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Inhibition of the ATP-Dependent Interaction of Actin and Myosin by the Catalytic Domain of the Myosin Light Chain Kinase of Smooth Muscle: Possible Involvement in Smooth Muscle Relaxation[1]

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Myosin light chain kinase (MLCK) phosphorylates the light chain of smooth muscle myosin enabling its interaction with actin. This interaction initiates smooth muscle contraction. MLCK has another role that is not attributable to its phosphorylating activity, *i.e.*, it inhibits the ATP-dependent movement of actin filaments on a glass surface coated with phosphorylated myosin. To analyze the inhibitory effect of MLCK, the catalytic domain of MLCK was obtained with or without the regulatory sequence adjacent to the C-terminal of the domain, and the inhibitory effect of the domain was examined by the movement of actin filaments. All the domains work so as to inhibit actin filament movement whether or not the regulatory sequence is included. When the domain includes the regulatory sequence, calmodulin in the presence of calcium abolishes the inhibition. Since the phosphorylation reaction is not involved in regulating the movement by MLCK, and a catalytic fragment that shows no kinase activity also inhibits movement, the kinase activity is not related to inhibition. Higher concentrations of MLCK inhibit the binding of actin filaments to myosin-coated surfaces as well as their movement. We discuss the dual roles of the domain, the phosphorylation of myosin that allows myosin to cross-bridge with actin and a novel function that breaks cross-bridging.

Key words: cross-bridge, MLCK, myosin, phosphorylation, smooth muscle.

Myosin light chain kinase (MLCK) is an important enzyme that phosphorylates the 20 kDa light chain (LC20) of smooth muscle myosin in the presence of Ca^{2+} and calmodulin (Ca^{2+}-CaM) converting from an inactive form to an active one (*1-3*). The catalytic site that exerts the kinase activity is located in the central part of the MLCK molecule (*4*). The regulatory site, which consists of pseudosubstrate and CaM-binding sequences, lies on the C-terminal side of the catalytic site. Ca^{2+}-CaM binds to the CaM-binding site and activates the kinase activity of the catalytic site by interacting with the pseudosubstrate sequence (*5-8*).

An *in vitro* motility assay using a myosin-coated glass surface has been developed to monitor the interaction of myosin with actin filaments. For smooth muscle myosin, LC20 phosphorylation is a prerequisite for motility (*9-11*). In the absence of ATP actin filaments are observed to attach to the myosin-coated glass surface. When Mg-ATP is present, the movement of actin filaments can be detected. This ATP-dependent movement of actin filaments is inhibited when the filaments are mixed with MLCK (*12-14*). This inhibition is relieved by Ca^{2+}-CaM, but may not be attributable to the kinase activity of MLCK because the myosin is fully phosphorylated, leaving little room for additional phosphorylation (*15*).

Since MLCK binds to actin filaments (*16-22*), it is very possible that the inhibition and its relief are related to the actin-binding activity of MLCK (*23-25*). The actin-binding fragments obtained by the cleavage of MLCK reproduce the inhibitory effect observed with the parent MLCK (*26*). By producing various recombinant actin-binding fragments of MLCK, we have demonstrated the active involvement of the N-terminal, residues 1-41, of MLCK (*26*). However, we eventually obtained a result that was not explained by the actin-binding activity of MLCK; wortmannin and ML-9, inhibitors of MLCK, worked so as to relieve the inhibition imposed by MLCK. Because they did not appear to interact with the actin-binding site of MLCK but with its catalytic site, we reexamined the effect of the catalytic site by the *in vitro* motility assay.

MATERIALS AND METHODS

Purification of Proteins—Actin was prepared from the acetone powder of chicken breast muscle (*27*) and used as actin filaments after polymerization. Myosin was prepared from fresh chicken gizzard according to Ikebe and Hart-

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Abbreviations: CaM, calmodulin; LC20, 20 kDa light chain of myosin; MLCK, myosin light chain kinase; TM, tropomyosin.

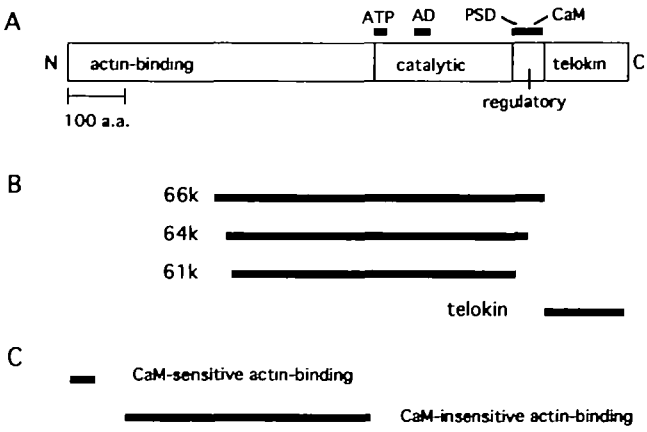


Fig. 1. **Schematic illustration showing the alignment of MLCK and its fragments used in our experiments.** (A) Domain structure of chicken gizzard MLCK based on the cDNA sequence of Olson *et al.* (*4*). ATP binding-, active (substrate-binding), pseudosubstrate-, and CaM-binding domains are indicated as ATP, AD, PSD, and CaM, respectively. (B) Fragments containing the catalytic domain examined in this report. For details of these fragments, see the text. (C) CaM-sensitive and insensitive actin-binding sequences reported in our previous report (*26*). These sequences have been estimated by experiments with fragments of bovine stomach MLCK expressed in bacteria. Positions of the corresponding sequences of chicken gizzard MLCK (Met^{1}-Pro^{41} for CaM-sensitive and Ala^{98}-Asp^{517} for CaM-insensitive sequence, respectively) are shown.

