THE EFFECT OF PHOSPHORYLATION OF GIZZARD MYOSIN ON ACTIN ACTIVATION

A. Gőrecka[1], M. O. Aksoy[2] and D. J. Hartshorne[2]

[1]Nencki Institute of Experimental Biology, 3, Pasteur Str. Warsaw, 02-093

[2]Departments of Biological Sciences and Chemistry Carnegie-Mellon University, Pittsburgh, Pa. 15213

Received May 18,1976

SUMMARY

Gizzard myosin is phosphorylated by a kinase found in chicken gizzards. The 20,000 dalton light chains are the only subunits to show an appreciable extent of ^{32}P incorporation. Phosphorylation requires trace amounts of Ca^{2+}. The Mg^{2+}-ATPase activity of gizzard myosin in the phosphorylated form is activated to an appreciable extent by skeletal actin, whereas the activation of the non-phosphorylated myosin is very low. These results suggest that the Ca^{2+}-sensitive regulatory mechanism of gizzard actomyosin is mediated via a kinase. In the presence of Ca^{2+} the onset of contraction and the resultant increase of the Mg^{2+}-ATPase activity we suggest is due, at least partly, to the phosphorylation of the 20,000 dalton light chains. Whether or not Ca^{2+} binding by myosin is also essential remains to be established.

INTRODUCTION

The mechanism of regulation of smooth muscle actomyosin by Ca^{2+} is the subject of some controversy. There are basically two theories, one favoring a troponin-like protein (1-3) and the other suggesting that the myosin molecule fulfills the regulatory function (4-7). Our recent results, although in favor of myosin-linked regulation have suggested that an additional factor is implicated. We proposed (8) that this involves a Ca^{2+}-dependent phosphorylation of one of the components of the actomyosin complex, and the possibility was raised that a kinase forms part of the regulatory mechanism in smooth muscle.

The preparation of kinase that we used was basically the same fraction that Ebashi et al. (1) designated gizzard native tropomyosin. It is interesting that the effect of this fraction on the Mg^{2+}-ATPase activity of desensitized gizzard actomyosin was to cause an activation in the presence of Ca^{2+} and to have very little

effect in the absence of Ca^{2+} (2,8). This is quite different from the effects produced in the skeletal muscle system using troponin and tropomyosin (9). It has been noted by several investigators (7,10,11) that the Mg^{2+}-ATPase activity of smooth muscle myosin was not activated effectively by actin. Since the crude kinase preparation activated the Mg^{2+}-ATPase activity of gizzard actomyosin and as this was accompanied by a phosphorylation of the actomyosin (8) it was thought that phosphorylation may be a prerequisite for actin activation. Before this hypothesis could be accepted, however, it was necessary to identify the site of phosphorylation, which might be expected to be the myosin molecule. This prediction was realized, and in this communication it is shown that the 20,000 dalton light chains of myosin are phosphorylated. Our results indicate also that phosphorylated myosin is activated by actin to a greater extent than the non-phosphorylated form. These data suggest that the regulatory mechanism in gizzard actomyosin is mediated via a kinase rather than a troponin-like component.

MATERIALS AND METHODS

Myosin was prepared using a combination of two methods. The initial part of the preparation followed the procedure of Bailin and Bárány (12). Treatment with ribonuclease and chromatography on DEAE-cellulose were omitted. The second part of the method employed the purification techniques outlined by Sobieszek and Bremel (13). Myosin was dissolved in 0.6M KCl and clarified by centrifugation at 100,000xg for 1 hr. The supernatant was diluted with 9 volumes of cold distilled water, and the precipitated myosin was collected by centrifugation at 5,000xg for 10 minutes. The pellets were suspended in 60mM KCl and ATP and EDTA were added to a final concentration of 10mM and 1mM, respectively. This solution was centrifuged at 40,000xg for approximately 20 hrs. $MgCl_2$ was added to the supernatant to a final concentration of 40mM. This step precipitated the myosin which was collected by low speed centrifugation and dialysed against 20mM KCl, 10mM tris-HCl (pH 7.6) 0.2mM dithiothreitol.

Actin from skeletal muscle and tropomyosin from chicken gizzard were prepared as outlined previously (14). The crude kinase from chicken gizzards was prepared following the procedure used by Ebashi et al. (1) to isolate native tropomyosin. The original method was modified slightly as we used a fraction obtained between 37 and 55 percent ammonium sulfate saturation.

