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Inhibitory Effect of the Catalytic Domain of Myosin Light Chain Kinase on Actin-Myosin Interaction: Insight into the Mode of Inhibition[1]

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The catalytic domain of myosin light chain kinase (MLCK) not only exerts kinase activity to phosphorylate the 20 kDa light chain but also inhibits the actin-myosin interaction. The site of action of this novel role of the domain has been suggested to be myosin [Okagaki *et al.* (1999) *J. Biochem.* 125, 619-626]. In this study, we have analyzed the amino acid sequences of MLCK and myosin that are involved in the inhibition. The ATP-binding peptide of Gly^{526}-Lys^{548} of chicken gizzard MLCK exerted the inhibitory effect on the movement of actin filaments on a myosin-coated glass surface. However, the peptide that neighbors the sequence failed to inhibit the movement. The inhibition of the ATP-binding peptide was confirmed by measuring ATPase activities of the myosin. The inhibition by parent MLCK of the movement was relieved by the 20 kDa light chain, but not by the 17 kDa myosin light chain. The peptide of the 20 kDa light chain sequence of Ser^{1}-Glu^{29} also relieved the inhibition. Thus, the interaction of the ATP-binding sequence with the 20 kDa light chain sequence should cause the inhibition of the actin-myosin interaction. Concerning the regulation of the inhibition, calmodulin relieved the inhibitory effect of MLCK on the movement of actin filaments. The calmodulin-binding peptide (Ala^{796} Ser^{815}) prevented the relief, suggesting the involvement of this sequence. Thus, the mode of regulation by Ca^{2+} and calmodulin of the novel role of the catalytic domain is similar, but not identical, to the mode of regulation of the kinase activity of the domain.

Key words: catalytic domain, MLCK, myosin light chain, myosin, phosphorylation.

We have recently reported that the catalytic domain of MLCK exerted an inhibitory effect on the actin-myosin interaction (*1*). Because the myosin used for detecting the effect was fully phosphorylated, the effect was not attributable to the kinase activity of the domain. Thus, the role of the domain is novel and awaits detailed characterization in the light of the mode of phosphorylation of myosin.

The first event that occurs during myosin phosphorylation is the binding of ATP to the ATP-binding site of the catalytic domain. Then, the γ phosphate of ATP is tranferred to the Ser^{19} and Thr^{18} of the 20 kDa light chain of myosin (LC20). This kinase activity of the catalytic domain of MLCK is kept at basal level by the pseudosubstrate sequence. The complex of Ca^{2+} and calmodulin (Ca^{2+}-CaM) relieves this autoinhibition of the activity by the pseudosubstrate sequence (*2-4* for reviews).

The inhibitory effect of the catalytic domain on the actin-myosin interaction was prevented by reagents that affect ATP-binding to MLCK, such as wortmannin and ML-9, and Ca^{2+}-CaM abolished the inhibition (*5*). Therefore, the sites of action of the catalytic domain and myosin are expected to be similar to those involved in the kinase activity. However, unlike the kinase activity, the inhibitory effect was observed regardless of whether the pseudosubstrate sequence was included in the catalytic domain (Fig. 6, C and D, in Ref. *1*). In this study, we synthesized several polypeptides of the catalytic domain related to the above properties of MLCK in order to identify the sites of action.

MATERIALS AND METHODS

Purification of Proteins—Actin was prepared from acetone powder of chicken breast muscle (*6*) and was used as actin filaments after polymerization. Myosin was prepared from fresh chicken gizzard (*7, 8*) with slight modifications and used after phosphorylation in the presence of MLCK, CaM, and Ca^{2+} (*9*) unless otherwise specified. MLCK (*10*) was prepared from frozen chicken gizzard as described. CaM from bovine brain (P2277) was purchased from Sigma (St. Louis, MO, USA). LC20 and myosin light chain of 17 kDa (LC17) were purified from chicken gizzard myosin

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Abbreviations: CaM, calmodulin; LC20, 20 kDa light chain of myosin; LC17, 17 kDa light chain of myosin; MLCK, myosin light chain kinase.

according to Perrie and Perry (*11*) with slight modifications.