shorne (*28*) with slight modifications. MLCK (*20*) and tropomyosin (TM) (*29*) were prepared from frozen chicken gizzard as described. CaM from bovine brain (P2277) was purchased from Sigma (St. Louis, MO, USA). Fragments containing the catalytic domain of MLCK were obtained by tryptic cleavage of MLCK according to Ikebe *et al.* (*6, 7*) with slight modifications. MLCK was cleaved with *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin, and the fragments were purified with DEAE-Toyopearl 650s (Toso, Tokyo) followed by Superose 12 (Pharmacia, Uppsala, Sweden). Fragments of 66, 64, and 61 kDa corresponded to Lys^{276}-Arg^{825}, Thy^{283}-Arg^{797} (or Lys^{793}), and Ala^{287}-Lys^{776} of MLCK, respectively. These sequences were aligned with the full length MLCK sequence as shown in Fig. 1B.

Reagents—Kinase inhibitors, ML-9 (120809) and staurosporine (390041), were purchased from Seikagaku-Kogyo (Tokyo). Other reagents were purchased from Wako Chemical (Osaka) or Kanto Chemical (Tokyo).

Motility Assay—The *in vitro* motility assay on a myosin-coated surface was performed as described previously (*11*). In short, nitrocellulose-coated coverslips were coated with myosin phosphorylated by MLCK in the presence of Ca^{2+}-CaM. We then constructed a flow cell by overlaying the coated surface on an uncoated glass slide. We introduced actin filaments labeled with phalloidin-rhodamine in buffer A [3 nM phalloidin-rhodamine actin, 50 mM KCl, 20 mM Imidazole (7.5), 1 mM ATP, 3 mM $MgCl_2$] supplemented with anti-oxidization reagents (25 mM DTT, 0.216 mg/ml glucose oxidase, 0.036 mg/ml catalase, 4.5 mg/ml glucose) to the flow cell. In the actual experiments, we further added the specified concentrations of MLCK, its catalytic fragments, or TM. In some cases, we also added 0.5 mM EGTA and 20 nM CaM for low Ca^{2+} conditions denoted as EGTA-CaM, or 0.5 mM $CaCl_2$ and 20 nM CaM for high Ca^{2+} conditions denoted as Ca^{2+}-CaM. MLCK alone has the ability to inhibit the movement of actin filaments in the absence of CaM (*12*), but we added CaM in all cases to compare movement in Ca^{2+} and in EGTA. The flow cell was observed under a fluorescence microscope, and the movement of actin filaments was recorded on a video recorder through an SIT camera (Hamamatsu Photonics C2400, Hamamatsu). The assay was performed at 25°C. Records of the movement of actin filaments were traced and the sliding velocity was calculated. Each velocity data point was the average of 25 actin filaments. Standard deviations are shown as vertical bars.

As will be described later, we observed actin filaments that were not moving but just attached to the myosin-coated surface. To quantify the attachment of actin filaments that were moving or not moving, we counted the number of actin filaments on the surface in an $80 \times 100\ \mu m^2$ field.

Quantification of LC20 Phosphorylation of Myosin Immobilized to a Glass Surface—A solution of 3 nM actin filaments in buffer A supplemented with 6 nM MLCK with the addition of EGTA-CaM or Ca^{2+}-CaM was placed in the flow cell and then incubated at 25°C for 30 min. The coverslip was removed after the incubation and washed with 8 M urea and 40 mM Tris-HCl (8.8), and the solution was subjected to glycerol-urea PAGE (*11*). The electrophoresed gels were stained with Silver Stain II Kit Wako (291-50301, Wako Chemical, Osaka), scanned densitometrically (EPSON GT-6500ART scanner and Power Macintosh 6300/120 computer), and quantified with an NIH image (ver. 3.0).

Actin-Binding Assay—To quantify the binding of MLCK to actin filaments, actin filaments (12 μM) and various amount of MLCK were mixed in a buffer containing 50 mM KCl, 20 mM Tris-HCl (7.5), 2 mM $MgCl_2$, and incubated at 25°C for 30 min. To examine the effect of TM, 2 μM TM (1/7 molar ratio to actin) was included. The mixture was centrifuged at $100{,}000 \times g$ for 60 min, and the precipitates and supernatants were subjected to SDS-PAGE. The electrophoresed gels were stained with Coomassie Brilliant Blue, and the actin, MLCK, and TM protein bands were densitometrically quantified as above.