Incorporation of ^{32}P from γ-labelled [^{32}P] ATP was measured by the method described previously (8).

ATPase activity was assayed in a medium containing 50mM KCl, 10mM $MgCl_2$, 2.5mM ATP, 25mM tris-HCl (pH 7.6). When required, 1mM 2,2'-ethylenedioxybis [ethyliminodi (acetic acid)], EGTA, was added to remove free Ca^{2+}. Other assay conditions were as given earlier (14).

The method of Fairbanks et al. (15) was used for SDS polyacrylamide electrophoresis.

RESULTS

The site of phosphorylation was identified by subjecting a phosphorylated sample of gizzard actomyosin to SDS gel electrophoresis and then determining the ^{32}P content in different regions of the gel. Shown in Fig. I is a scan of a stained gel and the ^{32}P content associated with each component. The only subunit with a significant extent of ^{32}P incorporation was the 20,000 dalton light chain of myosin (designated in Fig. I as L.C.2).

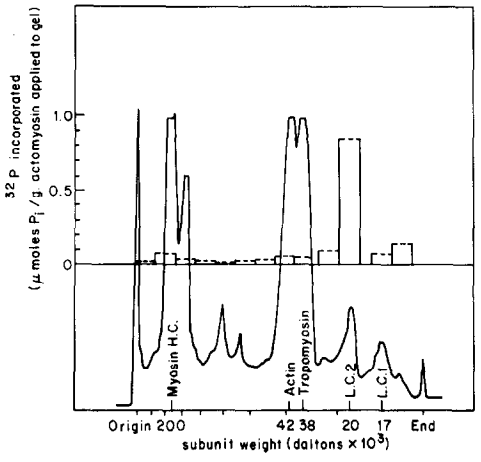

Fig. 1. The distribution of ^{32}P incorporation in gizzard actomyosin as determined by SDS gel electrophoresis. Phosphorylation was carried out as described previously (8) through the heating step (90°C for 20 min.). The actomyosin was then dialysed exhaustively against 10mM Tris-HCl (pH 7.6) and applied to SDS polyacrylamide gels (15). 10 gels were prepared, 55 μg protein was applied to each. All gels were stained (15) and one gel was scanned at 550 nm. The protein bands from the other 9 gels were cut out and each component combined in a scintillation vial. 0.7ml of 30% H_2O_2 was added and the samples were heated at 50-55°C for 5 hrs. Scintillation liquid was added and the samples were counted (8).

The effect of the kinase preparation on the Mg^{2+}-ATPase activity of a mixture of gizzard myosin and skeletal actin, is shown in Fig. 2. Shown also in this figure is the extent of ^{32}P incorporation associated with each mixture. At low concentrations of kinase, in the presence of Ca^{2+}, the Mg^{2+}-ATPase activity and the

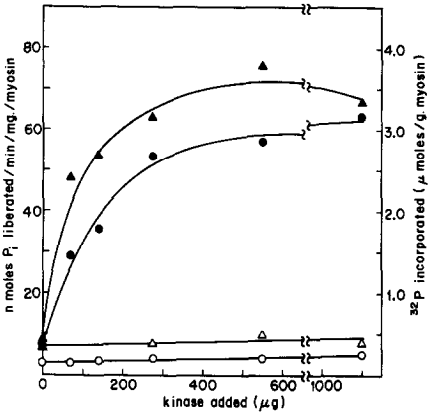

Fig. 2 The effect of kinase on the phosphorylation and Mg^{2+}-ATPase activity of gizzard myosin and skeletal actin. Assay conditions for both ATPase activity and phosphorylation were: 10mM $MgCl_2$, 50mM KCl, 25mM tris-HCl (pH 7.6), 2.5mM ATP. Myosin, 0.76mg. Actin 0.58mg. (●,○) ATPase activity. (▲,△) ^{32}P incorporation. Solid and open symbols denote assays done in the absence and presence of 1mM EGTA, respectively.

level of ^{32}P incorporation increased rapidly. The increase became less marked at higher concentrations of kinase. An important feature of this system is that in the absence of Ca^{2+} (i.e. the presence of EGTA) the Mg^{2+}-ATPase activity of the actomyosin was not affected by the addition of kinase and neither was the extent of ^{32}P incorporation. This confirms our earlier findings (8) that the phosphorylation is Ca^{2+} dependent. The suggestion that was made previously (8) that a relationship existed between Ca^{2+} sensitivity and phosphorylation of the actomyosin complex may now be refined to identify the site of phosphorylation as the myosin molecule.

