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Inhibitory Effect of Phosphorylated Myosin Light Chain Kinase on the ATP-Dependent Actin-Myosin Interaction

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Myosin light chain kinase (MLCK) phosphorylates the regulatory light chain of myosin in the presence of Ca^{2+} and calmodulin (Ca^{2+}-CaM) so that myosin can interact with actin filaments. MLCK has another activity that is not attributable to this kinase activity, i.e., it inhibits the ATP-dependent movement of actin filaments on a myosin-coated glass surface. MLCK itself can be phosphorylated at site A and site B with a few kinases. The phosphorylation at site A reduces kinase activity. However, we have no knowledge as to how phosphorylation of MLCK affects the inhibitory activity of MLCK. When MLCK was phosphorylated at site B, it exerted an inhibitory effect on the movement in much lower concentrations. When Ca^{2+}-CaM or ML-9 was present, the inhibition was reduced. The reduction was less when the movement was arrested by the MLCK phosphorylated at site B. This observation was explained by the increase in the affinity of MLCK to myosin upon the phosphorylation at site B.

Myosin light chain kinase (MLCK) phosphorylates the regulatory light chain of smooth muscle myosin in the presence of Ca^{2+} and calmodulin (Ca^{2+}-CaM), thereby activating the myosin to interact with actin filaments (1–3 for reviews). The catalytic domain is responsible for this kinase activity.

The regulatory domain, which neighbors the catalytic domain, regulates the activity not only by binding Ca^{2+}-CaM but also by undergoing phosphorylation by various protein kinases, including protein kinase A (PKA) (4), protein kinase C (5, 6), CaM kinase II (7–9), and p21-activated kinase (10). Because PKA is the first kinase that phosphorylates MLCK, the sites of phosphorylation are well-characterized (11, 12). There are two phosphorylatable residues, Ser^{815} at site A and Ser^{828} at site B in the regulatory domain (see Fig. 1A for chicken gizzard MLCK) (3). Site A phosphorylation reduced the affinity of MLCK to CaM and eliminates the kinase activity (4). However, site B phosphorylation exerted no appreciable effect on the activity and requires further characterization (13).

A novel regulatory role of MLCK that we have previously reported is that it inhibits the actin-myosin interaction without changing the level of myosin phosphorylation (14–16). This inhibitory effect has been explained exclusively by the actin-binding activity of MLCK (17 for review). However, the interaction of the catalytic domain with myosin light chain has recently been shown to exert a similar inhibition (18), raising the question as to which domain, actin-binding or catalytic domain, plays the major role in inhibiting the total MLCK.

In this study, we examined the effect of phosphorylation of MLCK with special reference to the inhibition by MLCK of the actin-myosin interaction.

MATERIALS AND METHODS

Purification of proteins. Actin was prepared from the acetone powder of chicken breast muscle (19) with slight modifications (20) and used as actin filaments after the polymerization. The concentration was expressed by that of the monomeric form. Myosin was prepared from fresh chicken gizzard (21) with slight modifications (22) and used after the phosphorylation by MLCK in the presence of Ca^{2+}-CaM unless otherwise stated. The monophosphorylated nature myosin at Ser^{19} was examined by the glycerol-urea polyacrylamide gel electrophoresis (PAGE) (22, 23). MLCK was prepared from frozen chicken gizzard by the method of Adelstein and Klee (1981) with slight modifications (24). CaM from bovine brain (P2277) and the catalytic subunit of PKA from bovine heart (1529-307) were purchased from Sigma (St. Louis, MO, USA) and from Boehringer Mannheim (Mannheim, Germany), respectively. ML-9 (120809), an inhibitor for MLCK, was purchased from Seikagaku-Kogyo (Tokyo, Japan).

