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Phosphorylation of Myosin Light Chain and the Actin-Activated ATPase Activity of Adrenal Medullary Myosin[1]

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Myosin light chain kinase was partially purified from bovine adrenal medulla. A polypeptide of Mr 165,000 dalton was identified as kinase by using anti-gizzard myosin light chain kinase IgG on immunoreplica. Phosphorylation of medullary myosin was Ca^{2+}- and calmodulin-dependent. The phosphorylated myosin was showed to enhance the actin-activated Mg^{2+}-ATPase activity. In contrast, the myosin ATPase activity was dramatically decreased by dephosphorylation of myosin.

It has been shown that the secretion of catecholamines from chromaffin cells requires Ca^{2+} and ATP (*1*). By analogy with the 'excitation-contraction coupling' in muscle, these secretory processes have been called 'stimulus-secretion coupling' (*2*). In the past decade, large amounts of evidence have been presented supporting the involvement of contractile proteins in the secretory processes. The adrenal medulla is favorable for elucidating the 'stimulus-secretion coupling.' Indeed, actomyosin-like protein (*3*, *4*), myosin (*5–7*), and actin (*8*) have been isolated from adrenal medulla. Until now, there was no evidence concerning the regulatory mechanism of adrenal medullary actomyosin. Recently, we isolated Ca^{2+}-sensitive actomyosin from adrenal medulla which is superprecipitable and shows Mg^{2+}-activated ATPase activity (in preparation). In this communication, we report that Ca^{2+}- and calmodulin-dependent myosin light chain kinase has been partially purified from adrenal medulla and the phosphorylation of medullary myosin is a key event of the actin-myosin interactions in this tissue.

The preparation of Ca^{2+}-sensitive actomyosin from bovine adrenal medulla was carried out essentially according to the method used for preparing platelet actomyosin (*9*). Myosin was extracted and isolated from Ca^{2+}-sensitive actomyosin by the method of Ebashi (*10*) and further purified by Sepharose 4B column chromatography with a high ionic strength buffer (0.6 M KCl, 20 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol, and 0.1 mM EGTA). By densitometric scanning, the medullary myosin was estimated to be >95 percent pure. Details of the purification procedures for myosin are given elsewhere.

The Ca^{2+}- and calmodulin-dependent myosin light chain kinase was partially purified as follows.

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Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate.

The fraction containing the kinase was extracted from bovine adrenal medulla and fractionated with ammonium sulfate (20–60% saturation) by the same method as described in Ref. *11*. The fraction obtained was dissolved in buffer (20 mM Tris-HCl, pH 7.5, and 0.1 mM dithiothreitol) containing 100 mM KCl and 0.2 mM $CaCl_2$, and centrifuged at 105,000 × *g* for 60 min. The clear supernatant was applied to a calmodulin-coupled Sepharose 4B column. The column was extensively washed with the above buffer and then with the buffer containing 500 mM KCl and 0.2 mM $CaCl_2$. Calmodulin-binding proteins were eluted with the buffer containing 100 mM KCl and 1 mM EGTA. The eluted fractions were diluted with 2 vols. of the buffer and applied to a DEAE-cellulose column which had been equilibrated with the buffer containing 30 mM KCl and 0.1 mM EGTA. The proteins were eluted with a linear gradient of 30 to 400 mM KCl (in 20 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol, and 0.1 mM EGTA). The peak fraction containing kinase activity, which was monitored using chicken gizzard myosin light chain as a substrate (*12*), was eluted at 180 mM KCl and used as the crude kinase fraction. The specific kinase activity was 0.5 μmol of P_i transferred/min/mg protein and Ca^{2+} and calmodulin were required for the enzyme action.

Actin (*13*) and calmodulin (*14*) were purified from rabbit skeletal muscle and bovine brain, respectively. Gizzard myosin light chain kinase and myosin light chain phosphatase were prepared as described in Refs. *11* and *15*. Protein was determined by the microbiuret method (*16*).

The medullary myosin contains at least two species of light chains with apparent Mr of 20,000 (LC 1) and 16,500 (LC 2) daltons, as determined by SDS polyacrylamide gel electrophoresis (Fig. 1b). The medullary LC 2 migrated slightly faster than the gizzard light chain (Mr 17,000). The medullary myosin light chains appeared to be composed of equal amounts of LC 1 and LC 2. In addition, a small amount of a contaminant of Mr 19,000 daltons was detected on SDS-gels. At present, it is uncertain whether this polypeptide is a third species of light chain or not. A Mr 165,000 dalton polypeptide in the crude kinase fraction was the only polypeptide which cross-reacted with anti-gizzard myosin light chain kinase (a Mr 135,000 dalton protein) IgG on immunoreplica (Fig. 1e). This polypeptide corresponded to about 27 percent of the total protein in the crude kinase fraction determined by densitometry.

