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Catalytic fragment of protein kinase C exhibits altered substrate specificity toward smooth muscle myosin light chain

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Smooth muscle myosin light chain (LC) can be phosphorylated by myosin light chain kinase (MLCK) at Ser^{19} and Thr^{18} and by protein kinase C (PKC) at Thr^{9} and Ser^{1} or Ser^{2} under the in vitro assay conditions. Conversion of PKC to the spontaneously active protein kinase M (PKM) by proteolysis resulted in a change in the substrate specificity of the kinase. PKM phosphorylated both sets of sites in LC recognized by MLCK and PKC as analyzed by peptide mapping analysis. The PKM-catalyzed phosphorylation of these sites was not greatly affected by a MLCK inhibitor, ML-9, nor by the activators of MLCK, Ca^{2+} and calmodulin.

Protein kinase C; Myosin light chain; Phosphorylation

1. INTRODUCTION

Phosphorylation of the 20 kDa smooth muscle myosin light chains (LC) is recognized as a key process in the regulation of smooth muscle concentration [1,2]. Both myosin light chain kinase (MLCK), a Ca^{2+}/calmodulin-activated protein kinase, and protein kinase C (PKC), a Ca^{2+}/phospholipid-activated protein kinase, have been shown to phosphorylate LC and have been implicated in the regulation of the actin-activated Mg^{2+}-ATPase activity [2]. MLCK phosphorylates smooth muscle LC at Ser^{19} and Thr^{18} in sequence resulting in an increase in the actin-activated Mg^{2+}-ATPase activity [3–5]. In contrast, PKC phosphorylates Thr^{9} and Ser^{1} or Ser^{2} with a resulting decrease in the Mg^{2+}-ATPase activity of myosin that has already been activated by MLCK [5–7]. Phosphorylation of the LC by PKC also decreases the rate of subsequent phosphorylation of the LC by MLCK [6]. PKC-catalyzed phosphorylation of myosin LC has been suggested to inhibit the contractile response of smooth muscle in vivo by counteracting the activation by MLCK [5,6].

It has been reported that incubation of human platelets with thrombin or phorbol 12-myristate,13-acetate (PMA), an activator of PKC, caused the phosphorylation of myosin LC at 2 sets of sites recognized separately by PKC and MLCK under the in vitro assay conditions [8]. It was suggested that treatment of the platelets with PMA also resulted in an increase in $[Ca^{2+}]_i$ and thereby caused an activation of MLCK. Because treatment of human platelets with phorbol esters also enhances the generation of PKM [9], we tested the possibility of whether PKM alone could phosphorylate the LC at both sets of sites recognized by PKC and MLCK. In this report we compare the phosphorylation of smooth muscle LC by MLCK, PKC and PKM. Our results show that PKM catalyzes the phosphorylation of the LC to a higher stoichiometry than that by PKC or MLCK. Peptide mapping analysis revealed that PKM phosphorylated both sets of sites recognized by PKC and MLCK. The PKM-catalyzed phosphorylation of these sites was not greatly inhibited by a MLCK inhibitor, ML-9, nor activated by the activators of MLCK, Ca^{2+} and calmodulin. These results indicate that the substrate specificity of PKC can be altered following its conversion to PKM.

Abbreviations: PKC, protein kinase C; PKM, the catalytic fragment of PKC; MLCK, myosin light chain kinase; LC, the light chain subunit of myosin; PMA, phorbol 12-myristate, 13-acetate; DAG, diacylglycerol; PS, phosphatidylserine.