Peptides Used in This Experiment—The following peptides of chicken gizzard MLCK sequence (*12*) were synthesized using a MilliGen 9050 peptide synthesizer and purified according to the manufacturer's instructions for Fmoc [*N*-(9-fluorenyl)methyloxy carbonyl] chemistry: the ATP-binding peptide GSGKFGQVFRLVEKKTGKVWAGKF, corresponding to Gly^{526} to Lys^{548} (*12*); the peptides neighboring the ATP-binding sequence on the N-terminal and C-terminal sides, EVNYQTVTINTEQKVSDVYNIEERL and FKYSAKEKENIRDEISIMNCLHH, corresponding to Glu^{501} to Leu^{525} and Phe^{550} to His^{573}, respectively (*12*); the CaM-binding peptide ARRKWQKTGHAVRAIGRLSS, corresponding to Ala^{796} to Ser^{815} (*13, 14*); the pseudosubstrate peptide DTKNMEAKKLSKDRMKKYMA, corresponding to Asp^{777} to Ala^{796} (*13–15*). The LC20 peptide SKRAKAKTTKKRPQRATSNVFAMFDQSQ, corresponding to Ser^{1} to Glu^{29}, was also synthesized (*16*).

Biochemical Experiments—*In vitro* motility assay with myosin-coated surface was performed as described previously (*9*). In short, coverslips treated with nitrocellulose were coated with myosin and placed with the coated surface downward on an uncoated glass slide to constructed a flow cell, into which were introduced actin filaments labeled with phalloidin-rhodamine in buffer A [3 nM phalloidin-rhodamine actin, 50 mM KCl, 20 mM imidazole (pH 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). In the experiments, specified concentrations of MLCK and peptide mimetics were added, and in some experiments, 0.5 mM EGTA and 20 nM CaM for low Ca^{2+} conditions, or 0.5 mM $CaCl_2$ and 20 nM CaM for high Ca^{2+} conditions, were also added. Although MLCK itself had ability to inhibit the movement of actin filaments in the absence of CaM (*17–19*), CaM was included in order to compare the 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 video tape through an SIT camera (Hamamatsu Photonics C2400, Shizuoka). The assay was performed at 25°C. Records of the movement of actin filaments were traced and sliding velocity was calculated. Each data point of the velocity was an average of 25 actin filaments. Values of standard deviation were shown as vertical bars associated with data points.

The actin-activated ATPase activity of myosin was measured under the conditions of 0.05 μM phosphorylated myosin, 60 mM KCl, 5 mM $MgCl_2$, 0.5 mM ATP, 1 mM DTT, 20 mM Tris-HCl (pH 7.5), and 0.2 mM EGTA in the presence of 2 μM actin. The phosphate liberated in 10 min at 25°C was quantified in duplicate or triplicate by the malachite green method (*20*). We ensured that the rate of ATP hydrolysis was steady and that the specific activity of the ATPase did not differ from our previously reported values. Therefore, we expressed the activity as a normalized ATPase activity (*21, 22*).

The interaction between the ATP-binding peptide and myosin was measured by surface plasmon resonance with the IAsys Cuvette System (Fisons Applied Sensor Technology, Cambridge, UK). The ATP-binding peptide was immobilized to the cuvette of carboxymethylated dextran matrix *via* *N*-hydroxycuccinimide and 1-ethyl-3-(3 dimethyaminopropyl) carbodiimide. The binding constant of the interaction was obtained according to the manufacturer's protocol in 60 mM KCl, 5 mM $MgCl_2$, 0.5 mM ATP, 1 mM DTT, 20 mM Tris-HCl (pH 7.5) and 0.2 mM EGTA at 25°C

RESULTS

Effect of ATP-Binding Peptide on the Actin-Myosin Interaction—Because of the suggested involvement of the ATP-binding sequence of MLCK in the inhibition by MLCK (*1*), we synthesized the ATP-binding peptide (*12*). As a control, we also synthesized the C-peptide and the N-peptide, whose sequences are those neighboring the ATP-binding sequence at the C-terminal and N-terminal, respectively (*12*). The effect of ATP-binding peptide on the movement of actin filaments on a myosin-coated glass surface is shown by open circles in Fig. 1. At concentrations above 0.5 mM, the ATP-binding peptide abolished the movement. The inhibition seemed to be specific for ATP-binding peptide, because the N-peptide and C-peptide, both of which lack the ATP-binding sequence, did not affect the movement. We also measured the actin-activated ATPase activity in the presence of various concentrations of these peptides. As shown in Fig. 2, the N- and C-peptides failed to affect the activity, while the ATP-binding peptide inhibited the activity, confirming the result with the movement of the actin filaments.

