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PHOSPHORYLATION OF MYOSIN LIGHT CHAIN BY PROTEASE ACTIVATED KINASE I

Polygena T. Tuazon, James T. Stull* and Jolinda A. Traugh

Department of Biochemistry
University of California
Riverside, California 92521

*Department of Pharmacology and Moss Heart Center
University of Texas
Health Science Center
Dallas, Texas 75235

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Protease activated kinase I from rabbit reticulocytes has been shown to phosphorylate the P-light chain of myosin light chains isolated from rabbit skeletal muscle. The enzyme is not activated by Ca^{+2} and calmodulin or phospholipids. Protease activated kinase I is not inhibited by trifluoperazine at concentrations up to 200 μM or by the antibody to the Ca^{2+}, calmodulin-dependent myosin light chain kinase from rabbit skeletal muscle. Two-dimensional peptide mapping of chymotryptic digests of myosin P-light chain show the site phosphorylated by the protease activated kinase is different from that phosphorylated by the Ca^{2+}, calmodulin-dependent myosin light chain kinase.

INTRODUCTION

The 20,000 dalton light chain of myosin from a number of muscle and nonmuscle cells has been shown to be specifically phosphorylated by the Ca^{2+}, calmodulin-dependent myosin light chain kinase (for review, see 1,2). These myosin light chains have also been reported to be phosphorylated by myosin light chain kinases that are independent of Ca^{2+} and calmodulin, including those from proliferative myoblasts (3), bovine mammary glands (4), human platelets (5), and rabbit alveolar macrophages (6). The relationship of the latter enzymes to the Ca^{2+}, calmodulin-regulated protein kinase has not been defined and it has been suggested that the enzyme from platelets could be a degradation product of the Ca^{2+}-regulated enzyme (7). Recently, tryptic digestion of the Ca^{2+}-dependent myosin light chain kinase from chicken gizzard and rabbit skeletal muscle (8) and chymotryptic digestion of myosin light chain kinase from turkey gizzard in the presence of calmodulin

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(9) has been shown to result in activation of the enzyme with a resultant loss of the requirement for Ca^{2+} and calmodulin.

Tahara and Traugh (10) have recently purified a cAMP-independent, Ca^{2+}-independent protein kinase from rabbit reticulocytes which is activated by limited proteolysis. This enzyme, referred to as protease activated kinase I, has been shown to phosphorylate histones 2B and 4 (10), eukaryotic initiation factors 3 and 4B (11) and 40S ribosomal protein S10 (12). In this paper, we show the phosphorylation of myosin light chain from rabbit skeletal muscle by protease activated kinase I. Phosphorylation of the P-light chain by protease activated kinase I is compared to the phosphorylation reaction catalyzed by the Ca^{2+}, calmodulin-dependent myosin light chain kinase.

MATERIALS AND METHODS

Trypsin (DCC-treated) and soybean trypsin inhibitor were obtained from Sigma. Chymotrypsin A_4 was obtained from Boehringer Mannheim and trifluoperazine dihydrochloride from Smith-Kline and French Laboratories. Precoated plastic-backed thin layer cellulose sheets (20 x 20 cm, 0.16 mm thick, without fluorescent indicator) were purchased from Eastman. [γ-^{32}P]ATP was prepared as previously described (13). Antibody to myosin light chain kinase was prepared in New Zealand White rabbit by subcutaneous injection of highly purified myosin light chain kinase.

Protease activated kinase I was purified from rabbit reticulocytes by chromatography on DEAE-cellulose, phosphocellulose and hydroxylapatite as described by Tahara and Traugh (10) and stored in buffer A (15 mM potassium phosphate, pH 6.8; 20 mM 2-mercaptoethanol; 1 mM EDTA; 3 mM EGTA; 0.02% NaN_3) at 4°C. Myosin light chain kinase was purified to apparent homogeneity from rabbit muscle as described by Yazawa and Yagi (14). Mixed myosin light chains were prepared from fresh rabbit muscle (15) and calmodulin was purified from bovine brain obtained from Pel-Freez according to the procedure of Dedman et al. (16).

Protease activated kinase I was assayed with myosin light chains as substrate in an incubation mixture (0.030 ml) containing; 50 mM Tris-HCl (pH 7.5), 10 mM magnesium chloride, 30 mM 2-mercaptoethanol, 0.4 mM [γ-^{32}P]ATP (100-300 cpm/pmol), the indicated concentrations of myosin light chains and trypsin-activated or non-activated enzyme. Incubations were at 30°C for 10 min. Activation of the enzyme by limited digestion with trypsin in 1 mM HCl was carried out as described by Tahara and Traugh (10) with the exception that bovine serum albumin was omitted and incubation was for 30 sec. For the non-activated control, 1 mM HCl was added to the preincubation mixture instead of trypsin and HCl. Proteolysis was terminated by the addition of a 10-fold excess of soybean trypsin inhibitor. Incorporation of $^{32}P_i$ into myosin light chain was monitored by electrophoresis in 15% polyacrylamide gels in the presence of SDS, followed by autoradiography (13). The bands corresponding to phosphorylated light chain were excised from the gel and counted after solubilizing the protein in SDS as described previously (17).