Phosphorylation of MLCK by the catalytic subunit of PKA. MLCK (8 μM in the final concentration) was phosphorylated at site B in the presence of 0.5 mM $CaCl_2$ and 8 μM CaM by 0.2 μM of the catalytic subunit of PKA in 0.4 mM ATP, 10 mM $MgCl_2$, and 30 mM Tris-HCl (pH 7.5) (4) unless otherwise specified. In some experiments, we used 0.4 mM [γ-^{32}P]ATP (0.2 Ci/mmol) in stead of cold ATP and subjected it to sodium dodecyl sulfate (SDS)-PAGE (see below). The protein

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Abbreviations: CaM, calmodulin; MLCK, myosin light chain kinase; PKA, protein kinase A.

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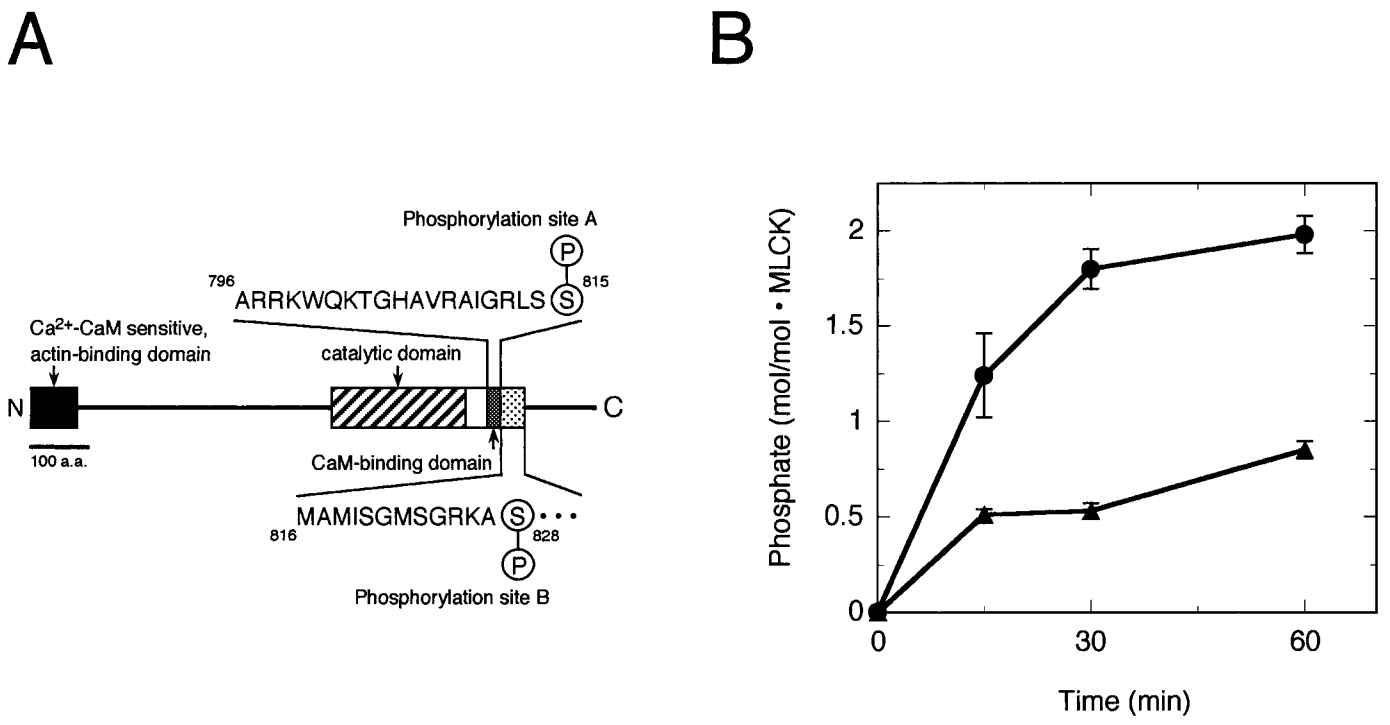

FIG. 1. Phosphorylation of MLCK by PKA. (A) Domain structure of chicken gizzard MLCK based on the cDNA sequence of Olson et al. (32). PKA phosphorylates sites A and B (11, 12). (B) Time course of phosphorylation in the presence (triangles) and in the absence (circles) of 0.5 mM $CaCl_2$ and 8 μM CaM. The extent of phosphorylation (ordinate) was examined by using [γ-^{32}P]ATP as described under Materials and Methods. Abscissa, incubation time; bars, SEM (n = 3). N.B. Site B was phosphorylated only when site A located in the regulatory domain was occupied by CaM in the presence of Ca^{2+} (12). In the absence of Ca^{2+}-CaM, both sites A and B were phosphorylated.

bands of MLCK were excised from the electrophoresed gel and their radioactivities were counted by a liquid scintillation counter (WALLAC 1410, Pharmacia, Uppsala, Sweden).