RESULTS

Changes in the Phosphorylation State of Myosin during the Motility Assay—LC20 was furnished with two sites that are phosphorylated by MLCK in the presence of Ca^{2+}-CaM. The myosin used for the *in vitro* motility assay in the present study was a mixture of singly and doubly phosphorylated myosin (lane 2 of Fig. 2). MLCK inhibited the sliding movement on surfaces coated with phosphorylated myosin. The concentration of MLCK that abolished movement was 6-9 nM. In the presence of Ca^{2+}-CaM, no inhibition was observed at lower MLCK concentrations; rather, MLCK worked so as to stimulate the movement slightly. These observations are quite compatible with our previous data (*12, 13*). The myosin-coated surfaces were exposed to various assay solutions containing MLCK in the presence of Ca^{2+}-CaM, and the myosin was subjected to glycerol-urea PAGE. As shown in lanes 3-5 of Fig. 2, we failed to detect any changes in the level of LC20 phosphor-

ylation, indicating that no additional phosphorylation occurred during the experiment.

Effect of Kinase Inhibitors on the Inhibition of Motility—We examined how the inhibition of actin filament movement by MLCK is modified in the presence of various concentrations of wortmannin and ML-9, typical inhibitors of the kinase activity of MLCK (*30–32*). As shown in Fig. 3A and 3B by the filled circles, the inhibition was relieved by wortmannin or ML-9 in a dose-dependent manner. However, these inhibitors in themselves do not affect the actin-myosin interaction as shown by the squares in Fig. 3, A and B. The effects of wortmannin and ML-9 are reversible; when the solution introduced in the flow cell was replaced by solutions without inhibitors, the sliding movement stopped within 20 s (data not shown). Since the kinase activity of MLCK remained low under these conditions, *i.e.*, in the absence of Ca^{2+}-CaM, the relief provided by these inhibitors is not attributable to the inhibition of the activity.

The kinase activity of MLCK is activated by Ca^{2+}-CaM, an activity that is inhibited by wortmannin and ML-9. The effect of these inhibitors on the movement of actin filaments in Ca^{2+}-CaM (open circles in Fig. 3, A or B) was slightly inhibitory, but the movement was never lower than the control, suggesting that the sites of the action of wortmannin and ML-9 are different from the CaM-binding domain. However, this effect is also not caused by the inhibition of the MLCK kinase activity as in the absence of Ca^{2+}-CaM, because the phosphorylated state of myosin is not altered during the experiment as described in the preceding section (Fig. 2).

Despite the fact that ML-9 shows a striking effect on the regulation of movement, it has no effect on the binding of MLCK to actin filaments either in EGTA-CaM or in Ca^{2+}-CaM (Fig. 3C).

Staurosporine, another inhibitor of MLCK (*33*), cancels the regulation of movement at similar concentrations as wortmannin without affecting the binding of MLCK to actin filaments (data not shown).

The studies with wortmannin, ML-9, and staurosporine suggest that the inhibitory effect is not mediated by the actin-binding activity of MLCK. We, therefore, reexamined the inhibitory effect of MLCK from the viewpoint that MLCK might act other than as an actin-binding protein.

Effects of TM on the Inhibitory Effect of MLCK—TM is an actin-binding protein and is expected to modulate the actin-binding properties of MLCK; actin filaments in smooth muscle cells are associated with TM. Therefore, it is important to know whether or not the inhibitory effect of MLCK occurs even in the presence of TM. As shown in Fig. 4A, TM enhances the actin-binding of MLCK (compare lane 2 with lane 4 as well as lane 6 with lane 8) in the presence and absence of Ca^{2+}-CaM, confirming the data of Sellers and Pato (*17*). Therefore, assuming that the inhibi-

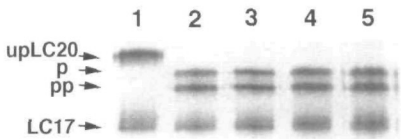


Fig. 2. **Phosphorylation of LC20 on a myosin-coated surface.** The glycerol urea PAGE pattern of released myosin light chains from a coverslip in contact with a solution of actin filaments (3), actin filaments plus MLCK in EGTA-CaM (4), or actin filaments plus MLCK in Ca^{2+}-CaM (5). Unphosphorylated myosin (1), and phosphorylated myosin (2) used in this experiment were also applied to the same gel. On the left, the position of unphosphorylated, monophosphorylated, and diphosphorylated LC20, and the 17 kDa light chain are marked by arrows as upLC20, p, pp, and LC17, respectively. For details, see "MATERIALS AND METHODS."