It must be noted that the movement was inhibited by a lower concentration than the ATPase activity. We attribute the discrepancy to the difference in the amount of myosin used for the motility assay and ATPase measurement: the latter was much higher than the former. The partial nature of inhibition in Fig. 2 is often observed for the ATPase activity (compare Fig. 1 with Fig. 2 in Ref. *19*). This is attributable to the ATPase activity that is not coupled with motility, as reported by Takiguchi *et al.* (*23*).

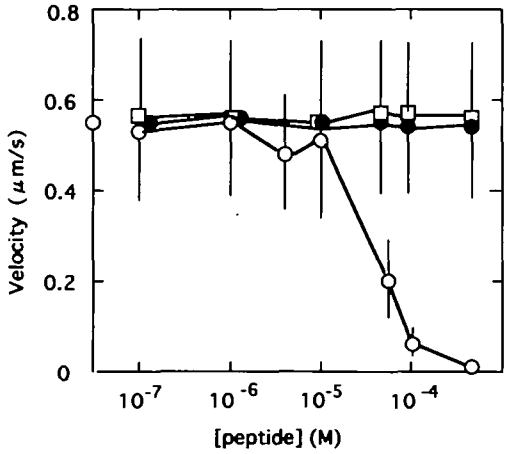

Fig. 1. **Effect of ATP-binding peptide and its related peptides on the movement.** Velocity of sliding movement with 20 nM CaM in 0.5 mM EGTA was plotted against concentration of these peptide. ATP-binding peptide (open circles), N-peptide (closed circles), and C-peptide (open squares). For details, see "MATERIALS AND METHODS."

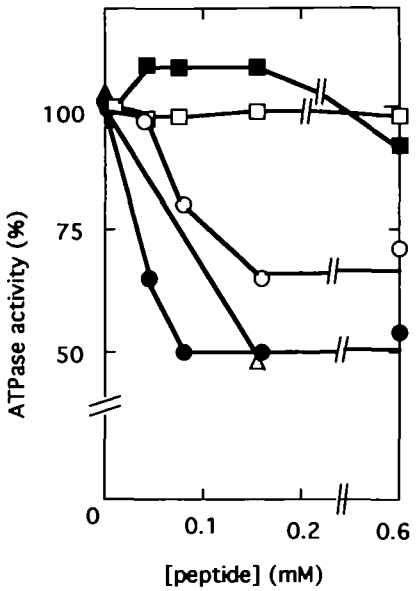

Fig. 2. **Effect of ATP-binding peptide on the ATPase activity.** Actin-activated ATPase of phosphorylated myosin was measured in the presence of various concentrations of peptide. Data of three independent experiments with the ATP-binding peptide are shown by open and closed circles, and triangles, and data of experiments with N- and C-peptides by open and closed cirles, respectively.

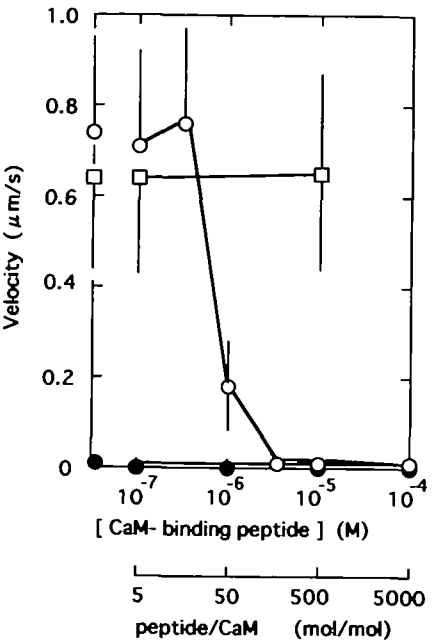

Fig. 3. **Effect of CaM-binding peptide on the relief of the inhibition.** The effect was examined in the presence of 10 nM MLCK and 20 nM CaM either in 0.5 mM Ca^{2+} (open circles) or in 0.5 mM EGTA (closed circles). The ratio of peptide to CaM is indicated at the bottom of the figure. The peptide alone did not affect the movement at any concentration (open squares).