Motility assay. *In vitro* motility assay of a myosin-coated surface was performed as previously described (25). In short, to the coverslips coated with myosin, we introduced 3 nM actin filaments labeled with rhodamine-phalloidin in a buffer consisting of 30 mM imidazole (pH 7.5), 50 mM KCl, 1 mM ATP, 3 mM $MgCl_2$, Ca^{2+}-EGTA buffer, and anti-oxidization reagents (25 mM DTT, 0.216 mg/ml glucose oxidase, 0.036 mg/ml catalase, and 4.5 mg/ml glucose). We further added specified concentrations of unphosphorylated or phosphorylated MLCK to the above buffer. We observed the movement of actin filaments on a myosin-coated surface under a fluorescence microscope equipped with an SIT camera (Hamamatsu Photonics C2400, Shizuoka, Japan). The average velocity was measured using an image analyzer (Image Σ-III, Nippon Avionics, Tokyo, Japan).

Actin-binding assay. To quantify the binding of MLCK to actin filaments (26), various amounts of MLCK were mixed and incubated at 25°C for 60 min with 3 μM actin filaments in a buffer containing 60 mM KCl, 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 0.1 mM EGTA. The mixture was then centrifuged at 140,000 × g for 60 min. The precipitates and supernatants were next separately subjected to SDS-PAGE (see below) followed by the densitometry.

Other methods. SDS-PAGE was carried out using the method of Laemmli (27) with slight modifications (26) so that we could check the purity (>95%) of proteins. Protein concentrations were determined by the methods of Bradford (28) with bovine serum albumin as the standard. The interaction between MLCK and myosin was measured by surface plasmon resonance with the IAsys Cuvette System (Fisons Applied Sensor Technology, Cambridge, UK). The LC20 peptide, which was synthesized according to the primary structure of regulatory myosin light chain (29), was immobilized to the cuvette of carboxymethylated dextran matrix *via* *N*-hydroxycuccinimide and 1-ethyl-3-(3 dimethyaminopropyl) carbodiimide. The binding of MLCK was examined according to the manufacturer's protocol in 50 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.5) and 0.2 mM EGTA at 25°C.

RESULT

Phosphorylation of MLCK. MLCK was phosphorylated by a catalytic subunit of PKA in the presence and absence of Ca^{2+}-CaM. As shown in Fig. 1B, the incorporated amount of phosphate was increased with incubation time. The maximal amounts (mol · Pi/mol · MLCK) were 1.95 ± 0.21 (mean ± SEM, n = 3) in the absence of Ca^{2+}-CaM (circles) and 0.85 ± 0.05 (mean ± SEM, n = 3) in the presence of Ca^{2+}-CaM (triangles). The experiment results agree well with those of previous report that stated MLCK is diphosphorylated in the absence of Ca^{2+}-CaM and monophosphorylated in its presence (4).