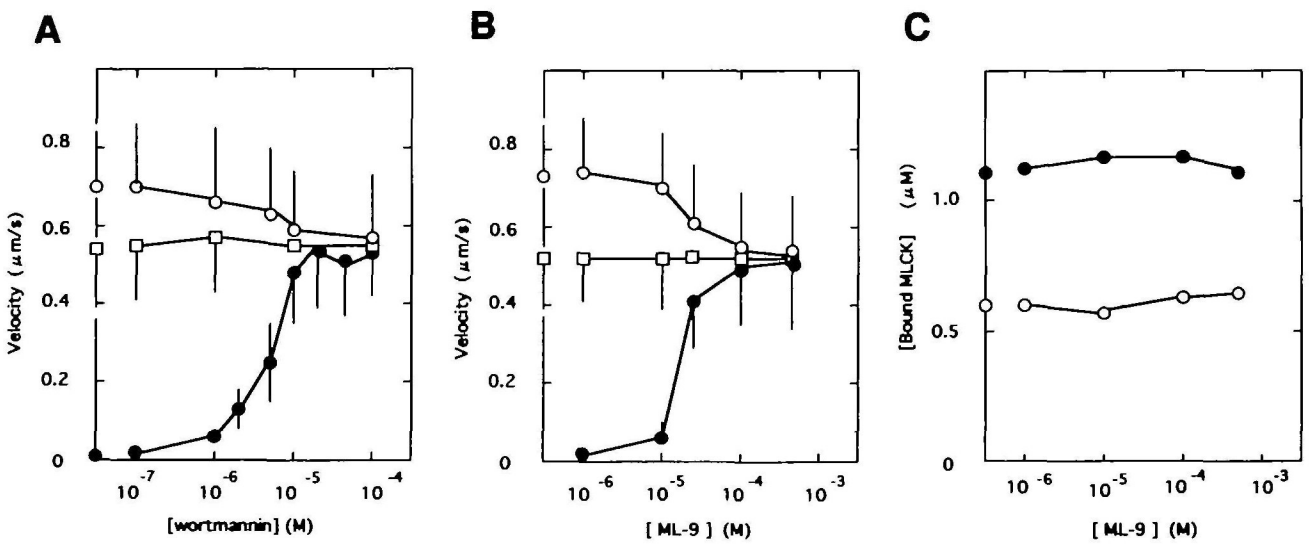
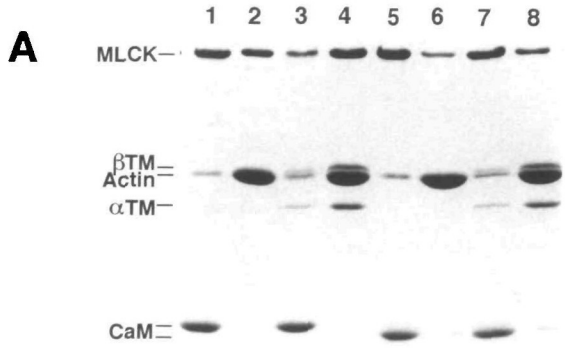


Fig. 3. **Relief of the inhibition of movement by kinase inhibitors.** (A and B) To a solution of 3 nM actin filaments and 6 nM MLCK, various concentrations of wortmannin (A) or ML-9 (B) were added and then perfused on a myosin-coated surface in the presence of MLCK and EGTA-CaM (closed circles), MLCK and Ca^{2+}-CaM (open circles), or in the absence of MLCK (open squares). Data points on the ordinate indicate the values in the absence of inhibitors. (C) The binding of MLCK to actin filaments was examined by mixing various amounts of ML-9 to a solution of 12 μM actin filaments and 4 μM MLCK. The amount of MLCK bound to actin filaments was examined by centrifugation assay, in the presence of 0.5 mM EGTA and 10 μM CaM (closed circles) or of 0.5 mM $CaCl_2$ and 10 μM CaM (open circles). Data points on the ordinate indicate the values in the absence of ML-9.