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2. MATERIALS AND METHODS

The following materials were obtained from the indicated sources: Histone IIIS, EGTA and soybean trypsin inhibitor from Sigma; [γ-^{32}P]ATP, and [^{125}I]protein A from DuPont-New England Nuclear; phosphatidylserine (PS) and dioleoylglycerol from Avanti Polar Lipids; peptide $K13SNH_2$ corresponding to the amino acid sequence of turkey gizzard myosin light chain 11–22 (KKRPQRATSNVF) [10] from Peninsula Laboratories; calmodulin from Calbiochem; Ampholine solutions from Pharmacia LKB Biotechnology; TPKC-trypsin from Worthington; phosphocellulose P-81 paper from Whatman; Silica Gel 60 thin-layer plate from EM Science; ML-9 (1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine) from Seikagaku America Inc.; and rabbit anti-turkey gizzard MLCK antiserum was kindly provided by Dr. Mary D. Pato. Rat brain PKC [11] and the catalytic fragment of PKC, PKM [12], were purified to near homoge-

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neity as described previously. MLCK [13] and myosin LC, a mixture of the 20 and 17 kDa LCs [14], were purified from turkey gizzard.

Phosphorylation of the LC was carried out at 30°C in a basic reaction mixture containing 30 mM Tris-HCl buffer, pH 7.5, 6 mM $MgCl_2$, 0.2 mM [γ-^{32}P]ATP, 0.4 mg/ml LC. Additional ingredients were added to the basic reaction mixture for the assay of the various protein kinases: 50 μg/ml PS, 10 μg/ml DAG and 0.4 mM $CaCl_2$ for PKC; 0.4 mM EGTA for PKM; and 0.1 μM calmodulin and 0.16 mM $CaCl_2$ for MLCK. Reactions were initiated by the addition of the kinases. Measurement of ^{32}P-incorporation into the protein substrate and the peptide K13SNH$_2$ was as previously described [15].

Digestion of ^{32}P-labeled LC by trypsin was carried out in 50 mM NH_4HCO_3, pH 8.5, for 16 h at 30°C. Isoelectric focusing of the ^{32}P-labeled tryptic peptides on polyacrylamide gel (1 mm thickness) was carried out at 4°C for 60 min with a power setting of 30 W and maximal voltage setting of 1500 V using 1 M H_3PO_4, as the anode solution and 1 M NaOH as the cathode solution. A plate of gel was prepared from 25 ml of a solution containing 6 ml of 29.1% (w/v) acrylamide solution, 6 ml of 0.9% (w/v) *N,N'*-methylenebisacrylamide solution, 9 g urea, and 0.5 ml each of Ampholine, pH 2.5–4, 3.5–5 and 5–8. Gel was polymerized by the addition of 10 μl of TEMED and 80 μl of 10% ammonium persulfate. ^{32}P-labeled peptides were detected by autoradiography of the dried gel. Peptide mapping with thin-layer silica gel plates was carried out by a previously described method [7].