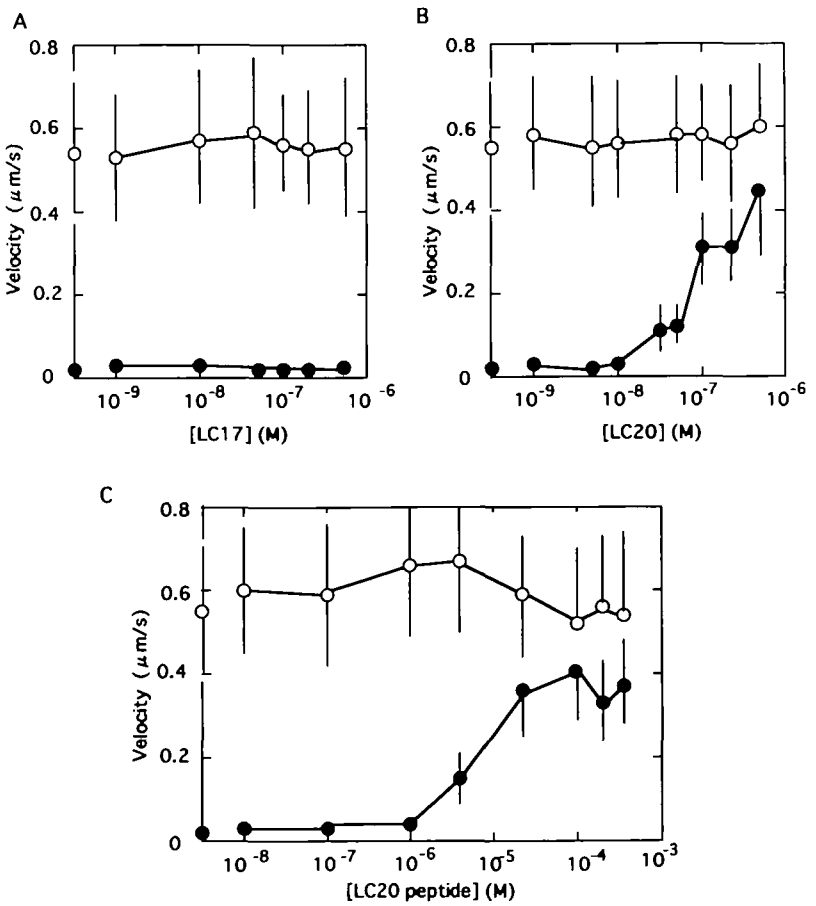

Fig. 4. **Effect of light chains on the movement.** The effect was examined in the presence of various concentrations of isolated LC20 (A) and LC17 (B). Synthetic peptide of LC20 was also examined (C). Closed and open circles represent respectively the presence and absence of 10 nM MLCK in EGTA-CaM.

Interaction of ATP-Binding Peptide with Myosin—Sellers and Pato measured the binding of MLCK to myosin (*24*). When myosin was in a dephosphorylated form, MLCK bound to myosin with a high affinity. However, the binding activity was reduced upon phosphorylation. Because we used phosphorylated myosin in the present study, we examined whether or not the ATP-binding peptide interacts with myosin by detecting surface plasmon resonance of the IAsys cuvette with immobilized ATP-binding peptide. The resonance increased with increase in myosin concentration. The dissociation constant was calculated as 8.9×10^{-5} M.

Effect of CaM-Binding and Psudosubstrate Peptides—In the previous report (*1*), the 66 kDa fragment of MLCK, which was composed of catalytic domain associated with CaM-binding and pseudosubstrate sequences (*12-15*), inhibited the movement of actin filaments, and the inhibition was abolished by Ca^{2+}-CaM. Therefore, we were interested in whether or not the CaM-binding and pseudosubstrate sequences are involved in the inhibition and the subsequent relief.