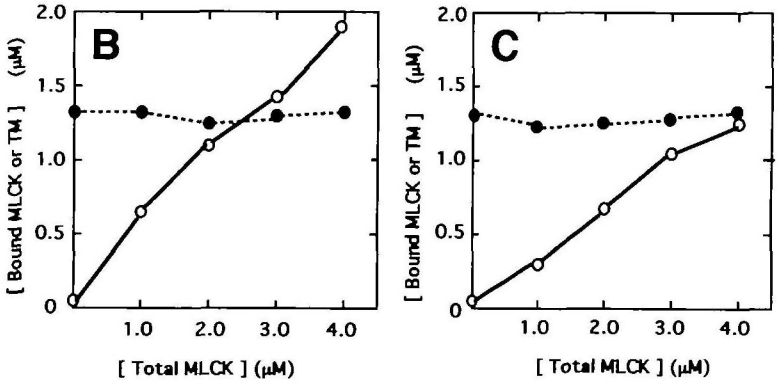


Fig. 4. **Effect of TM on the binding of MLCK to actin filaments.** (A) SDS-PAGE pattern of supernatants (1, 3, 5, 7) and precipitates (2, 4, 6, 8) of the centrifugation assay. Actin filaments incubated with 4 μM MLCK, 0.5 mM EGTA, and 10 μM CaM (1, 2) or 4 μM MLCK, 0.5 mM $CaCl_2$, and 10 μM CaM (5, 6). Actin filaments complexed with TM incubated with 4 μM MLCK, 0.5 mM EGTA, and 10 μM CaM (3, 4) or with 4 μM MLCK, 0.5 mM $CaCl_2$, and 10 μM CaM (7, 8). Positions of MLCK, actin, α-TM, β-TM, and CaM are indicated at the left. (B) Various concentrations of MLCK were mixed with actin filaments complexed with TM in the presence of 0.5 mM EGTA and 10 μM CaM, and the amounts of bound MLCK (open squares) and TM (closed squares) were examined by centrifugation assay. (C) Various amounts of MLCK were mixed with actin filaments in the presence of TM, 0.5 mM $CaCl_2$, and 10 μM CaM. The amounts of MLCK (open squares) and TM (closed squares) were plotted against the concentration of MLCK. Note that MLCK and TM do not compete in binding to actin filaments either in EGTA-CaM or Ca^{2+}-CaM.

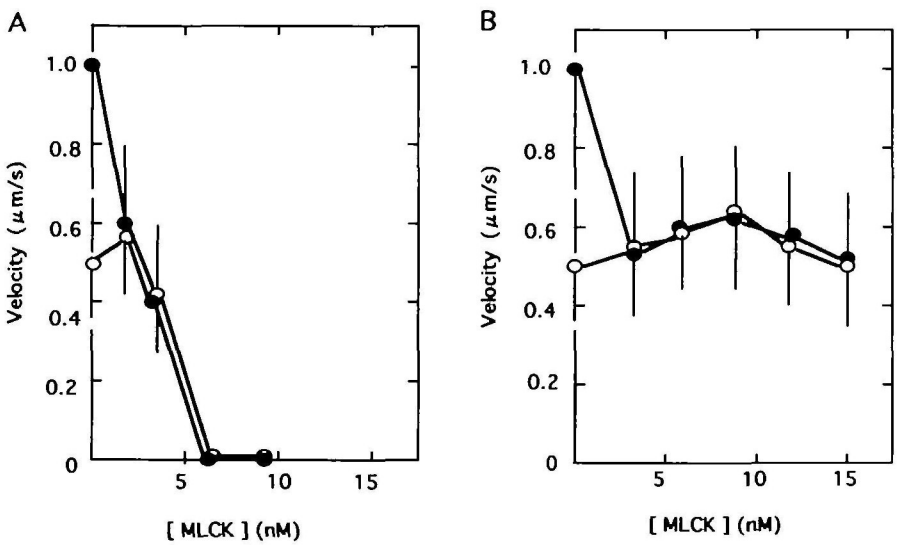


Fig. 5. **Effect of TM on the inhibition of movement by MLCK.** Sliding velocity of actin filaments in the presence of EGTA-CaM (A) or of Ca^{2+}-CaM (B), in the presence or absence of 0.5 nM TM (closed and open circles, respectively).

tory effect of MLCK is totally attributable to its actin-binding properties, the inhibitory effect is expected to be enhanced by TM. However, this was not the case; as shown in Fig. 5A, the inhibition by MLCK was not affected by TM (compare filled circles with open circles). The effect of TM itself must be stimulatory for the actin-myosin interaction (*11*), because the filled circles are located above the open circles as shown in Fig. 5. Even in the presence of MLCK and Ca^{2+}-CaM, the movement of actin filaments was not affected by TM (Fig. 5B). Thus, the effect of TM is another example that can not explain the inhibition by the actin-binding properties of MLCK.

Effect of the Catalytic Domain of MLCK on the Movement of Actin Filaments—We prepared three fragments (Fig. 1B); the longest fragment 66 kDa contains both the CaM-binding and pseudosubstrate sequences, the CaM-binding sequence is deleted in the 64 kDa fragment, and both the CaM-binding and pseudosubstrate sequences are absent from the 61 kDa fragment (*6, 7*). The effect of the 66 kDa fragment on the movement of actin filaments is shown in Fig. 6B. It inhibits the movement in a concentration-dependent manner, with total abolition observed at 6