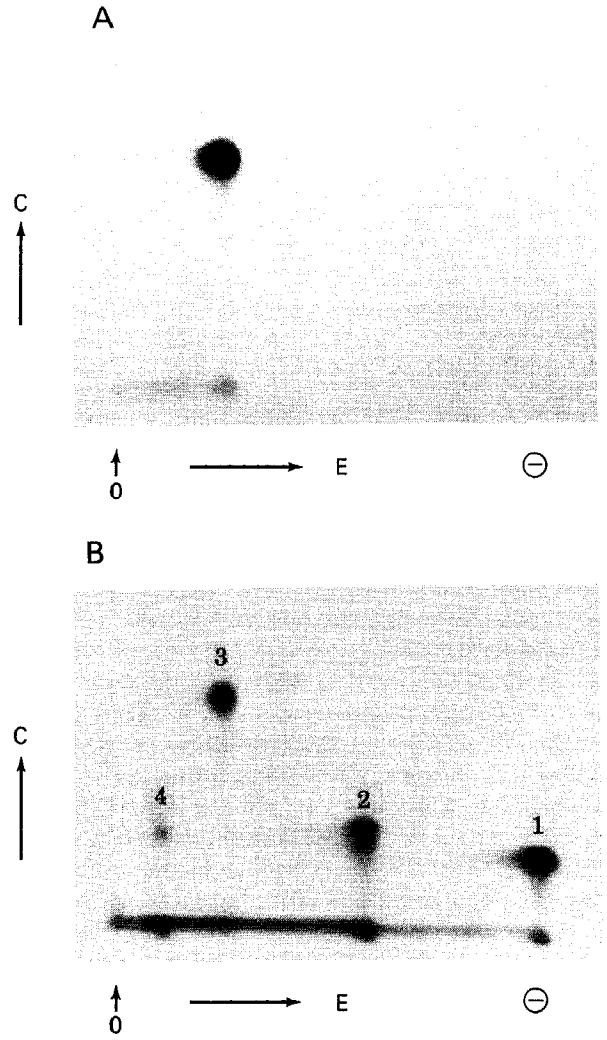

Fig. 1. Two-dimensional peptide mapping analysis of ^{32}P-labeled tryptic peptides derived from LC phosphorylated by MLCK and PKM. Myosin LC phosphorylated by (A) MLCK or (B) PKM in the presence of [γ-^{32}P]ATP was digested with trypsin and analyzed by electrophoresis on Silica Gel 60 in a buffer containing acetic acid:formic acid:H_2O (15:8:80, v/v/v) and thin-layer chromatography in *n*-butanol:pyridine:acetic acid:H_2O (195:150:30:20, v/v/v). The ^{32}P-labeled peptides were visualized by autoradiography.

3. RESULTS AND DISCUSSION

Incubation of the LC with purified MLCK resulted in the incorporation of approximately 0.8–1.2 mol ^{32}P/mol of LC with one distinct spot on the peptide map (Fig. 1A). Phospho-amino acid analysis revealed the presence of phosphoserine as a major radioactive constituent and phosphothreonine as a minor one, both of which have previously been identified as Ser^{19} and Thr^{18}, respectively [4,5]. PKC catalyzed the incorporation of 1.6–2.3 mol ^{32}P/mol into the LC in a Ca^{2+}/PS-dependent fashion. Peptide mapping analysis revealed the presence of 2 major tryptic phosphopeptides, in which one contains Ser^1 or Ser^2 and the other Thr^9 [7]. Phosphorylation of LC by PKM resulted in the incorporation of 2.2–3.1 mol ^{32}P/mol of LC and the reaction was not affected by the addition of Ca^{2+}/PS/DAG or Ca^{2+} and calmodulin. Tryptic peptide mapping analysis revealed the presence of several phosphopeptides, among them one which comigrated with that phosphorylated by MLCK (Fig. 1B, spot 3) and the rest (Fig. 1B, spots 1, 2 and 4) with those phosphorylated by PKC. Correspondence of the various tryptic phosphopeptides derived from the PKM-phosphorylated LC to those from MLCK- and PKC-phosphorylated ones was also confirmed by peptide mapping with isoelectric focusing on polyacrylamide gel (Fig. 2). A pI=4.5 tryptic ^{32}P-labeled peptide, containing predominantly Ser^{19} (corresponding to spot 3 in Fig. 1B), was detected following phosphorylation of the LC by MLCK (Fig. 2, lane 1). PKC catalyzed the phosphorylation of several ^{32}P- labeled peptides including pI=7.5, containing Thr^9 (corresponding to spot 1 in Fig. 1B), and pI=5.0 (corresponding to spot 2 in Fig. 1B) and 3.6 (corresponding to spot 4 in Fig. 1B), both of which contained a Ser residue. We had tentatively identified these latter two ^{32}P-labeled peptides based on their predicted pIs as Ac-Ser^1-Ser^2-Lys-Arg and Ac-Ser^1-Ser^2-Lys, respectively, resulting from alternate tryptic cleavage at Arg^4 and Lys^3 (Fig. 2, lane 2). Phosphorylation of LC by PKM (Fig. 2, lane 3) resulted in the labeling of all those peptides from LC phosphorylated by MLCK and PKC as well as an unidentified one having pI=3.8 (the band above pI=3.6).

