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ACTIVATION OF MYOSIN LIGHT CHAIN KINASE BY TRYPSIN

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SUMMARY

We have demonstrated that trypsin activated the activities of partially purified myosin light chain kinases both from chicken gizzard and from rabbit skeletal muscle. Controlled exposure to trypsin produced activation of the chicken gizzard kinase to a similar extent as was observed with calmodulin plus calcium. The activation by trypsin did not require calcium and was dependent on both the preincubation time with the protease and its concentration. Our result suggests that the limited proteolysis of the kinase by trypsin not only stimulates the activity of the kinase but also converts the kinase to the Ca^{2+}-calmodulin insensitive form.

INTRODUCTION

Increasing evidence indicates that calmodulin (Ca^{2+}-regulated modulator protein) can activate a number of different enzymes such as cyclic nucleotide phosphodiesterase (1, 2), the myosin light chain (3, 4) or the calmodulin-dependent protein kinase (5), brain adenylate cyclase (6), the Ca^{2+}-Mg^{2+}-ATPase of erythrocyte plasma membrane (7, 8), glycogen synthase kinase (9) and phosphorylase kinase (10). But, the precise mechanism of the activation by calmodulin has not been clarified. Ca^{2+}-dependent cyclic nucleotide phosphodiesterase is reported to be activated not only by Ca^{2+}-calmodulin but also by α-chymotrypsin (11) or trypsin (12, 13, 14).

In this paper, we demonstrate that the activities of both chicken gizzard myosin light chain kinase and rabbit skeletal muscle myosin light chain kinase are activated and lose Ca^{2+}-dependency by controlled exposure to trypsin.

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The abbreviations used are: MLCK, myosin light chain kinase;
EGTA, ethyleneglycol-bis-(β-amino-ethyl ether)-N,
N'-tetraacetic acid

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MATERIALS AND METHODS

Protein preparations: The myosin light chain kinase from chicken gizzard was obtained from 75 g of gizzards. Washed myofibrils were prepared and extracted by the method of Sobieszek and Bremel (15). Solid ammonium sulfate was added to the extract supernatant, and the fraction precipitating between 35 and 60% saturation was sedimented at 20,000 x g for 20 min. The precipitate was solubilized in 30 ml of 50 mM Tris-HCl (pH 7.0), 2 mM EGTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM $MgCl_2$, 60 mM KCl and dialyzed overnight against 3 liters of the same buffer. The supernatant collected at 30,000 x g for 20 min after dialysis was then applied to a column (1.9 x 32 cm) of DEAE-cellulose (Whatman). The column was washed with the same buffer containing 60 mM KCl and then eluted with 450 ml of the buffer in a linear gradient of KCl (0.06 to 0.5 M). The major peak of myosin kinase activity was applied to a column (2.6 x 83 cm) of Sepharose 4B, equilibrated with the same buffer used in dialysis. This fraction was used for the MLCK assays.

Calmodulin-deficient myosin light chain kinase from rabbit skeletal muscle was prepared by the method of Yazawa, M. and Yagi, K. (16).

The 20,000-dalton light chain of chicken gizzard myosin, which was used as substrate for the MLCK, was prepared by the method of Perrie and Perry (17). Further purification yielding a homogeneous and calmodulin deficient light chain was achieved by DEAE-ion exchange chromatography using a gradient described for the final step in the purification of calmodulin (18).

Bovine brain calmodulin was prepared as previously reported (19).

Trypsin (TRTPCK, treated with L-(tosylamide 2-phenyl)ethyl chloromethyl ketone to inhibit contaminant chymotryptic activity according to Kostka and Carpenter (20, 259 Units/mg) was from Worthington Biochemical Corp. and soybean trypsin inhibitor was from Sigma Chemical Corp.

Assay for MLCK activity: Unless specified otherwise, all kinase assays were preformed at 25°C in a final volume of 0.2 ml containing 20 mM Tris-HCl (pH 7.5) 0.2 mM [γ-^{32}P]ATP, 18 μM smooth muscle 20,000-dalton myosin light chain, 10 mM $MgCl_2$, and either 0.1 mM $CaCl_2$ or 1 mM EGTA. The kinase concentration from chicken gizzard was 3.0 μg/ml and from rabbit skeletal muscle was 5.0 μg/ml. The final concentration of calmodulin was 0.1 μM for chicken gizzard MLCK and 2.8 μM for skeletal muscle MLCK, respectively. Incubations were terminated at 1 min by the addition of 1 ml of 10% trichloroacetic acid.

Protein concentration was determined by the method of Lowry et al. (21).