The kinase activity of MLCK in the presence of Ca^{2+}-CaM was reduced when MLCK was diphosphorylated. The monophosphorylated MLCK exhibited an activity as high as that of unphosphorylated MLCK (data not shown). Because these observations confirm those of previous reports (4, 9), we concluded that diphosphorylated MLCK was phosphorylated at both site A (11)

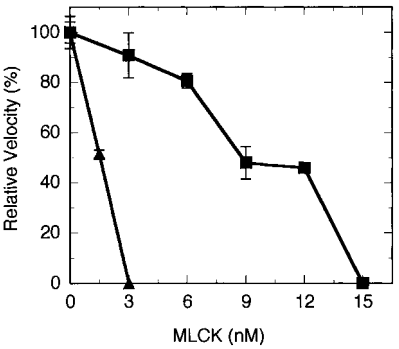

FIG. 2. Effect of MLCKs on the movement of actin filaments on the myosin-coated surface. Various concentrations of unphosphorylated MLCK (squares) or phosphorylated MLCK (triangles) were mixed with a solution of actin filaments. The mixtures were then applied to the myosin-coated surface in the presence of CaM and EGTA so that the movement of actin filaments was observed. N.B. MLCK phosphorylated at site B inhibited the movement of actin filaments more effectively than did unphosphorylated MLCK. Ordinate, relative velocity of the movement of actin filaments; abscissa, concentrations of MLCK; bars, SEM (n = 3).

and site B (12) and monophosphorylated MLCK at site B (4). In the present study, we used the monophosphorylated MLCK as phosphorylated MLCK.

Effect of MLCK on the movement of actin filaments. Figure 2 shows how the movement of actin filaments was affected by MLCK. The movement was inhibited half-maximally when unphosphorylated MLCK was present at about 10 nM. The cessation of the movement was observed in the presence of 15.0 ± 3.0 nM of unphosphorylated MLCK (mean ± SEM, n = 3) (squares in Fig. 2). These data agree well with our data reported previously (14, 15).

When MLCK was phosphorylated at site B, the inhibitory effect was pronounced. As shown by triangles in Fig. 2, the half-maximal inhibition was detectable in the presence of 2–4 nM of unphosphorylated MLCK. The concentration that eliminated the movement was 3.83 ± 0.36 nM (mean ± SEM, n = 3) for phosphorylated MLCK.

Reduced inhibition by Ca^{2+}-CaM. We previously reported that Ca^{2+}-CaM reduced the inhibitory effect of MLCK (14, 15). As shown in Fig. 3A, Ca^{2+}-CaM induced the movement of actin filaments which had been arrested by MLCK. When MLCK was unphosphorylated (squares in Fig. 3A), we observed the movement at 1 mol · CaM/mol · MLCK, confirming the results of previous reports (14, 15). The concentration required for the maximal activation exceeded 2 mol · CaM/mol · MLCK.

The effect of Ca^{2+}-CaM on the phosphorylated MLCK at site B was much weakened. As shown by triangles in Fig. 3A, CaM at three-fold excess over MLCK on a molar basis was not sufficient to negate the inhibition imposed by phosphorylated MLCK. Five-fold excess of CaM was required to observe the movement of actin filaments. Maximal velocity was observed above 10 molar excess of CaM. As described above, the affinity of Ca^{2+}-CaM to phosphorylated MLCK was not affected. We consider that the increased requirement of Ca^{2+}-CaM is due to the increase in the affinity to phosphorylated MLCK (see Discussion).

The myosin used in the assay was phosphorylated at Ser^{19} of the regulatory light chain, leaving the possibility of the additional phosphorylation at Thr^{18} (1–3 for reviews). We wondered if the reduction of movement might be attributable to the additional phosphorylation by MLCK, the kinase activity of which was activated by Ca^{2+}-CaM. The kinase activity of unphos-