Phosphorylation of the LC by MLCK and PKC usually reached a near-maximum level of 1 and 2 mol ^{32}P/mol, respectively, within 1 h of incubation under the assay conditions (Fig. 3). Although the initial rate of phosphorylation of the LC by PKC and PKM were comparable, the PKM-catalyzed reaction always exhibited a second phase of slower ^{32}P-incorporation to a

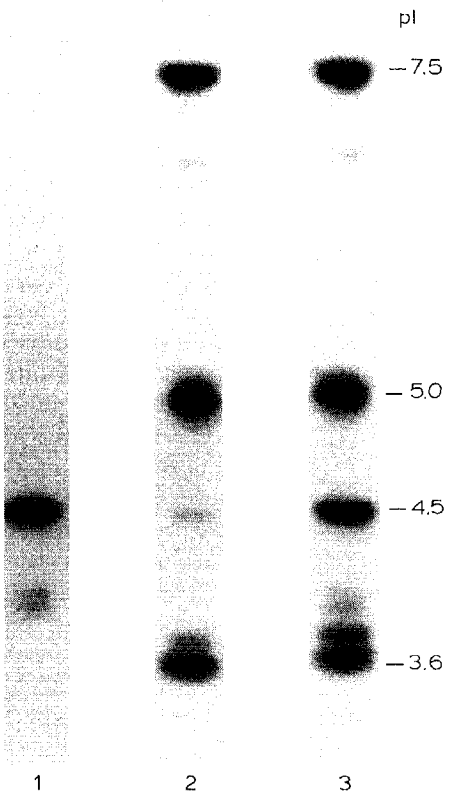

Fig. 2. Isoelectric focusing analysis of the ^{32}P-labeled tryptic peptides derived from LC phosphorylated by MLCK, PKC and PKM. Tryptic phosphopeptides from myosin LC phosphorylated by MLCK (lane 1), PKC (lane 2), and PKM (lane 3) were separated by isoelectric focusing on polyacrylamide gel followed by autoradiography of the dried gel as described in section 2. The apparent isoelectric points of the ^{32}P-labeled peptides were assigned after determination of the pH gradient of the gel by a surface electrode.

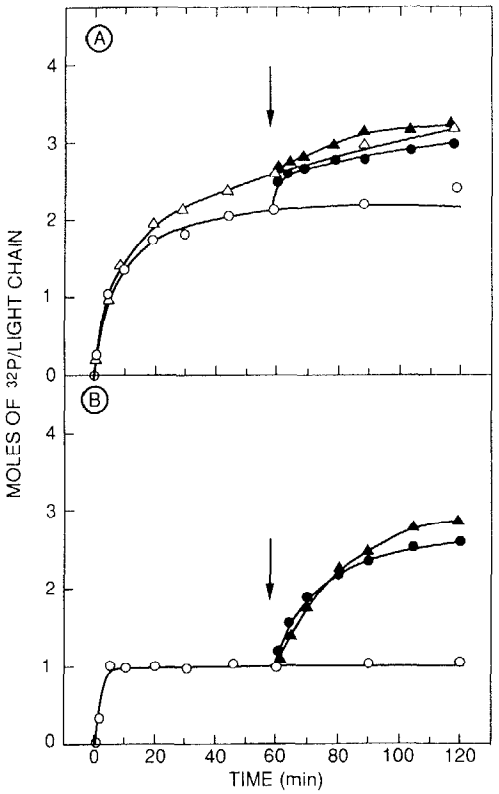

Fig. 3. Sequential phosphorylation of myosin LC by the combination of PKC, PKM and MLCK. (A) LC was incubated with PKM (△) or PKC (○) under the standard assay conditions. After 60 min of incubation an aliquot from each reaction mixture was taken and supplemented with MLCK (▲,●). (B) LC was initially phosphorylated by MLCK (○) for 60 min and followed by PKC (●) or PKM (▲).