RESULTS

Effect of preincubation time with trypsin and concentration of trypsin on activation of the chicken gizzard kinase activity are shown in Fig. 1. At the concentrations of 2, 5 and 10 μg/ml of trypsin, the activation of the MLCK from chicken gizzard was observed in time-dependent fashion.

As shown in Fig. 2, incubation of the chicken gizzard MLCK with trypsin for 6 min at 25°C caused activation of the MLCK in a dose-dependent manner at the concentrations ranging from 1.0 to 100 μg/ml of trypsin.

Maximum stimulation was observed in the presence of 100 μg/ml trypsin which caused approximately 80-fold increase in the MLCK activity. This increase in

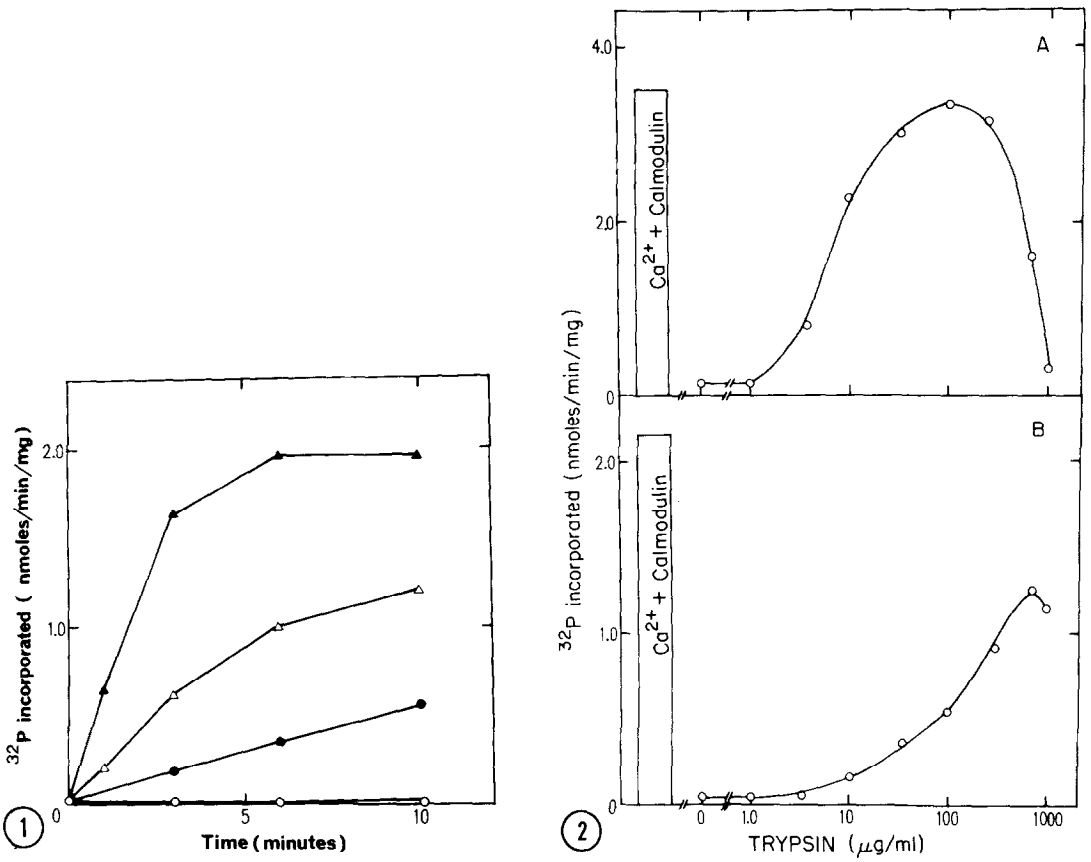

Fig. 1 Trypsin induced-activation of the myosin light chain kinase from chicken gizzard. The myosin kinase (6.0 μg/ml) was incubated in a total volume of 0.1 ml without (○) or with trypsin, 2(●), 5(△) or 10(▲) μg/ml in the presence of 1 mM EGTA. At the indicated time, trypsin inhibitor (500 μg) was added and ^{32}P incorporation into the 20,000-dalton light chain of chicken gizzard myosin was assayed under standard condition. Each point represents the mean of duplicate assay tubes.