If one takes the maximum level of phosphorylation of the myosin molecule from Fig. 2 as 3.6 moles/10^6g myosin, then this is equivalent to approximately 1.7 moles P incorporated per mole myosin. Since there are probably 2 moles of the 20,000 dalton light chain per mole of gizzard myosin (7) it is reasonable to expect that the maximum extent of phosphorylation is two moles P per mole myosin, i.e. one per 20,000 light chain. The reason why this level is not obtained experimentally is due most likely to the presence of a phosphatase.

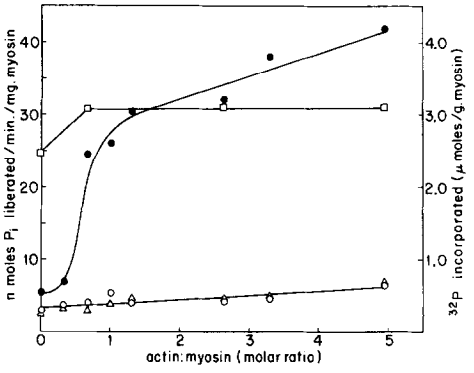

Fig. 3 The effect of varying actin concentrations on the phosphorylation and Mg^{2+}-ATPase activity of gizzard myosin. Assay conditions as in Fig. 2. Kinase, 0.27mg. ATPase assays done in the absence (●) and presence (○) of 1mM EGTA. (△) ATPase assays done in the absence of kinase. (□) ^{32}P incorporation done in the presence of kinase and absence of EGTA.

The effect of the kinase on the activation of gizzard myosin by actin can be shown more effectively in Fig. 3. For this experiment a constant amount of myosin and kinase was used and the amount of actin was varied. The Mg^{2+}-ATPase activity of the gizzard myosin was activated significantly by relatively low concentrations of actin. Again, it should be pointed out that this activation was achieved only in the presence of kinase and in the presence of Ca^{2+} (Fig. 3). When gizzard tropomyosin was used in place of the kinase no activation was observed.

A significant finding, illustrated in Fig. 3, is that in the presence of the kinase the activation by actin occurred at a reasonable stoichiometry of actin to myosin. The Mg^{2+}-ATPase activity increased rapidly up to an equimolar ratio and then increased more gradually with excess actin. The level of ATPase activity that is obtained with a mixture of gizzard myosin, skeletal actin and kinase is similar to that found with gizzard actomyosin isolated as the intact complex (8), assuming for the latter an actin to myosin molar ratio of between 2 and 3.

The extent of ^{32}P incorporation was determined for some of the experimental points and is shown in Fig. 3. The influence of actin on the phosphorylation of myosin is not significant. Although a slight increase was observed on the addition

of actin, it is clear that the major extent of phosphorylation of myosin occurred in the absence of actin.

DISCUSSION

The above results indicate that phosphorylated myosin is activated by actin more efficiently than non-phosphorylated myosin. This finding could account for the variable extents of actin activation reported in the literature (7,10,11), as one might expect a relationship between the degree of actin activation and the kinase content in the myosin preparation. The possibility that the activation of ATPase activity is an artifact due to the action of a "pseudo-ATPase" (a linked kinase-phosphatase system) is unlikely. If a "pseudo-ATPase" system was responsible for the effect, the activation by the kinase of the Mg^{2+}-ATPase activity of myosin alone should approach that of the actin-myosin complex. This was not the case. We were also concerned with the possibility that actin might influence the phosphorylation of myosin. This is unlikely, as it was shown in Fig. 3 that the extent of ^{32}P incorporation was essentially independent of the concentration of actin.

There are many reports in the literature on the phosphorylation of muscle or muscle-like proteins (16). In general, however, phosphorylation is not accompanied by an alteration of function. An exception to this is the report of Adelstein and Conti (17) in which it was demonstrated that phosphorylation of platelet myosin increased the extent of actin activation. These observations are similar to ours except that the platelet kinase is not Ca^{2+} dependent. The 20,000 dalton light chain of platelet myosin also was phosphorylated (17,18).