stoichiometry of >3 mol ^{32}P/mol (Fig. 3A). Addition of MLCK after an initial phosphorylation of the LC with PKC resulted in a further ^{32}P-incorporation at a rate slower than that by MLCK alone. However, addition of MLCK following the phosphorylation of the LC with PKM resulted in a relatively low level of increment. The final levels of phosphorylation by PKM alone, PKM plus MLCK and PKC plus MLCK were nearly identical. Phosphorylation of the LC by MLCK and followed by the addition of either PKC or PKM resulted in a similar extent of enhancement in the ^{32}P-incorporation (Fig. 3B). The rates of phosphorylation of the LC by PKC or PKM under these conditions were slower than their respective ones without prior incubation with MLCK. These results indicate that phosphorylation of LC by PKC or PKM retards the subsequent phosphorylation by MLCK and prior phosphorylation of the LC by MLCK retards the phosphorylation by PKC or PKM. The tryptic phosphopeptides derived from the phosphorylated LC under these various assay conditions were also analyzed by two-dimensional and isoelectric focusing peptide mappings and confirmed that PKM can phosphorylate those sites recognized by PKC and MLCK.

The sites in LC phosphorylated by MLCK are located within the sequence of P^{14}QRATS^{19}NVFAM^{24}, which is also likely to be a substrate of PKC. A synthetic peptide, K13SNH$_2$, corresponding to the amino acid sequence of turkey gizzard myosin LC 11–22 (KKRPQRATSNVF) was tested as a substrate of PKC and PKM. PKC phosphorylated the peptide with a K_m = 17.0 ± 0.13 μM and V_{max} = 2373 ± 110 nmol/min/mg and PKM with a K_m = 17.3 ± 0.28 μM and a V_{max} = 3019 ± 298 nmol/min/mg. The V_{max} values of this substrate for PKC and PKM are approximately 50% of their respective values with histone IIIS. Phosphoamino acid analysis revealed that the serine residue was phosphorylated by both kinases, indicating that the Ser residue in the peptide K13SNH$_2$, corresponding to the Ser^{19} of the LC was the phosphorylation site. This result indicates that Ser^{19} of the LC, which has been identified as the phosphorylation site of MLCK, is also a potential phosphorylation site of PKC and PKM. The relatively low activity of PKC as compared to PKM toward this site in the intact LC may be due to steric hindrance encountered between PKC and LC.

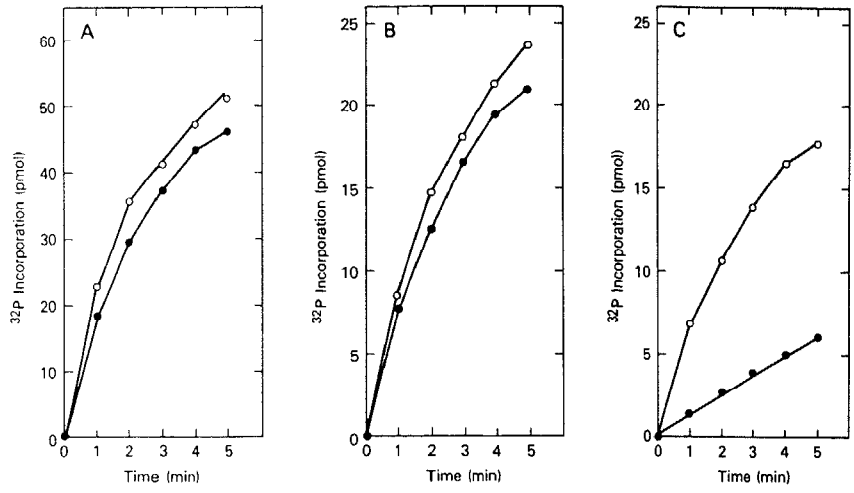

Fig. 4. Effect of ML-9 on the phosphorylation of the LC by PKC, PKM and MLCK. LC was phosphorylated by (A) PKC, (B) PKM or (C) MLCK in the presence (●) or absence (○) of 30 μM of ML-9. Samples were taken for the analysis of ^{32}P-incorporation.