Myosin light chain kinase activity was measured in a reaction mixture (0.030 ml) containing; 50 mM Tris-HCl (pH 7.4), 30 mM 2-mercaptoethanol,

10 mM magnesium chloride, 0.4 mM ATP, 267 nM calmodulin, 0.1 mM $CaCl_2$ and the indicated amounts of myosin P-light chain and myosin light chain kinase. For reactions conducted in the absence of calcium and calmodulin, 1 mM EGTA was added. Incorporation of $^{32}P_i$ into myosin light chain was monitored by counting gel slices following polyacrylamide gel electrophoresis as described above.

Two-dimensional phosphopeptide maps were carried out as previously described (17).

RESULTS

The phosphorylation of isolated myosin light chain from rabbit skeletal muscle by protease activated kinase I was compared to that catalyzed by the Ca^{2+}, calmodulin-dependent myosin light chain kinase from skeletal muscle. The P-light chain was phosphorylated by the myosin light chain kinase upon addition of Ca^{2+} and calmodulin and was identified by polyacrylamide gel electrophoresis in sodium dodecyl sulfate followed by autoradiography (Figure 1). This same light chain was phosphorylated by protease activated kinase I in the presence of EGTA following limited digestion of the enzyme with tryp-

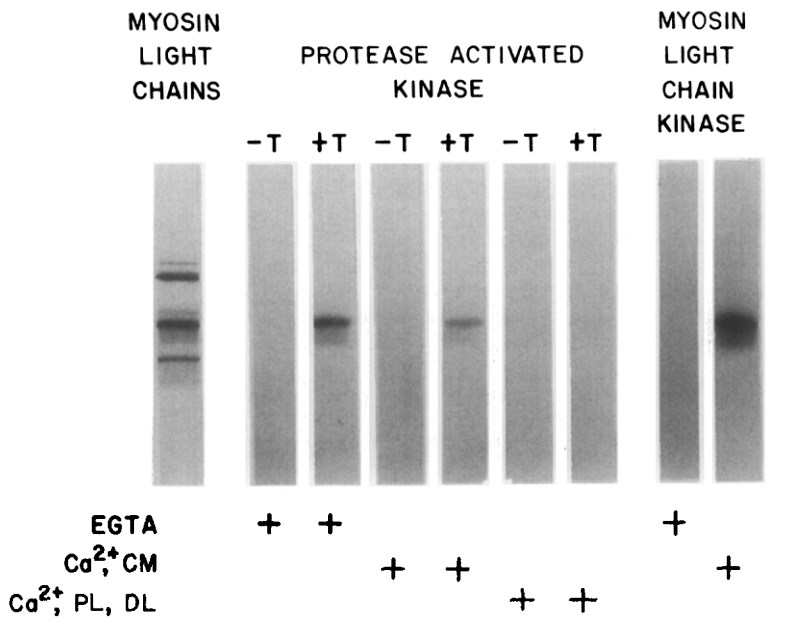

Figure 1. Phosphorylation of isolated myosin light chain from skeletal muscle by protease activated kinase I and myosin light chain kinase. Myosin P-light chain from skeletal muscle (342 pmol) was phosphorylated with myosin light chain kinase (MLCK; 0.005 µg) or protease activated kinase I (PAK I; 3.6 µg) in a 0.030 ml reaction mixture as described in Methods. EGTA, 1 mM; $CaCl_2$, 0.5 mM; calmodulin (CM), 275 mM; phosphatidyl inositol, 0.12 mg/ml, phosphatidylserine, 0.12 mg/ml (PL) and diolein (DL), 0.012 mg/ml were added as indicated. Reaction mixtures were analyzed on 15% polyacrylamide gels containing SDS. Autoradiograms are shown; the stained gel is at the far left.