Fig. 2 Effect of trypsin and calmodulin plus Ca^{2+} on myosin light chain kinases from chicken gizzard (A) and rabbit skeletal muscle (B). The myosin kinases from chicken gizzard (6.0 μg/ml) and skeletal muscle (10.0 μg/ml) were incubated in a total volume of 0.1 ml with trypsin as indicated concentrations ranging from 1.0 to 1000 μg/ml at 25°C in the presence of 1 mM EGTA. A trypsin inhibitor (500 μg) was added after 6 min for smooth muscle MLCK and after 2 min for skeletal muscle MLCK and ^{32}P incorporation into myosin light chain was assayed under standard condition. Maximum stimulation of each MLCK activity by calmodulin plus Ca^{2+} is also shown in the figure. Saturating amounts (0.1 μM for smooth muscle MLCK and 2.8 μM for skeletal muscle MLCK) of purified calmodulin from bovine brain were used.

the activity of MLCK was comparable to the activation observed with calcium and calmodulin. However, addition of trypsin over 400 μg/ml produced reduction of the stimulation of the kinase activity (Fig. 2).

TABLE 1

Effect of calmodulin, Ca^{2+} and EGTA on the myosin light chain kianse activities from smooth muscle (chicken gizzard) and skeletal muscle (rabbit) after treatment with trypsin. The myosin kinases from chicken gizzard (6.0 μg/ml) and rabbit skeletal muscle (10.0 μg/ml) were incubated with trypsin as indicated concentrations in the presence of 1 mM EGTA at 25°C and incubations were terminated by addition of soybean trypsin inhibitor (500 μg) after 6 min for smooth muscle MLCK and after 2 min for skeletal muscle MLCK. And the non-treated and trypsin-treated enzymes were assayed with addition as indicated for 1 min at 25°C under standard condition. Saturating amounts (0.1 μM for smooth muscle MLCK and 2.8 μM for skeletal muscle MLCK) of purified calmodulin from bovine brain were used.

Additions	Myosin light chain kinase activity (^{32}P nmoles/min/mg)[a]			
	Smooth muscle (chicken gizzard)		Skeletal muscle (rabbit)	
	No trypsin	100 μg/ml trypsin	No trypsin	1 mg/ml trypsin
1 mM EGTA	0.04	3.38	0.02	1.17
1 mM EGTA + Calmodulin	0.04	3.48	0.02	1.14
100 μM Ca^{2+}	0.04	3.42	0.03	1.16
100 μM Ca^{2+} + Calmodulin	3.52	3.00	2.20	1.01

[a] Results are shown as nmol ^{32}P transferred per mg chicken gizzard myosin light chain per min and each value represents the mean of duplicate assay tubes.

Moreover, trypsin produced significant activation of the MLCK activity from rabbit skeletal muscle as also shown in Fig. 2. This is not in agreement with the result by Waisman et al. (5).

The trypsin-treated myosin kinase form chicken gizzard was no longer activated by exposure to calcium plus calmodulin as demonstrated in Table 1. The additional effects of maximally effective doses of calmodulin and trypsin in the presence of calcium were not demonstrated. The calcium-calmodulin stimulated enzyme was inhibited by addition of ethyleneglycol-bis-(β-aminoethylether)-N, N', -tetraacetic acid (EGTA). The trypsin-treated enzyme was no longer inhibited by EGTA, suggesting that limited proteolysis by trypsin converts the enzyme into Ca^{2+}-insensitive form. The same characteristics of the MLCK from skeletal muscle are also shown in Table 1.

DISCUSSION

The results indicate that trypsin activates the myosin light chain kinases both from chicken gizzard and from skeletal muscle, and maximum protease activation causes 80-fold increase in the chicken gizzard myosin kinase activity,

which is comparable to the activation with calmodulin and calcium. Activation of phosphorylase kinase by proteolysis has been reported by several workers (22, 23). Moreover, a proenzyme of protein kinase which can be converted to the active form by trypsin or Ca^{2+}-dependent neutral protease from many mammalian tissues has been reported (24). Calmodulin-dependent cyclic nucleotide phosphodiesterase can be converted to an active calmodulin independent form with decrease in molecular weight by proteolytic degradation (11, 14). However, Waisman et al. report that the calmodulin-dependent protein kinase (myosin light chain kinase) from rabbit skeletal muscle is not activated by trypsin (5). This inconsistancy with our result is not clear at the present time but may be due to the higher concentration of trypsin necessary to activate the skeletal muscle MLCK.

There have been reported to be two kinds of the myosin light chain kinases, Ca^{2+}-dependent and Ca^{2+}-independent enzymes, isolated from human platelet by Adelstein et al. (25, 26). In addition, the estimated molecular weight of the calcium-dependent kinase was 105,000 and that of the calcium-independent species was 78,000 (25, 26). As Adelstein et al. described, it is possible that limited proteolysis of the native enzyme may account for the observed differences in molecular weight and calcium dependence.

The above results suggest that the role of proteolysis as a regulatory mechanism of this enzyme is not only to stimulate the activity but also to change the enzyme property.

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