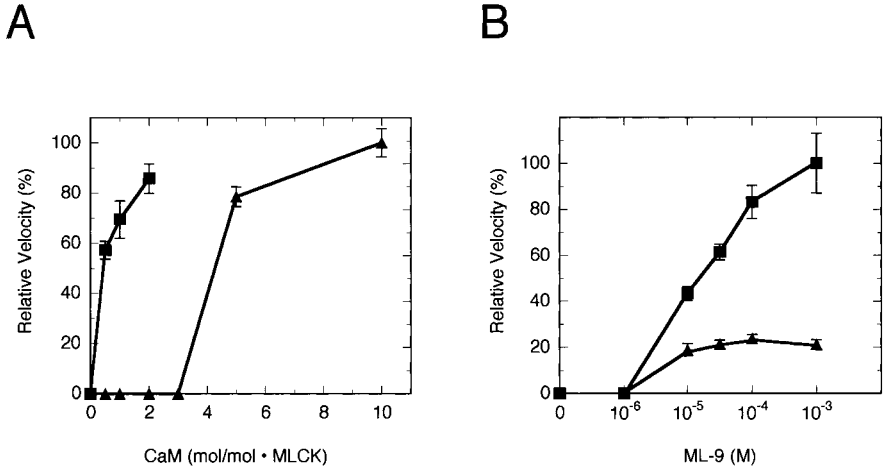

FIG. 3. Reduction of inhibition induced by MLCKs by CaM (A) and ML-9 (B). Fifteen nM of unphosphorylated MLCK (squares) or 3 nM of phosphorylated MLCK (triangles) was mixed with a solution containing actin filaments and various concentrations of CaM or ML-9. The mixtures were then introduced onto the myosin-coated surface to observe the motility of actin filaments. Ordinate, relative velocity of the movement of actin filaments; abscissa, concentrations of CaM or ML-9; bars, SEM (n = 3).

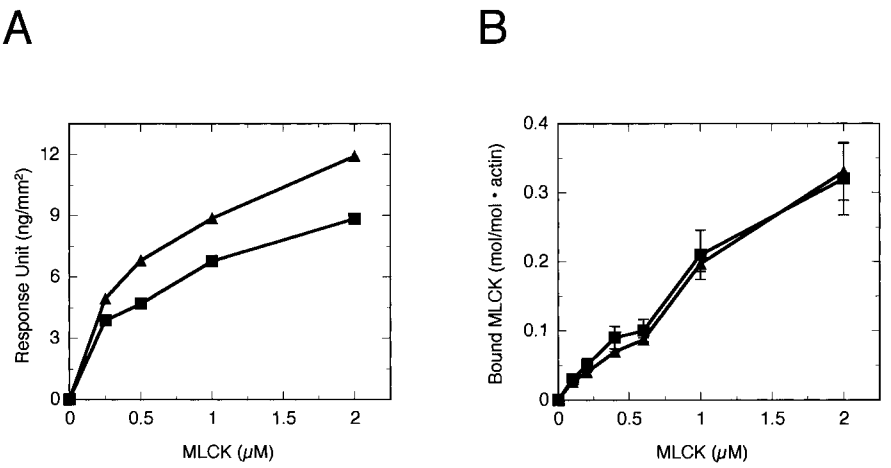

FIG. 4. Binding of MLCK to myosin (A) and actin filaments (B). (A) LC20 peptide, which was synthesized according to the primary structure of myosin light chain (29), was immobilized to the IAsys cuvette. Surface plasmon resonance from the cuvette was detected by adding unphosphorylated MLCK (squares) or phosphorylated MLCK (triangles). Ordinate, surface plasmon resonance; abscissa, concentrations of MLCK. (B) Actin filaments were mixed with various concentrations of unphosphorylated MLCK (squares) or phosphorylated MLCK (triangles). MLCK was then precipitated together with actin filaments by centrifuging the mixture. Ordinate, the amounts of MLCK bound to actin filaments; abscissa, concentrations of MLCK; bars, SEM (n = 3).

phorylated MLCK is much the same as that of MLCK phosphorylated at site B (4, 9). If this is the case, a similar reduction would be expected. However, the reduced inhibition induced by unphosphorylated MLCK is more effective than that imposed by phosphorylated MLCK, ruling out the above possibility.

Reduction by ML-9. We previously reported MLCK inhibitors such as ML-9 (30) reduce the inhibition induced by MLCK (18). As shown by squares in Fig. 3B, we confirmed that the movement of actin filaments, which had been eliminated by unphosphorylated MLCK, was detected in the presence of ML-9. However, only slow movement of actin filaments was observed when their movement had been arrested by MLCK phosphorylated at site B.

It must be noted that the effect of ML-9 is not attributable to the inhibition of kinase activity of MLCK but to its myosin-binding activity (18). We later demonstrates that the latter activity is enhanced upon phosphorylation at site B (Fig. 4A).