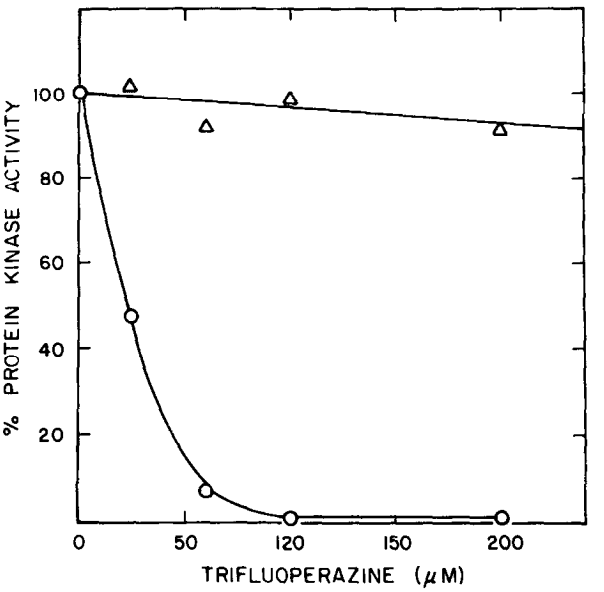

Figure 2. Effect of trifluoperazine on the phosphorylation of myosin light chain by protease activated kinase I and myosin light chain kinase. Myosin light chains (342 pmol) were phosphorylated in 0.060 ml reaction mixtures by the activated form of protease activated kinase I (Δ) (1.6 μg) with 1 mM EGTA or by purified myosin light chain kinase (o) (0.01 μg) with Ca^{2+} (0.42 mM) and calmodulin (138 nM) as described in Methods. Varying concentrations of trifluoperazine was added as indicated. Reactions were analyzed on 15% polyacrylamide gels containing SDS and the phosphorylated light chain was identified by autoradiography. The P-light chain was excised from the gel, solubilized in 0.5 ml of 1% SDS and counted with toluene scintillation cocktail containing Triton X-100 (17).

sin. Without prior activation by trypsin, no phosphorylation was observed either in the presence or absence of Ca^{2+} and calmodulin or Ca^{2+} and phospholipids. Addition of Ca^{2+} and calmodulin or phospholipids to the activated form of the activated protease kinase did not enhance, but rather diminished, the phosphorylation of myosin light chain.

The effect of trifluoperazine on the phosphorylation of the myosin P-light chain is shown in Figure 2. The Ca^{2+}, calmodulin-dependent myosin light chain kinase was inhibited 50% by 28 μM trifluoperazine and completely inhibited by 100 μM. Protease activated kinase was not affected at concentrations of trifluoperazine up to 200 μM.

The effect of antibody prepared against myosin light chain kinase on the activity of the protease activated kinase is shown in Figure 3. It can be seen that myosin light chain kinase activity was inhibited 50% by 0.05 μl of

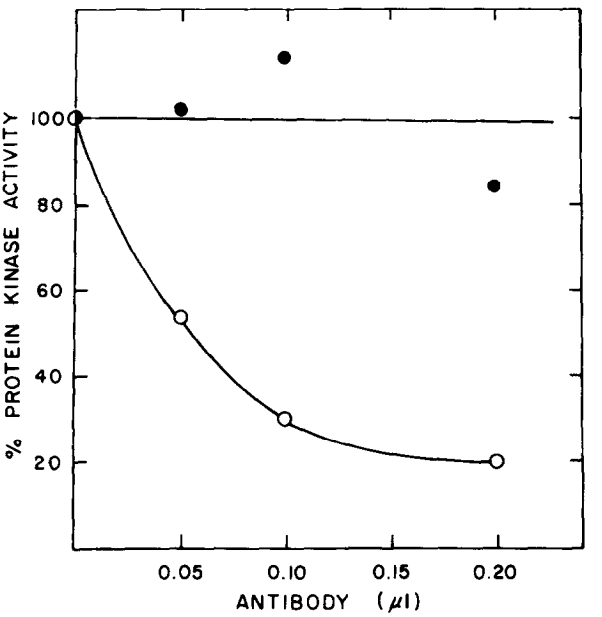

Figure 3. Effect of antibody to myosin light chain kinase on the phosphorylation of myosin light chain by protease activated kinase and myosin light chain kinase. Phosphorylation of myosin light chain (342 pmol) by protease activated kinase I (●) with 1 mM EGTA or by myosin light chain kinase (o) with 0.42 mM Ca^{2+} and 138 mM calmodulin was carried out in 0.060 ml reaction mixtures. Increasing amounts of antibody were added as indicated. Incorporation of $^{32}P_i$ into the P-light chain was quantitated by counting gel slices from 15% polyacrylamide gels containing SDS. For control reactions, i.e. no antibody added, $^{32}P_i$ incorporated into the P-light chain was 29 and 33 pmol by the protease activated kinase and myosin light chain kinase, respectively.

the antiserum. Using two different concentrations of protease activated kinase, no significant effect on the activity was seen by adding observed at concentrations of antiserum up to 0.20 μl.

