and additional ATP added to 2.5 mM. After centrifugation at 50,000 g for 18 hours the supernatant was diluted with 8 volumes of cold distilled water and the precipitated myosin collected by centrifugation at 10,000 g for 15 mins. The pellet was dissolved in 0.1 M $MgCl_2$, 5 mM ATP and 10 mM potassium phosphate (pH 7.0) and three cycles of centrifugation and precipitation as described by Ebashi (11) were applied. A disadvantage of the myosin preparation is that the heavy chains were usually slightly degraded. In the experiments shown in Fig. 3 about 9% of the heavy chains were proteolysed. Conventional protease inhibitors plus antipain or leupeptin were without influence. The effect of proteolysis was to increase slightly the Mg^{2+}-ATPase activity of myosin alone and myosin plus actin.

Gizzard actin was prepared from the supernatant following the precipitation of actomyosin. Actin paracrystals were formed at 150 mM $MgCl_2$, collected by centrifugation at 14,300 g for 20 mins. and dialyzed against 60 mM KCl, 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$ and 0.2 mM dithiothreitol. Gizzard tropomyosin was separated from the crude kinase preparation (12) by fractionation between 50 and 70% ammonium sulfate saturation and subsequent precipitation at 30 mM $MgCl_2$ (13). This tropomyosin fraction was used for the experiments of Fig. 3.



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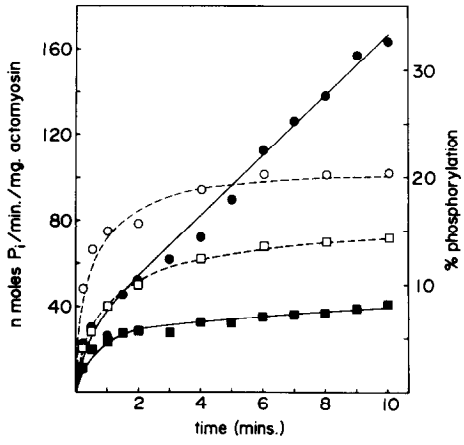

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Fig. 1. The Mg^{2+}-ATPase activities of natural actomyosin (O,●) and washed actomyosin (△). Assays carried out in approx. 5 x 10^{-5} M Ca^{2+} (O,△), and in the presence of 1 mM [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid (●). Both actomyosins were at 1 mg/ml. Other conditions as in METHODS.

Fig. 2. The effect of purified and crude myosin light chain kinase on the phosphorylation and Mg^{2+}-ATPase activity of washed actomyosin. Phosphorylation with purified kinase (□) and crude kinase (O); ATPase activity with purified kinase (■) and crude kinase (●). Actomyosin, 1 mg/ml., calmodulin 15 µg/ml., myosin light chain kinase apoenzyme 2 µg/ml., crude kinase, 0.1 mg/ml. Other conditions as in METHODS.

For further purification tropomyosin was applied to a hydroxyapatite column (Bio-Gel HTP) in 1 M KCl, 2 mM potassium phosphate (pH 7.0) and eluted with a 2 to 200 mM phosphate gradient. Two peaks were eluted, one at approx. 60 mM phosphate and the tropomyosin peak at 100 to 120 mM phosphate. The purified MLCK (14) and calmodulin (15) were prepared as outlined previously.

Assays were carried out in 60 mM KCl, 4 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.6), 1 mM γ-labeled ^{32}P ATP as described earlier for ATPase activity (16) and phosphate incorporation (14). SDS-polyacrylamide gradient gels (7.5 to 15% acrylamide) were carried out according to Laemmli (17).

RESULTS AND DISCUSSION

The time courses of ATP hydrolysis by the natural actomyosin and the washed actomyosin are shown in Fig. 1. In the presence of Ca^{2+} the kinetics for the natural actomyosin were non-linear and showed an initial rapid phase followed by a slower linear phase, the rate for the latter was 15 nmoles P_i $min^{-1}mg^{-1}$ actomyosin. In the absence of Ca^{2+} a rapid initial hydrolysis was

