Biochem. J. (1991) 275, 679–684 (Printed in Great Britain)

Activation of smooth muscle myosin light chain kinase activity by a monoclonal antibody which recognizes the calmodulin-binding region

Yoshihiko ARAKI and Mitsuo IKEBE*
Department of Physiology and Biophysics, Case Western Reserve University, School of Medicine,
Cleveland, OH 44106, U.S.A.

The regulatory domain of smooth muscle myosin light chain kinase (MLCK) was studied using monoclonal antibodies. Of the 22 monoclonal antibodies tested, a monoclonal antibody designated LKH-18 was found to activate MLCK in the absence of Ca^{2+}/calmodulin. This activation was even greater when an Fab fragment of LKH-18 was used. Consequently, the actin-dependent smooth muscle myosin ATPase activity and the superprecipitation of actomyosin were significantly activated by MLCK plus LKH-18, even in the absence of Ca^{2+}/calmodulin. The antibody-binding site was studied using proteolytic fragments and synthetic peptide analogues of MLCK. Immunoblot analysis revealed that LKH-18 reacted with the 66 kDa calmodulin-dependent active fragment but not with the 64 kDa inactive fragment or with the 61 kDa calmodulin-independent active fragment. Furthermore, LKH-18 reacted with MLCK-(796–815)-peptide but not with MLCK-(786–801)-peptide or with MLCK-(796–807)-peptide. Therefore the LKH-18-binding site was assigned to amino acid residues 808–815 of MLCK, which are thought to be a part of the calmodulin-binding site. The present results suggest that the binding of ligand to this region induces a conformation change in MLCK and that this abolishes the action of the inhibitory region which exists next to the *N*-terminal side of the calmodulin-binding site.

INTRODUCTION

Phosphorylation of the 20 kDa light chain (LC_{20}) of smooth muscle myosin is thought to be an integral component of the regulatory mechanism of the smooth muscle contractile apparatus (Adelstein & Eisenberg, 1980; Hartshorne, 1987). Phosphorylation of the light chain is catalysed by the Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK). One of the most important problems in the study of the structure–function relationships of MLCK, and indeed other calmodulin-dependent enzymes, is how the Ca^{2+}/calmodulin complex activates enzymic activity. For MLCK, this problem is greatly assisted by the recent determination of the complete amino acid sequence for both gizzard MLCK (Olson *et al.*, 1990) and skeletal muscle MLCK (Takio *et al.*, 1986; Roush *et al.*, 1988).

A number of studies have suggested that MLCK contains a regulatory region composed of inhibitory and calmodulin-binding sites. However, the exact positions of these sites are debated. A peptide containing the cyclic AMP-dependent protein kinase phosphorylation site and strong calmodulin-binding affinity has been isolated by Lukas *et al.* (1986). Its sequence correlates with residues 797–816 of the MLCK sequence (Olson *et al.*, 1990). Kemp *et al.* (1987) originally suggested that the calmodulin-binding region of gizzard MLCK acts as a pseudosubstrate inhibitor, on the basis of a similarity in the number and arrangement of basic residues in the MLCK molecule and LC_{20}. For skeletal muscle MLCK, it has been proposed that an inhibitory region exists between the catalytic site and the calmodulin-binding site (Edelman *et al.*, 1985). For gizzard MLCK it was shown subsequently that the regulatory domain is composed of two regions: an inhibitory (pseudosubstrate) region and a calmodulin-binding region (Pearson *et al.*, 1988; Ikebe *et al.*, 1987, 1989; Ikebe, 1990). It was demonstrated that the proteolysis of the kinase yielded a 64 kDa inactive fragment, which was converted to a 61 kDa active unregulated kinase by further proteolysis (Ikebe *et al.*, 1987). This suggests that the inactive 64 kDa fragment contains an inhibitory region which is composed of a relatively short amino acid sequence. Synthetic peptides based on parts of the calmodulin-binding site were also found to be potent inhibitors of the active calmodulin-independent MLCK fragment (Ikebe, 1990). These findings suggested that generation of the Ca^{2+}/calmodulin-independent active fragment occurred as a result of the cleavage on the *N*-terminal side of inhibitory region. The location of the inhibitory region was suggested by two groups. Pearson *et al.* (1988) suggested that the production of the inactive fragment resulted from cleavage at Arg 808, and that further digestion to produce the calmodulin-independent form removed the sequence from Ser-787 to Arg-808. Ikebe *et al.* (1989) reported that the *C*-terminal amino acids of the inactive 64 kDa fragment and the 61 kDa calmodulin-independent active fragment are Lys-793 or Arg-797 and Lys-776 respectively, and the inhibitory region was identified as amino acids 776–793.