As shown by the squares in Fig. 3, the CaM-binding peptide itself did not exert any effect on the movement of actin filaments. At 10 nM, MLCK abolished the movement (filled circle in the ordinate), an observation that confirms our previous report (Fig. 5A in Ref. *1*). In EGTA, the CaM-binding peptide did not release the inhibition (filled circles). However, as reported previously, 20 nM CaM relieved the inhibition. When CaM-binding peptide was mixed with CaM, the relief was prevented in a dose-dependent manner (open circles).

Similar experiments were carried out with the pseudosubstrate sequence, which is included in the 66 kDa fragment described above. The pseudosubstrate sequence did not release the inhibition in EGTA or affect the recovery of the movement in the presence of Ca^{2+}-CaM (data not shown). Thus, only the CaM-binding sequence is effective for the relief of the inhibition.

Taking these experiments togethers, we conclude that Ca^{2+}-CaM relieves the inhibition *via* the CaM-binding sequence, without contribution of the psudosubstrate sequence.

Effect of Myosin Light Chains on the Inhibition of the Actin-Myosin Interaction—In the previous report, we did not refer to the site of myosin with which MLCK interacted to inhibit the actin-myosin interaction. To address this subject, we prepared LC17 and LC20 from chicken gizzard myosin and examined whether they abolished the inhibi-