The medullary myosin was incubated with the medullary crude kinase fraction or purified gizzard kinase in the presence of ATP with or without Ca^{2+} and calmodulin. Urea-gel electrophoresis revealed that one of the light chains of the medullary myosin (LC 1) was phosphorylated by both of the two kinase preparations only in the presence of Ca^{2+} and calmodulin (Fig. 2). The phosphorylated light chain migrated faster than the unphosphorylated one. The identification of the phosphorylated polypeptide separated on SDS- and urea-gels was also confirmed by using [^{32}P]ATP (data not shown). The phosphorylation experiment showed that the medullary myosin is a substrate for the medullary and gizzard kinases. Treatment with myosin light chain phosphatase from gizzard muscle resulted in the dephosphorylation of phosphorylated medullary LC 1 (data not shown). Analysis by urea-gel electrophoresis showed that the purified medullary myosin used in this experiment was already phosphorylated to the extent of about twenty percent of LC 1, without the incubation. Surprisingly, LC 2 was subdivided into two differently migrating polypeptides (LC 2a and LC 2b) on urea-gels. LC 2a and LC 2b were not phosphorylated by the two kinase preparations. By measuring the staining intensity, LC 2a and LC 2b were determined to be present in equal amounts. From these results, it appeared that the medullary myosin comprises at least three species of light chains of Mr 20,000 (LC 1) and Mr 16,500 (LC 2a and LC 2b) in a 2 : 1 : 1 molar ratio.

In the absence of F-actin, the Mg^{2+}-ATPase activities of medullary myosin in a low ionic strength buffer (60 mM KCl) were extremely low, 1–4 nmol/min/mg protein. Increasing amounts of added F-actin enhanced the Mg^{2+}-ATPase activity in the presence of kinase preparations and calmodulin with or without Ca^{2+} (Fig. 3). The same results were obtained on using medullary and gizzard kinases. The specific actin-activated Mg^{2+}-ATPase activity was estimated to be about 26–28 nmol/min/mg myosin under the conditions where the kinases were active in the presence of Ca^{2+}. Under the activated conditions for kinase activity, the medullary myosin was found to be phosphorylated to the extent of more than 90 percent of

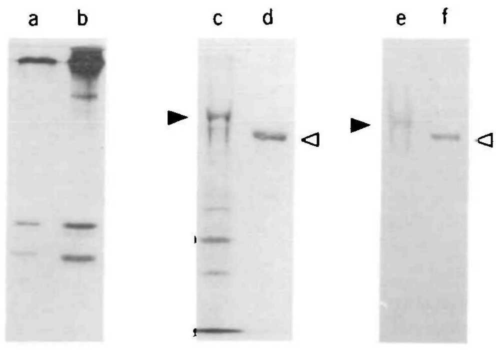

Fig. 1. Identification of myosin light chains and myosin light chain kinase from adrenal medulla. SDS-polyacrylamide gel electrophoreses of gizzard (a) and medullary myosin (b), the crude kinase fraction from adrenal medulla (c) and purified gizzard myosin light chain kinase (d). Electrophoreses were carried out in 15% (a, b) or 7.5% (c, d) polyacrylamide gels in the buffer system of Laemmli (*25*). Gels were stained with Coomassie brilliant blue and destained with 7% acetic acid. Immunoreplica of (c) and (d), which corresponded to (e) and (f), respectively, was performed by using anti-gizzard myosin light chain kinase as in Ref. *26*.

total LC 1, when determined by urea-gel electrophoresis. The level of Mg^{2+}-ATPase activity was comparable with that of smooth muscle actomyosin (*17*, *18*). In contrast, the actin-activated Mg^{2+}-ATPase activity was 11–12 nmol/min/mg myosin in the absence of Ca^{2+} where the kinases were inactive and the phosphorylated LC 1 remained constant, about twenty percent of total LC 1, before and after the ATPase measurement under these assay conditions. These results indicate that the actin-dependent enhancement of Mg^{2+}-ATPase activity with the inactive state of the kinase might be due to the partial phosphorylation of LC 1 during the purification procedure. In fact, the actin-activated Mg^{2+}-ATPase activity was observed to be much lower in the presence of gizzard myosin light chain phosphatase (2–3 nmol/min/mg myosin, Fig. 3). In agreement with our results, Creutz demonstrated that the Mg^{2+}-ATPase of medullary myosin was activated by the addition of F-actin (*7*). In this report, however, he did not consider the phosphorylation of myosin. It is likely that the myosin used was partially phosphorylated. In our preliminary experiment, the actin-activated Mg^{2+}-ATPase activity was further enhanced with combination with bovine brain tropomyosin which was prepared according to the method of Bretscher