To investigate in more detail the nature and functions of the regulatory region in MLCK and the mechanism by which calmodulin activates MLCK, we generated 22 clones producing monoclonal antibodies (mAbs) against smooth muscle MLCK. One of these antibodies, LKH-18, was found to interact with the calmodulin-binding site and significantly activate kinase activity. Using the antibody as a probe, we studied the regulatory mechanism of smooth muscle MLCK.

EXPERIMENTAL

Materials

Protein purification. The following proteins were isolated by

---

Abbreviations used: MLCK, myosin light chain kinase; LC_{20}, the 20 kDa light chain of myosin; mAb, monoclonal antibody; IC_{50}, concn. causing 50 % inhibition of binding.

* To whom correspondence should be addressed.

the methods in the respective references: MLCK (Walsh *et al.*, 1983) and myosin (Ikebe & Hartshorne, 1985*a*) from frozen turkey gizzard; LC_{20} from gizzard myosin (Hathaway & Haeberle, 1983); F-actin from rabbit skeletal muscle (Driska & Hartshorne, 1975); and calmodulin from bull testes (Walsh *et al.*, 1983). The preparation of 66 kDa, 64 kDa and 61 kDa fragments of MLCK was carried out as described previously (Ikebe *et al.*, 1987, 1989).

Preparation of calmodulin-binding peptide. MLCK peptides were synthesized as the *C*-terminal amide form by the Merrifield solid-phase procedure, as described by Kemp *et al.* (1987).

Production of mAbs

Purified MLCK (100 μg) mixed with an equal volume (100 μl) of complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO, U.S.A.) was injected subcutaneously twice, with a 1 week interval between, into female BALB/c mice (5–6 weeks old). After the second injection, one intraperitoneal booster injection of 100 μg of MLCK alone was given. This final injection could be administered up to 4 months after the initial injection.

The spleen was removed 4 days after the last immunization and used for hybridization. Spleen cells were fused to the myeloma cell line SP2/0 Ag14 (Schulman *et al.*, 1978) using the standard method (Oi & Herzenberg, 1980) with modifications as described by Araki *et al.* (1987) and Higashihara *et al.* (1989). Cloning of hybridoma was carried out as described previously (Higashihara *et al.*, 1989). Screening of antibody-producing cells was carried out using e.l.i.s.a. as described previously (Higashihara *et al.*, 1989). The antibody-producing hybridoma clone obtained was recloned twice by the dilution plating technique using BALB/c mouse splenocytes as the feeder layer (Higashihara *et al.*, 1989). An established hybridoma clone was cultured in RPMI medium containing 10% (v/v) fetal calf serum or injected intraperitoneally into BALB/c mice. The cultured supernatant and the ascitic fluid were used as the mAb source.

Electrophoresis and immunoblot analysis

The molecular masses of the antigens recognized by the mAbs were determined by enzyme immunostaining of the protein after blotting of peptides to a nitrocellulose membrane from an SDS/PAGE gel. Sample proteins in 10 mM-Tris/HCl (pH 6.8)/6% SDS/4% β-mercaptoethanol/25% glycerol were boiled for 3 min (Weber *et al.*, 1972). The proteins separated by gradient SDS/PAGE (7.5%–20% gels) (Laemmli, 1970) were transferred to nitrocellulose membrane sheets by the method of Towbin *et al.* (1976). The immunological reactivity of the transferred proteins with the mAb was assayed using a peroxidase-conjugated second antibody system (Higashihara *et al.*, 1989).

Purification of mAb and its Fab fragment

IgG class antibody was purified by ammonium sulphate precipitation followed by DEAE-Sephacel chromatography as previously described (Higashihara *et al.*, 1989). The Fab fragment of mAb (IgG_1) was purified as follows. The purified mAb (LKH-18) was digested by incubation with pre-activated papain (Higashihara *et al.*, 1989) (25:1, w/w) for 60 min at 37 °C in 30 mM-Tris/HCl (pH 7.5)/1 mM-$MgCl_2$/20 mM-dithiothreitol. After incubation, the reaction was stopped by the addition of iodoacetic acid (pH 7.5) to 10 mM. The reaction mixture was applied to a DEAE-Sephacel column (Sigma). The flow-through fractions containing Fab fragments were collected and dialysed exhaustively against 30 mM-Tris/HCl (pH 7.5)/1 mM-$MgCl_2$. SDS/PAGE (reducing) revealed that the purity was greater than 95% (data not shown).

Isotyping of monoclonal antibodies was determined by using a Screen/Isotyping Kit (Boehringer-Mannheim, Indianapolis, IN, U.S.A.) according to the manufacturer's protocol.