In skeletal muscle, the Ca^{2+} dependent control system operates by regulating the actin myosin interaction, and in the absence of Ca^{2+} by effecting a dissociation of the two proteins (19,20). This is the function of troponin and tropomyosin. In smooth muscle (if one generalizes the situation found in gizzard) there appears to be a different mechanism. There is no control exerted through the thin filaments, and the regulatory mechanism is centered on the myosin molecule (4-7). The situation, however, is not simply a myosin-linked system such as that found, for example, in the molluscs (21). Myosin alone is not sufficient for regulation for, as we have

shown, a kinase is implicated. An additional factor must also be present to allow the kinase to serve a regulatory function, namely, a phosphatase which removes the phosphate groups from the myosin. Gizzard actomyosin preparations do contain a phosphatase, as the dephosphorylation of myosin can be observed following the removal of free Ca^{2+}. The balance of the kinase and phosphatase obviously is a critical feature of the regulatory mechanism and this must be resolved in the future. Another point which is not clear at this time is the role of Ca^{2+} binding to myosin. It was shown previously that gizzard myosin binds Ca^{2+} (7) but it is not known if this is an essential part of the mechanism and is required in addition to the phosphorylation.

ACKNOWLEDGMENTS

This work was supported by grant HL-09544 from the National Institutes of Health

REFERENCES

1. Ebashi, S., Iwakura, H., Nakajima, H., Nakamura, R. and Ooi, Y. (1966) Biochem. Z. 345, 201-211.
2. Ebashi, S., Toyo-oka, T. and Nonomura, Y. (1975) J. Biochem. (Tokyo) 78, 859-861.
3. Carsten, M. E. (1971) Arch. Biochem. Biophys. 147, 353-357.
4. Bremel, R. D. (1974) Nature 252, 405-407.
5. Bremel, R. D. and Sobieszek, A. (1975) Biochemistry of Smooth Muscle, N. L. Stephens (ed.) in press, University Park Press, Baltimore.
6. Mrwa, U. and Rüegg, J. C. (1975) FEBS letters, 60, 81-84.
7. Hartshorne, D. J., Abrams, L., Aksoy, M., Dabrowska, R., Driska, S. and Sharkey, E. (1975) Biochemistry of Smooth Muscle, N. L. Stephens (ed.) in press, University Park Press, Baltimore.
8. Aksoy, M. O., Williams, D., Sharkey, E. M. and Hartshorne, D. J. (1976) Biochem. Biophys. Res. Commun. 69, 35-41.
9. Hartshorne, D. J. and Mueller, H. (1969) Biochim. Biophys. Acta 175, 301-319.
10. Bárány, M., Þárány, K., Gaetjens, E. and Bailin, G. (1966) Arch. Biochem. Biophys. 113, 205-221.
11. Yamaguchi, N., Miyazawa, Y. and Sekine, T. (1970) Biochim. Biophys. Acta 216, 414-421.
12. Bailin, G. and Þárány, M. (1971) Biochim. Biophys. Acta 236, 292-302.
13. Sobieszek, A. and Bremel, R. D. (1975) Eur. J. Biochem. 55, 49-60.
14. Driska, S. and Hartshorne, D. J. (1975) Arch. Biochem. Biophys. 167, 203-212.
15. Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606-2617.
16. Perry, S. V., Cole, H. A., Morgan, M., Moir, A. J. G. and Pires, E. (1974) IX FEBS Meeting, Budapest. Proc. Fed. Eur. Biochem. Soc. 163-176. Budapest and North Holland Publ. Co., Amsterdam and London.
17. Adelstein, R. S. and Conti, M. A. (1975) Nature 256, 597-598.
18. Daniel, J. L. and Adelstein, R. S. Biochemistry, in press.
19. Parker, L., Pyun, H-Y, and Hartshorne, D. J. (1970) Biochim. Biophys. Acta 223, 453-456.
20. Eisenberg, E. and Kielley, W. W. (1970) Biochem. Biophys. Res. Commun. 40, 50-56.
21. Lehman, W. and Szent-Györgyi, A. G. (1975) J. Gen. Physiol. 66, 1-30.