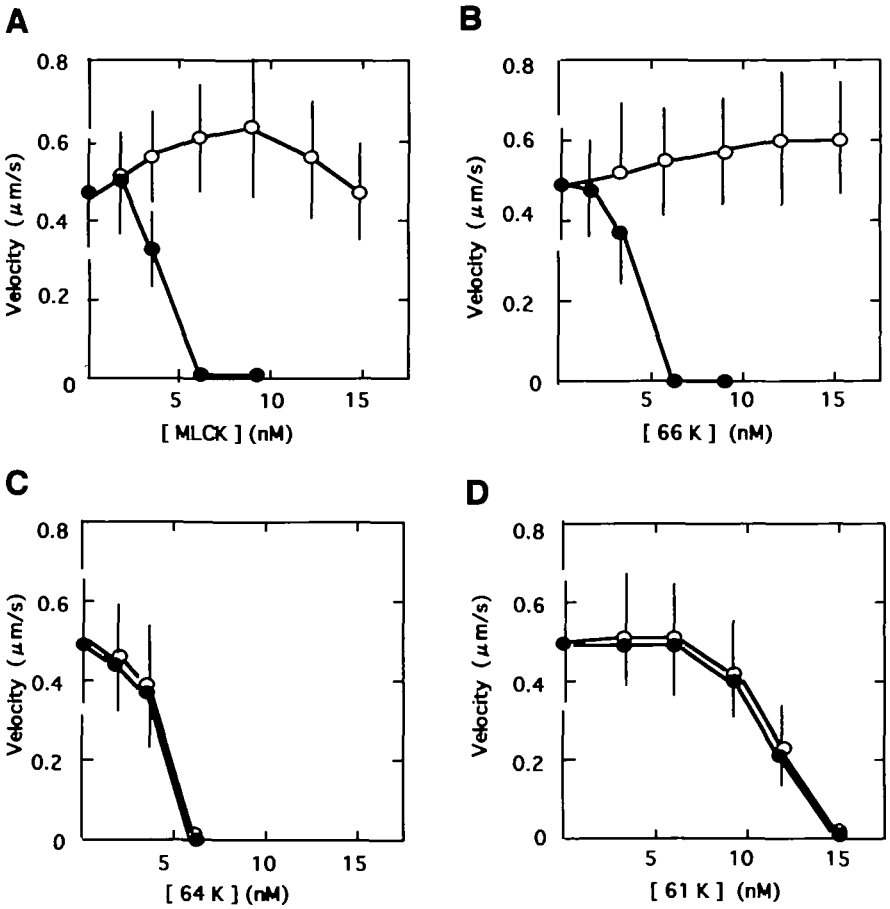


Fig. 6. **Effect of catalytic domain fragments on actin filament movement.** (A) intact MLCK, (B) 66 kDa fragment, (C) 64 kDa fragment, (D) 61 kDa fragment. In the presence of EGTA-CaM (closed circles) or Ca^{2+}-CaM (open circles).

nM. In the presence of Ca^{2+}-CaM, no inhibition was detectable. The inhibitory effect was also observed with the 64 and 61 kDa fragments (Fig. 6, C and D). However, their inhibitions were not prevented by Ca^{2+}-CaM. Thus the catalytic domain, the sole common sequence among the three fragments (Fig. 1B), is responsible for the inhibition. The CaM-binding sequence in the 66 kDa fragment relieved the inhibition by binding to CaM in the presence of Ca^{2+}. It must be noted that the kinase activities of the 66, 64, and 61 kDa fragments are not identical (*6, 7*). The 66 kDa fragment shows activity in the presence of Ca^{2+}-CaM. The activity of 64 kDa fragment is low whether or not Ca^{2+}-CaM is present. The activity of the 61 kDa fragment is always high without requiring Ca^{2+}-CaM. Nevertheless, all three fragments showed inhibitory effects on the actin-myosin interaction, indicating that the effects of these fragments are not associated with the kinase activity of the catalytic domain.

Effects of ML-9 and TM on the Inhibition by the 66 kDa and Other Fragments—The experiment shown in Fig. 7 examines whether or not the site of action of ML-9 is in the catalytic domain without the complication of the actin-binding activity of the parent MLCK. The inhibition by the 66 kDa fragment (filled circles) was relieved by increased concentrations of ML-9. Similar to the parent MLCK, the slight stimulation by MLCK in the presence of Ca^{2+}-CaM was abolished by ML-9 (open circles). The inhibitions by the 64 and 61 kDa fragments were also relieved by ML-9 at a concentration of 10^{-4} M (data not shown).

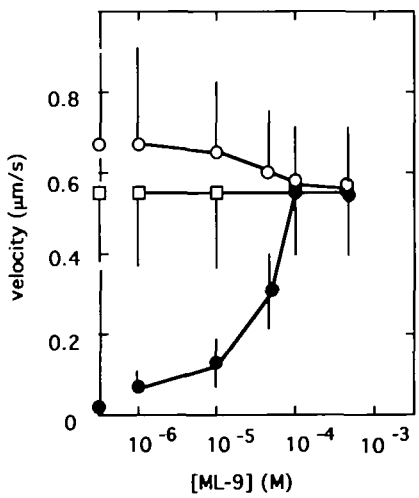


Fig. 7. **Effect of ML-9 on the regulation of actin filament movement by the 66 kDa fragment.** To a solution of 3 nM actin filaments and 6 nM MLCK, various concentrations of ML-9 were added and the mixtures were then perfused on a myosin-coated surface. In the presence of MLCK and EGTA-CaM (closed circles), or MLCK and Ca^{2+}-CaM (open circles). In the absence of MLCK (open squares). Data points on the ordinate indicate the values in the absence of ML-9.