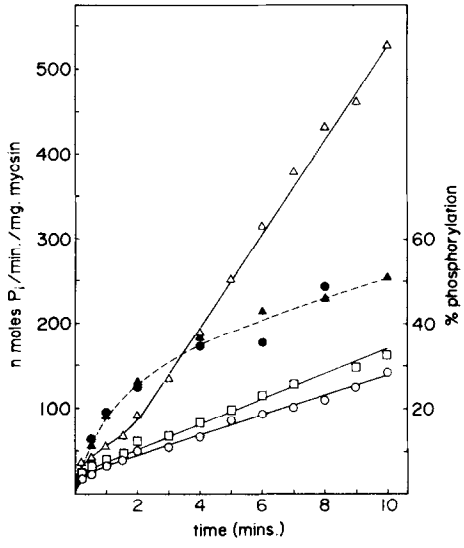

Fig. 3. The effect of phosphorylation on the Mg^{2+}-ATPase activity of reconstituted actomyosin. Gizzard myosin, 0.6 mg/ml., gizzard actin, 0.2 mg/ml., tropomyosin fraction 60 µg/ml., purified myosin light chain kinase apoenzyme 1 µg/ml., calmodulin, 15 µg/ml. ATPase assays, open symbols; phosphorylation assays, closed symbols. Mixtures used for assays were: myosin, tropomyosin, kinase plus calmodulin (O); myosin, actin, kinase plus calmodulin (□, ■); myosin, actin, tropomyosin fraction, kinase plus calmodulin (△, ▲). Other conditions as in METHODS.

still observed, although the second linear phase was reduced to approximately 0.5 nmole P_i min^{-1} mg^{-1} actomyosin. From a practical point of view this illustrates the importance of measuring the time course of ATP hydrolysis rather than fixed time points, as obviously the extent of Ca^{2+}-sensitivity would be partly dependent on the sampling time. In the presence of Ca^{2+} the ATPase activity of the washed actomyosin was very low and for the linear phase gave a rate of about 0.4 nmole P_i min^{-1} mg^{-1} actomyosin. The marked reduction of actin-activated ATPase activity as a result of the washing procedure agrees with the results obtained earlier (10).

The long range objective is to identify the components involved in the activation of the actin-activated ATPase activity, and the first factor which was considered is the effect of phosphorylation of myosin. The ATPase activity and phosphate incorporation of the washed actomyosin with either crude MLCK or, the purified MLCK apoenzyme plus calmodulin is shown in Fig. 2. The actomyosin

ATPase activity in the presence of the pure MLCK was activated only slightly and for the linear second phase a rate of 1.25 nmole P_i $min^{-1}mg^{-1}$ was obtained. In the presence of crude MLCK the linear ATPase rate was 14 nmoles P_i $min^{-1}mg^{-1}$ actomyosin. The extent of phosphate incorporation after 10 mins approached 20% and 14% of the total sites for the crude and pure MLCK, respectively. Contrasting to the relatively small difference in the extent of myosin phosphorylation the ATPase rates differed by about a factor of 11. Thus from a comparison of the two assay conditions it is clear that there is no simple relationship between the extents of ATPase activation and myosin phosphorylation. Further it is evident that the phosphorylation of myosin alone is not sufficient for full ATPase activity.

The same conclusion can be derived when a reconstituted actomyosin is used (Fig. 3). For a system containing gizzard myosin, gizzard actin and the purified MLCK the extent of actin-activated ATPase activity was relatively low and in the linear region of the curve was about 3 nmoles P_i $min^{-1}mg^{-1}$ myosin (the myosin ATPase activity in the absence of actin was subtracted). When the gizzard tropomyosin fraction (see METHODS) was added the ATPase activity increased and a linear rate of about 43 nmoles P_i $min^{-1}mg^{-1}$ myosin was obtained (again the ATPase rate for myosin alone was subtracted, see METHODS). The phosphorylation curves for the two systems, however, were identical. This clearly illustrates that for a given level of phosphorylation the ATPase activity of actomyosin can vary considerably depending on what additional components are present.

A qualitatively similar pattern to that described above was obtained earlier (10) and it was shown that a partially purified MLCK achieved a pronounced activation in the absence of tropomyosin and a further activation (2 to 3 fold) was observed in the presence of tropomyosin. Our results are quantitatively different and suggest that phosphorylation alone produces only a slight activation of ATPase activity.

There is sufficient evidence to propose that myosin phosphorylation is implicated in the regulatory mechanism of smooth muscle (1) although the results presented above suggest that phosphorylation alone will not allow the full activation of ATPase activity by actin. From earlier studies (8,10) and from the experiments shown in Fig. 3 it is reasonable to assume that tropomyosin is also involved. However, preliminary evidence indicates that tropomyosin alone is not sufficient. Gizzard tropomyosin purified by chromatography on hydroxyapatite (see METHODS) does not restore full ATPase activity to a reconstituted actomyosin and this was achieved only when an additional fraction was added. These results will be reported later in more detail. The identity of the "activating" factors and their relationship to leiotonin is obviously a priority for future research.

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