The preparation of PKM used in this study was prepared from a homogeneous PKC preparation and was tested to be free of contaminating MLCK by immunoblot analysis with rabbit anti-smooth muscle MLCK antibody. Under our experimental conditions the antibody could readily detect 10 ng of MLCK, however, without exhibiting any immunoreactivity toward 3 μg each of PKC and PKM. Furthermore, addition of calmodulin (0.1 μM) to the PKC or Ca^{2+} (0.4 mM) and calmodulin (0.1 μM) to the PKM reaction mixture did not change their respective phosphorylation sites on LC. These results indicated that the contamination of the immunoreactive MLCK was almost negligible in the preparations of PKC and PKM. Previously, MLCK was shown to be activated irreversibly by limited proteolysis with trypsin. The resulting catalytically active fragment of the MLCK is independent of Ca^{2+} and calmodulin. To exclude the possible contamination of the proteolytically-activated MLCK, which may be lacking the epitope for the anti-MLCK antibody, we tested the effect of ML-9, a selective inhibitor of MLCK [16], on LC phosphorylation by PKC, PKM and MLCK. Under the assay conditions containing 200 μM ATP, 30 μM ML-9 inhibited the initial rate of LC phosphorylation with PKC and PKM by 14 ± 2.2% and 17 ± 2.3%, respectively (Fig. 4A and B). In comparison, the MLCK- catalyzed phosphorylation of the LC was inhibited by 76 ± 2.3% in the presence of the same concentration of ML-9 (Fig. 4C). The ML-9-mediated inhibition of the PKM-catalyzed phosphorylation of Ser^{19} in LC, as analyzed by isoelectric focusing of the resulting ^{32}P-labeled tryptic peptides, was also much less than that of the MLCK-catalyzed one. The low level of inhibition of PKC and PKM by ML-9 is expected because this inhibitor is not absolutely specific for MLCK; the K_i of ML-9 for PKC and MLCK are 54 and 3.8 μM, respectively [16]. Thus, it is unlikely that the PKM preparations contain protease-activated MLCK.

The results presented in this report demonstrate that PKM can phosphorylate both sets of sites in the turkey gizzard LC recognized by PKC and MLCK. This conclusion is based on (i) the peptide mapping analysis of the LC phosphorylated by these kinases, (ii) measurement of the stoichiometries of ^{32}P-incorporation into LC catalyzed by these kinases, and (iii) the effects of a MLCK inhibitor, ML-9, on these kinase-catalyzed reactions. The PKM-catalyzed phosphorylation of these sites was also observed with LC associated with intact turkey gizzard heavy meromyosin, suggesting that PKM may phosphorylate LC in vivo. Recently, it has been suggested that PKC may share a common site with MLCK upon phosphorylation of an embryonic smooth muscle LC isoform [17]; however, it is unknown whether the low level of phosphorylation of the MLCK site by a partially purified PKC preparation results from the action of PKM. Previously, it has been demonstrated that PKM and PKC have similar relative activities for histone H1 and myelin basic protein [18]. In contrast, human fibrinogen is a better substrate for PKM than PKC [18]. Thus, smooth muscle myosin LC and human fibrinogen are a group of substrates that are preferentially phosphorylated by PKM as compared to PKC. The current study provides the first demonstration that PKM can, in fact, phosphorylate a new site in LC in addition to those recognized by PKC.

The isolated LC from smooth muscle has been shown to be a substrate for a number of kinases, including cAMP-dependent protein kinase [19], cGMP-dependent kinase [20], phosphorylase kinase [21], casein kinase-1 [21], casein kinase-2 [21], myosin I heavy chain kinase [22], protease-activated protein kinase 1 [23] as well as PKC and MLCK. Although isolated LC can be phosphorylated by these kinases in vitro, only MLCK and PKC are thought to play major roles in the regulation of the actin-activated Mg^{2+}-ATPase activity in vivo [2]. In human platelets [8] and neutrophiles [24] stimulation

of PKC by phorbol ester results in the generation of PKM, which could participate in the phosphorylation of cellular proteins including the LC. The actions of PKM inside the cells have also been demonstrated by microinjection of PKM into NIH3T3 cells that resulted in morphological changes of these cells comparable to those induced by PMA [25]. It seems likely that the PMA-induced generation of PKM could bring about a broader cellular responses, due to an enhanced kinase activity, than the activation of PKC induced by a ligand without causing its degradation.

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