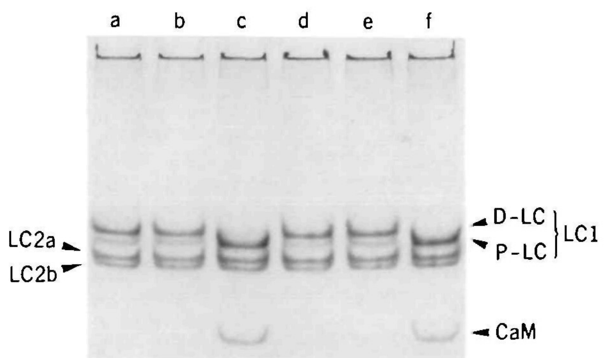

Fig. 2. Ca^{2+}- and calmodulin-dependent phosphorylation of medullary myosin light chains determined by urea-gel electrophoreses (*27*). The assay conditions were as follows: 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM dithiothreitol, 0.2 mM $CaCl_2$ or 1 mM EGTA, 0.5 mg/ml of medullary myosin, and 10 μg/ml of medullary kinase (a–c) or gizzard kinase (d–f) with or without calmodulin (16 μg/ml). (a) and (d), 1 mM EGTA; (b) and (e), 0.2 mM $CaCl_2$; (c) and (f), 0.2 mM $CaCl_2$ and calmodulin. LCl, myosin light chain of Mr 20,000; D-LC, dephosphorylated light chain; P-LC, phosphorylated light chain; LC2, myosin light chain of Mr 16,500; CaM, calmodulin.

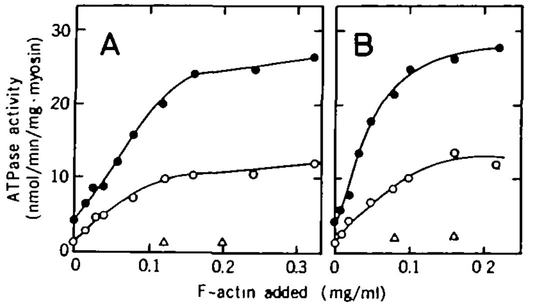

Fig. 3. Effect of Ca^{2+}- and calmodulin-dependent myosin light chain phosphorylation on the actin-activated Mg^{2+}-ATPase of medullary myosin. The medullary myosin (0.4 mg/ml) was incubated at 30°C for 5 min in a total vol. of 0.2 ml containing 20 mM imidazole HCl, pH 6.9, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 25 μg/ml of medullary kinase (A) or gizzard kinase (B), 20 μg/ml of calmodulin, and 0.1 mM $CaCl_2$ (●) or 1 mM EGTA (○). The actin-activated Mg^{2+}-ATPase activity in the presence of gizzard myosin light chain phosphatase (50 μg/ml) without kinases and calmodulin is also shown (△). F-actin was added as indicated. Incubation was started by the addition of 1 mM ATP. Phosphate was determined by the method of Youngburg and Youngburg (*28*).

and Weber (*19*).

There is controversy over the regulatory mechanism of smooth and non-muscle actomyosins. The most widely accepted view is that the myosin-linked regulation operates *via* phosphorylation of myosin light chains by a specific Ca^{2+}- and calmodulin-dependent kinase and dephosphorylation by myosin phosphatase (*20*). On the other hand, Ebashi *et al.* proposed that the actin-linked regulation in smooth muscle is achieved through a system based on some factors which confer the Ca^{2+}-sensitivity upon thin filaments, called leiotonins A and C (*21*). In non-muscle cells including brain (*22*), platelets (*23*), and thymus (*24*), it has been shown that myosin phosphorylation represents an obligatory process in the actin-myosin interaction.

In spite of the purification of contractile elements from adrenal medulla, there has been no report on the control mechanism of actomyosin in this tissue. The evidence we presented in this paper is the first concerning the Ca^{2+}-dependent regulatory mechanism in medullary actin-myosin interaction. We have concluded that myosin-linked regulation occurs in the medullary actomyosin *via* the Ca^{2+}- and calmodulin-dependent phosphorylation of myosin. Further studies are in progress to determine whether any factors other than myosin phosphorylation are required for the Ca^{2+}-dependent regulation of medullary actomyosin or not.

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