The effect of the 66 kDa fragment was also examined in the presence of TM. As shown in Fig. 8A, the inhibition

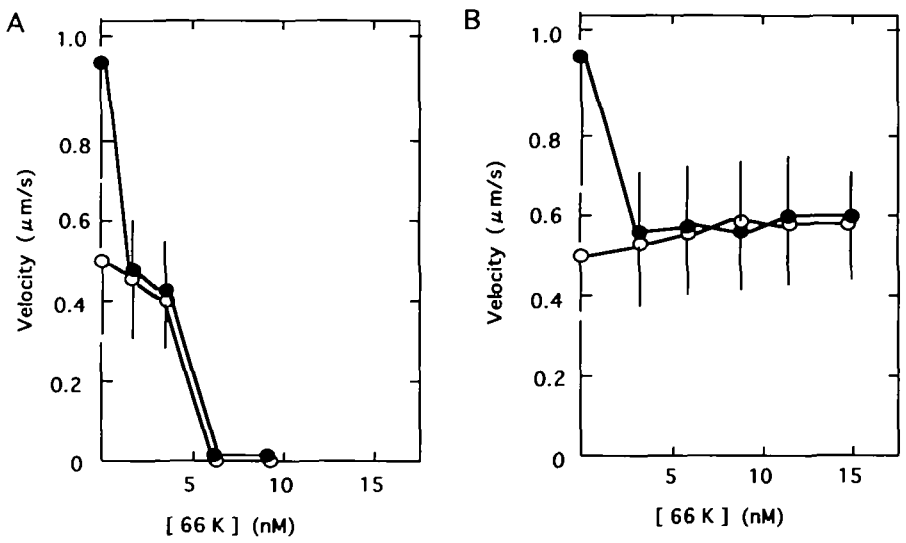


Fig. 8. **Effect of TM on the inhibition of actin filament movement by the 66 kDa fragment.** Sliding velocity of actin filaments in the presence of EGTA-CaM (A) or Ca^{2+}-CaM. In the presence or absence of 0.5 nM TM (closed and open circles, respectively).

imposed by the 66 kDa fragment was never affected by TM. Further, the movement of actin filaments in the presence of the 66 kDa fragment and Ca^{2+}-CaM was also not affected (Fig. 8B). Thus the observations made with the parent MLCK (Figs. 3 and 5) could be reproduced with the 66 kDa fragment.

DISCUSSION

In this paper, a novel role of the catalytic domain of MLCK was revealed in its inhibition of the actin-myosin interaction in a phosphorylation-independent manner, and this inhibition being relieved by Ca^{2+}-CaM. The importance of this novel role suggests a dual role of the catalytic domain. One is the well-known role of phosphorylating myosin at LC20 to allow myosin to interact with actin filaments (*1-3*). The second novel role is that it breaks the interaction. The former works at high and the latter at low Ca^{2+} concentrations.

Does a small amount of contaminating protein(s) inhibit the movement? MLCK was purified through two column chromatography steps, and fragments of 66, 64, and 61 kDa were further purified through two chromatography steps after tryptic digestion. If the inhibition is caused by contaminating protein(s) in our MLCK preparation, the protein(s) should be sensitive to CaM, because the suppression of movement is relieved by CaM. Candidates for such a protein include caldesmon and calponin. But higher concentrations of caldesmon and calponin than MLCK is required to inhibit the movement (*13*). Thus it is difficult to suppose that the suppression of movement is mediated by such contaminant(s).

Previously, we reported that the parent MLCK inhibits the actin-myosin interaction. Because N-terminal actin-binding fragments inhibited the interaction, we attributed the inhibition to the actin-binding domain (*23, 24, 26*). The sequences of these fragment correspond to Met^{1} to Asp^{517} of chicken gizzard MLCK (Fig. 1C). However, we have not yet examined the remaining sequence of MLCK that contains the catalytic, regulatory, and telokin domains. The positions of the above sequences are shown in Fig. 1, B and C. We have shown that the N-terminal CaM-sensitive actin-binding sequence (Fig. 1C) stops the movement at 60 nM (Fig. 5 of Ref. *26*). A CaM-insensitive actin-binding sequence (Fig. 1C) does not affect the movement (Fig. 3a of Ref. *26*). The 66 kDa fragment stops the movement at 6 nM (Fig. 6B), while telokin, which has the same amino acid sequence as the C-terminal 157 residues of MLCK (Fig. 1B and Ref. *34*), has little effect on the movement (data not shown). Thus our previous (*26*) and present studies cover the full amino acid sequence of gizzard MLCK (Fig. 1, C and B).

There may be a question as to why we did not use recombinant fragments instead of native, proteolytic fragments. We have tried to express fragments containing the catalytic domain (Fig. 1B) in *Escherichia coli*. However, the recombinants show very low affinities for CaM, and large amounts of CaM are required to activate their kinase activity. Therefore, we used the proteolytic fragments of MLCK in the present study.