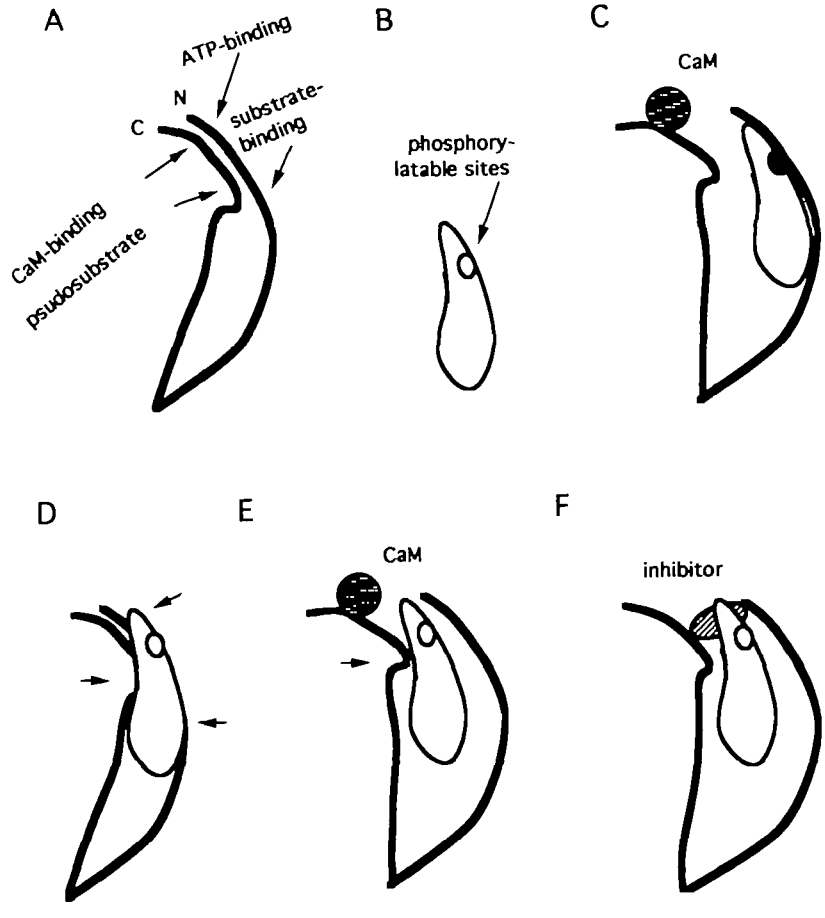

Fig. 5. **Schematic illustration to explain interaction of the catalytic domain of MLCK with LC20.** (A) Conformation of the catalytic domain in the absence of Ca^{2+}-CaM. Contact is made between the substrate-binding and pseudosubstrate sequences and between the ATP-binding and CaM-binding sequences. (B) Structure of LC20 showing phosphorylatable residues of Ser^{19} and Thr^{18} as a small circle. The phosphorylated and unphosphorylated states of these residues are discriminated as open and shaded circles, respectively. (C) Interaction of MLCK and unphosphorylated LC20 in the presence of Ca^{2+}-CaM. The cleft of catalytic domain is opened by Ca^{2+}-CaM for interaction of phosphorylatable residue of LC20 (shaded circle) with the substrate-binding sequence of MLCK (*12, 13, 25*). The catalytic domain activated by Ca^{2+}-CaM in this conformation phosphorylates LC20. (D, E, F) Interaction of the catalytic domain and phosphorylated LC20 reported in this paper. Phosphorylated LC20 is thought to interact with MLCK in a different way from unphosphorylated LC20, because phosphorylatable residues show only weak interaction with MLCK when the residue is phosphorylated. (D) Interaction of phosphorylated LC20 with the catalytic domain in the absence of Ca^{2+}-CaM. Phosphorylated LC20 is thought to be clipped in the cleft of the domain when the movement is inhibited by MLCK. The putative sites of interaction are marked with arrows. (E) Interaction of LC20 with the catalytic domain in the presence of Ca^{2+}-CaM. Once the cleft of the domain is opened by Ca^{2+}-CaM, the contact of LC20 with the cleft is released, and movement is restored. An arrow indicates a remaining site of weak interaction suggested by the slight activation in Ca^{2+}-CaM (*1*). (F) Interaction of LC20 with the catalytic domain in the presence of kinase inhibitor but in the absence of Ca^{2+}-CaM. Kinase inhibitors such as wortmannin and ML-9 not only relieved the inhibition but also cancelled the slight activation of the movement imposed by Ca^{2+}-CaM (Fig. 3 in Ref. *1*). Thus, it is certain that ATP-binding sequence is close to the CaM-binding sequence, as in the case of twitchin kinase, CaM-kinase I, or titin kinase. When kinase inhibitors that mimic ATP bind to or around the ATP-binding sequence, they deform the conformation around the ATP-binding sequence. This deformation also interferes with the CaM-binding sequence to relieve the contact of LC20 with the cleft, and the inhibition by MLCK is relieved.

tion of the actin-myosin interaction imposed by MLCK (Fig. 4, A and B).

LC17 neither affected the movement of actin filaments (open circles in Fig. 4A) nor relieved the inhibition by MLCK (filled circles in Fig. 4A). LC20 also failed to affect the movement (open circles in Fig. 4B), but it relieved the inhibition in a dose-dependent manner (filled circles in Fig. 4B).

To narrow down the site of action of MLCK to LC20, we synthesized the LC20 peptide including the sequence that works as a substrate for the catalytic activity (*25*), and examined its effect in a similar way as for LC20 (Fig. 4C). LC20 peptide itself did not affect the actin-myosin interaction, but relieved the inhibition of the actin myosin interaction imposed by MLCK. LC20 peptide reproduced the effect of LC20 at the higher concentration. This effect accords well with our idea that the ATP-binding sequence of MLCK interacts with the sequence of LC20 peptide.

DISCUSSION

In this study, we addressed the question of which parts of MLCK and myosin are involved in the inhibitory effect of MLCK on the actin-myosin interaction. We identified Gly^{526}-Lys^{548} as the MLCK sequence that interacts with myosin, Ala^{796}-Ser^{815} as the MLCK sequence that interacts with CaM, and Ser^{1}-Glu^{29} as the LC20 sequence that interacts with the catalytic domain of MLCK. These sequences are known to constitute the CaM-binding site that activates kinase activity of MLCK (*12, 13*), and the phosphorylatable site of LC20 (*25*), respectively. Ca^{2+}-CaM-dependent relief of the inhibition was completely abolished by the binding of CaM-binding peptide to CaM (Fig. 3).