Interaction of MLCK with myosin. We have reported that synthetic LC20 peptide interacts with MLCK to negate the inhibitory effect of MLCK (Fig. 4C in ref. 29). Because the peptide synthesized according to the primary structure of regulatory light chain of myosin (29), we considered that its interaction with MLCK was an indication of the MLCK-myosin interaction. As shown in Fig. 4A, the surface plasmon resonance with the IAsys cuvette conjugating LC20 peptide increased with the increase in the concentration of MLCK. The increase was much pronounced when MLCK was phosphorylated at site B, indicating that the phosphorylation increases the affinity of MLCK to myosin.

Actin-binding of MLCK. As described previously, both the myosin-binding activity and the actin-binding activity of MLCK inhibit the movement of actin filaments (14–16). It is possible that the phosphorylation of MLCK increases actin-binding activities so that the movement is inhibited by MLCK more effectively. To rule out this possibility, we mixed MLCK that had been phosphorylated to various extents with actin filaments and precipitated the bound MLCK together with actin filaments by centrifuging the mixture. As shown in Fig. 4B, the concentrations giving half-maximal binding are 0.85 ± 0.02 μM (mean ± SEM, n = 3) for unphosphorylated MLCK and 0.86 ± 0.06 μM (mean ± SEM, n = 3) for phosphorylated MLCK. The maximal amounts of MLCK bound to actin (mol · MLCK/mol · actin) were also same; 0.32 ± 0.05 (mean ± SEM, n = 3) for unphosphorylated MLCK and 0.33 ± 0.04 (mean ± SEM, n = 3) for phosphorylated MLCK. These data indicated that actin-binding properties are not only altered whether or not MLCK is phosphorylated. Thus, the increase of the inhibitory effect of MLCK due to the phosphorylation at site B can not be related to the actin-binding activity of MLCK.

DISCUSSION

In this study, the inhibitory effect of MLCK on the movement of actin filaments on a myosin-coated surface was enhanced when MLCK was phosphorylated at site B irrespective of the phosphorylation at site A. The enhancement was demonstrated by the following observations. First, a lower concentration of MLCK affected the movement upon phosphorylation (Fig. 2). Second, a higher concentration of CaM was required to

reduce the inhibition induced by the phosphorylated MLCK (Fig. 3A). Third, ML-9 reduced the inhibition by unphosphorylated MLCK, but not the inhibition by phosphorylated MLCK (Fig. 3B).

The inhibitory effect of MLCK on the movement has been attributed to actin-binding activity for two reasons. First, actin-binding fragments induced the inhibitory effect on the movement (31). Second, the inhibitory effect was obscured when the concentration of actin filaments was increased (16). However, the catalytic fragments also induced similar inhibition, whether or not the regulatory domain was included (18). The inhibition was reduced by ML-9, which interacts with the catalytic domain (29). Therefore, the catalytic domain works equally to induce the inhibition. The present data indicating that the affinity of MLCK to myosin (see below) was increased upon site B phosphorylation favors the importance of the catalytic domain in the inhibition of the movement by whole MLCK.

Reduction of the kinase activity of MLCK by the site A phosphorylation is explained that affinity of Ca^{2+}-CaM to MLCK is affected by the phosphorylation (4). However, the increased requirement of Ca^{2+}-CaM upon the site B phosphorylation (Fig. 3B) is not associated with the decreased affinity to Ca^{2+}-CaM. The direct measurement of the interaction of MLCK with myosin (Fig. 4A) indicates that the site B phosphorylation of MLCK increases its affinity to myosin.

The present data were exclusively obtained with the myosin phosphorylated at Ser^{19} of its regulatory light chain (see Materials and Methods). When we used the myosin phosphorylated at Ser^{19} and Thr^{18} of its light chain (22), higher concentrations of MLCKs were required to arrest the movement of actin filaments. It is of interest to examine how phosphorylated Thr^{18} residue of myosin light chain interacts with MLCK phosphorylated at site B.

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