It is well-established that MLCK initiates smooth muscle contraction by phosphorylating myosin at LC20. Therefore, wortmannin and ML-9, which are often used as reagents to inhibit phosphorylation, abolish smooth muscle contraction (*32*). The present study shows that wortmannin and ML-9 also antagonize the novel role of the catalytic domain, the inhibition of the actin-myosin interaction, in a phosphorylation-independent manner. Therefore, if this role is present *in vivo*, these inhibitors will enhance the contraction of smooth muscle. However, to our knowledge, such an effect has not yet been reported. We are interested in finding conditions that allow these inhibitors to stimulate contraction, which will in turn explain the physiological significance of the novel role of the catalytic domain. Another comment about these inhibitors is that the concentration that affects the kinase activity of MLCK is about 10-fold lower than the concentration that abolishes the novel role of MLCK. The value of the inhibition constant, IC_{50}, of wortmannin for the former has been reported to be 0.17 μM (*30*), and the concentration that gives half relief for the latter is 3-4 μM (Fig. 3A). Similarly, the IC_{50} value of ML-9 for the former is 3.8 μM (*32*), and for the latter it is 20-30 μM (Figs. 3B and 8). Therefore, we need further study to see whether these inhibitors interfere with the

TABLE I. **Effect of MLCK or the 66 kDa fragment on the attachment of actin filaments to a myosin-coated surface.** The number of actin filaments on the fixed area of the surface was counted by perfusing actin filaments (3 nM) and increasing the concentration of MLCK or the 66 kDa fragment in EGTA-CaM. When MLCK or the 66 kDa fragment were added at low concentrations, we observed that they abolished the movement of actin filaments on the myosin-coated glass surface as described in the text, while many actin filaments remained to be attached to the surface. Further increases in concentration caused the filaments to detach from the surface. However, this inhibition of attachment was not observed in the presence of Ca^{2+}-CaM up to 18 nM MLCK or 66 kDa fragment.

MLCK or 66 kDa fragment (nM)	Number of attached actin filaments[a]					
	MLCK[b]			66 kDa fragment[b]		
	Total	Moved	Unmoved	Total	Moved	Unmoved
0	100	92	8	113	79	34
3	74	64	10	—	—	—
6	65	5	60	73	12	61
9	27	0	27	19	0	19
12	5	0	5	4	0	4
15	0	0	0	0	0	0

[a]The series of experiments on MLCK was performed on the same surface by perfusing increasing the concentration of MLCK in a stepwise fashion. Experiments with the 66 kDa fragment were also performed similarly. [b]Experiments with MLCK and the 66 kDa fragment were performed on different surfaces.

binding of ATP to the ATP-binding site of MLCK (*32*).

In our myosin-coated surface assay, 15 pmol of myosin was in contact with the glass surface, and about 80%, *i.e.* 12 pmol, was bound to the surface. Supposing the MLCK concentration that inhibits the attachment of actin filaments to the surface and the volume of the flow cell for the assay to be 15 nM (Table I) and 60 μl, respectively, 0.9 pmol of MLCK should be present in the flow cell. Thus the molar ratio of myosin to MLCK that abolishes the interaction is calculated to be 13:1 (Table I). The molar ratios of myosin to MLCK in gizzard and arterial smooth muscle have been estimated to be 8.8:1 and 6.6:1, respectively (*18*). Therefore, the physiological relevance of the novel role is likely in terms of the concentration of MLCK in smooth muscle cell.

When the cytoplasmic concentration of Ca^{2+} in smooth muscle is elevated, MLCK phosphorylates myosin at LC20 and allows myosin to interact, *i.e.* to cross-bridge, with actin filaments. This cross-bridging causes smooth muscle contraction (*1, 2*). With the subsequent decrease in cytoplasmic Ca^{2+}, the cross-bridging is broken so that the smooth muscle relaxes (*1, 2*). The involvement of MLCK and its catalytic fragments in this breakage is clearly shown in Table I. At their lower concentrations, they stop the movement of actin filaments by interacting with myosin. At higher concentrations, they allow the filaments to detach from the myosin, suggesting the breaking of cross-bridges. Here it should be noted that the myosin is in a phosphorylated state, suggesting that the dephosphorylation of myosin is not a prerequisite for cross-bridge breakage. This suggestion is in good agreement with Tansay *et al.* (*35*) who reported that skinned smooth muscle that has contracted in the presence of okadaic acid is relaxed when the concentration of Ca^{2+} is reduced to 10^{-7} M, despite LC20 being fully phosphorylated. Indeed, various mechanisms for cross-bridge breakage have been suggested; for example, calcium channel blockers relax endothelin-1-induced contraction without any dephosphorylation of LC20 (*36*). An elevation in the extracellular concentration of Mg^{2+} induces a reduction in the intracellular concentration of Ca^{2+} and relaxation leaving LC20 to be phosphorylated (*37*). When a muscle strip is first contracted with Ca^{2+} and ATP and then exposed to Ca^{2+} and CTP, the muscle maintains stress with basal levels of LC20 phosphorylation. This phosphorylation-independent contraction is relaxed by CaM antagonists, suggesting that a CaM-like Ca^{2+} binding protein is important for latch bridge attachment (*38*).

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