We should not overlook the fact that a high concentration of ATP-binding peptide is required for abolishing the movement. This is partly attributable to the nature of the peptide; if the peptide were incorporated into MLCK, it should be effective at a low concentration. The other implication of the high concentration is that the ATP-binding site is not the sole site that exerts the inhibitory effect. This view is in conforms with recent studies on cAMP-dependent protein kinase; it has 11 or more sites of interaction with ATP (*26, 27*). We investigated the sequence from Glu^{501} to His^{573} of ATP binding peptide and its neighboring sequences (Figs. 1 and 2) and the sequence from Asp^{777} to Ser^{815} of pseudosubstrate and CaM-binding sequences (Fig. 3). Thus the additional important sequence, if it exerts, should lie between Pro^{574} and Lys^{776}.

We observed that wortmannin and ML-9 relieved the inhibition imposed by the parent MLCK (*1*), but the ATP-binding peptide did not. It is possible that these inhibitors affect different site(s) from ATP-binding sequence (*28, 29*).

The last comment on the ATP-binding peptide is its basic nature; its calculated pI is 11.11, while that of N- and C-peptide is 4.05 and 7.28, respectively. We could not rule out the complication associated with adjusting pH of peptide solution to pH 7.5, the condition used for the assays.

X-ray crystallography has revealed that the catalytic domains of protein kinases have complex structure. Thus, the geometrical configuration around the ATP-binding sequence should be considered in order to understand more fully the function of these kinases. Three dimensional structures of a few protein kinases, whose amino acid sequences are highly homologous to that of the catalytic domain of MLCK, demonstrate intramolecular interaction inside the catalytic domain of these kinases. Twitchin kinase shows a very complex structure composed of many helices, sheets, and connecting loops (*30, 31*). There are two tight contacts in the structure: between the ATP-binding site and the C-terminal side of the regulatory domain, and between the substrate-binding site and the N-terminal side of the regulatory domain (*30*). Similarly, there is contact between ATP-binding and CaM binding sites in CaM-kinase I (*32*). And the ATP-binding site of titin kinase makes contact with the regulatory site including the CaM-binding sequence (*33*). The authors of these findings speculate that these kinases adopt a closed structure in the absence of CaM or S-100, and that binding of CaM (for CaM-kinase I or titin kinase) or S-100 (for twitchin kinase) in the presence of Ca^{2+} to the regulatory sites opens the cleft of the substrate-binding domain, allowing substrates to interact with this domain.

Although the crystal structure of the catalytic and regulatory domains of MLCK has not yet been solved, these studies suggest that intramolecular interaction may take place between these domains. To explain our experimental data, we postulate the intramolecular interaction illustrated schematically in Fig. 5. The catalytic and regulatory domains of MLCK adopt a closed conformation, in which the cleft remains closed in the absence of Ca^{2+}-CaM (*13*), as depicted in Fig. 5A. The catalytic domain in this conformation inhibits the movement of actin filament by interacting with phosphorylated LC20 (Fig. 5D). When this interaction is released by Ca^{2+}-CaM, the inhibition of the movement is relieved (Fig. 5E).

The above scheme is derived from following observations in the present and reported studies. Figure 5A is modeled after the contact between the substrate-binding (Leu^{601}-Thr^{613}) and pseudosubstrate (Asp^{777}-Ala^{796}) sequences (*13*) and that between the ATP-binding (Gly^{526}-Lys^{548}) and CaM-binding (Ala^{796}-Ser^{816}) sequences (*30, 32, 33*). The interaction of LC20 with the catalytic domain, which was observed in Fig. 4, is shown in Fig. 5D. There are a several sites of interaction, shown by arrows, which are based on Refs. *34* and *35*, and Fig. 6D in Ref. *1*. Figure 5, E and F, explains the release of inhibition by Ca^{2+}-CaM (Fig. 3 and Ref. *1*) and by kinase inhibitors (*1*), respectively. The difference in shape of the catalytic domain is based upon the difference in the mode of action.

Under the appropriate ionic conditions, LC20 phosphorylation converts myosin from 10S to 6S form (*36*). Activity-structure hypothesis relates the conformational change of myosin with increased ATPase activity of myosin. Binding of MLCK to phosphorylated myosin at the ATP-binding sequence of the catalytic domain inhibits its ATPase activity (Fig. 2). It will be of interest to see whether the inhibition is associated with a similar structural change of myosin.

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