Maximal incorporation of 1 mol of phosphate per mol of P-light chain was obtained with the protease activated kinase. In order to compare the sites phosphorylated by the two enzymes, myosin P-light chain, maximally phosphorylated by either protease activated kinase I or myosin light chain kinase, was digested with chymotrypsin and the chymotryptic digests were analyzed by two-dimensional peptide mapping (Figure 4). Two chymotryptic phosphopeptides were obtained with the protease activated kinase. These were different from the chymotryptic phosphopeptides obtained with the Ca^{2+}-dependent myosin light chain kinase. This difference was confirmed by the composite peptide pattern obtained when a mixture of the two digests was analyzed.

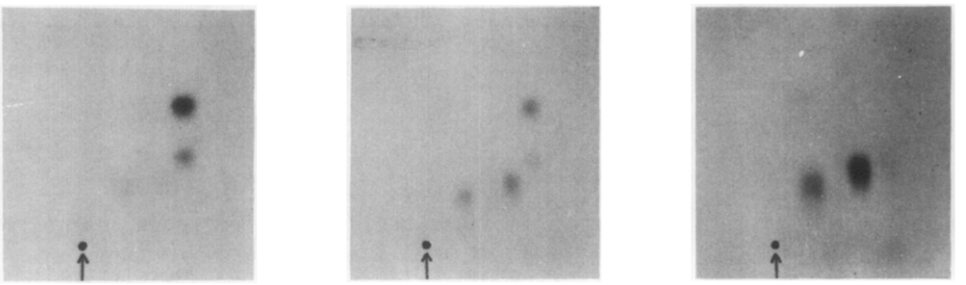

Figure 4. Identification of the chymotryptic phosphopeptides of myosin light chain phosphorylated by protease activated kinase I and myosin light chain kinase. Myosin P-light chain (900 pmol), phosphorylated maximally for 60 min in a 0.030 ml reaction mixture by protease activated kinase I (PAK I; 7.5 µg) or myosin light chain kinase (MLCK; 0.02 µg), was digested with chymotrypsin. The chymotryptic digests were analyzed by two-dimensional peptide mapping involving thin-layer electrophoresis at pH 6.4 with pyridine:acetic acid: water (10:0.4:90) in the first dimension and thin-layer chromatography in butanol:acetic acid:water (3:1:1) in the second dimension. The phosphopeptides were visualized by autoradiography at -70°C on Fuji X-ray film with intensifying screens.

Since two phosphopeptides were observed when one site was phosphorylated with myosin light chain kinase, it was not unexpected to observe two phosphopeptides using protease activated kinase I.

DISCUSSION

Protease activated kinase I has been shown to phosphorylate the same myosin light chain from skeletal muscle that is phosphorylated by the Ca^{2+}, calmodulin-dependent myosin light chain kinase. Ca^{2+} and phospholipids or calmodulin have no stimulatory effect on the inactive form of the protease activated kinase. In contrast to the Ca^{2+}, calmodulin-dependent myosin light chain kinase, the proteolytically activated enzyme is not inhibited by trifluoperazine. In this respect, it is also different from the Ca^{2+}, phospholipid-dependent protein kinase that has been reported to phosphorylate myosin light chain from gizzard and is inhibited 50% by 50 µM trifluoperazine (18).

We have shown that 1 mole of phosphate is incorporated per mole of P-light chain. The site phosphorylated by the protease activated kinase is different from that phosphorylated by the Ca^{2+}-dependent myosin light chain kinase, as evidenced by the chymotryptic phosphopeptides. The possibility that the protease activated kinase is a proteolytic product of the Ca^{2+},

calmodulin-dependent myosin light chain kinase is ruled out by the observation that the trypsin-treated myosin light chain kinase gives identical phosphopeptides to those obtained with the Ca^{2+}, calmodulin-dependent myosin light chain kinase (data not shown). Additional evidence is the observation that antibody to myosin light chain kinase does not inhibit the activity of protease activated kinase I. On the basis of the difference in phosphorylation sites and the interaction with trifluoperazine and antibody to myosin light chain kinase, as well as mode of activation, it appears that the protease activated kinase is a previously unidentified myosin light chain kinase. This enzyme is also distinct from the Ca^{2+}, phospholipid-activated protein kinase of Nishizuka and coworkers (19) as shown by a lack of activation by Ca^{2+} and phospholipids using myosin light chain and substrate specificity with histone (10). Recently protease activated kinase I has been purified from rabbit skeletal muscle (20), chicken liver and brain (K. S. Morley and J. A. Traugh, unpublished results) and shown to phosphorylate myosin P-light chain.

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