Activation of MLCK activity by LKH-18 and its Fab fragment

MLCK LC_{20} and LKH-18 (or its Fab fragment) were incubated simultaneously in the presence of EGTA at 25 °C for 30 min. After incubation, phosphorylation of LC_{20} was assayed as described by Walsh *et al.* (1983). The rate of phosphorylation was determined by the calculation of the initial phase of the reaction. An ATPase assay (Ikebe & Hartshorne, 1985*b*) was carried out as described previously after a 30 min incubation of myosin, F-actin, MLCK and LKH-18 (or its Fab fragment) in the presence of EGTA at 25 °C. Superprecipitation was monitored at 660 nm using a Perkin–Elmer Lambda 4A UV/VIS spectrophotometer. Conditions are given in the Figure legends.

Inhibition of MLCK mAb(LKH-18) binding by synthetic peptides

A purified LKH-18 solution (10 μg/ml; 25 μl) was mixed with equal amounts of various concentrations of synthetic peptide (serially diluted from an original concentration of 1 mM). The mixture was incubated at room temperature for 1 h in MLCK-coated wells of an e.l.i.s.a. plate. The binding of MLCK and LKH-18 was estimated by an e.l.i.s.a. method. The inhibition titre was expressed as the concentration of inhibitor which prevented 50% of MLCK/LKH-18 binding (IC_{50}) in the e.l.i.s.a. system.

Assay of binding of LKH-18 (Fab fragment) to MLCK in the presence of Ca^{2+}/calmodulin

MLCK, LKH-18 (Fab fragment) and 100 μl of calmodulin-conjugated Sepharose 4B (capacity 16 nmol of calmodulin-binding protein/ml) were incubated at room temperature for 30 min in 1 ml of solution containing 30 mM-Tris/HCl (pH 7.5), 1 mM-$MgCl_2$ and 0.1 mM-$CaCl_2$. After incubation, the mixture was centrifuged (13600 *g*; 5 min) and the same volume of supernatant or pellet was applied to SDS/PAGE. Conditions are given in the Figure legends.

RESULTS

Epitope of LKH-18

A total of 22 hybridomas which secreted antibodies (LKH-1-22) against MLCK were established. The isotypes of these antibodies were identified as IgG (κ light chain) (Table 1). The immunoreactivities of these antibodies against gizzard MLCK and its fragments were determined using e.l.i.s.a. and immunoblotting methods as shown in Table 1. The antibodies could be classified into several groups in terms of their reactivity against different MLCK fragments and were used as a functional probe of MLCK. Among the 22 antibodies, we found that one monoclonal antibody, designated LKH-18 (IgG_1), markedly activated native MLCK activity in the absence of Ca^{2+}/calmodulin. The epitope of LKH-18 was studied by employing tryptic peptide mapping. We have previously reported that the tryptic proteolysis of MLCK in the absence of Ca^{2+}/calmodulin initially yields a 64 kDa inactive peptide containing an inhibitory region, but not a calmodulin-binding region, and that this is further proteolysed to give a 61 kDa constitutively active peptide (Ikebe *et al.*, 1987). In the presence of Ca^{2+}/calmodulin, tryptic proteolysis produces a 66 kDa Ca^{2+}/calmodulin-dependent active peptide (Ikebe *et al.*, 1989). The immunoreactivity of LKH-18 against the 66 kDa, 64 kDa and 61 kDa tryptic peptides was examined by immunoblotting (Fig. 1). LKH-18 recognized only

Table 1. Characterization of mAbs against MLCK

The 64 kDa, 61 kDa and 23 kDa fragments are major fragments produced by trypsin digestion of MLCK in the absence of Ca^{2+}/calmodulin (Ikebe *et al.*, 1987); '130 kDa' is native MLCK. ND, not determined.

mAb	Subclass of Ig	Reactivity					
		Immunoblot				E.l.i.s.a.	
		130 kDa	64 kDa	61 kDa	23 kDa	130 kDa	64 kDa
LKH 1	IgG$_1$	ND	−	−	+	+	−
2	IgG$_1$	+	−	−	−	+	−
3	IgG$_1$	+	−	−	+	+	−
4	IgG$_{2a}$	+	−	−	−	+	−
5	IgG$_1$	ND	+	+	+	+	+
6	IgG$_1$	+	−	−	−	+	−
7	IgG$_{2b}$	ND	+	+	−	+	+
8	IgG$_1$	+	−	−	−	+	−
9	IgG$_1$	+	−	−	−	+	+
10	IgG$_{2b}$	+	+	+	−	+	+
11	IgG$_1$	ND	−	−	−	+	+
12	IgG$_1$	+	−	−	−	+	+
13	IgG$_1$	ND	−	−	−	+	+
14	IgG$_{2b}$	ND	+	+	−	+	+
15	IgG$_1$	ND	+	+	−	+	+
16	IgG$_1$	+	+	+	+	+	+
17	IgG$_1$	+	−	−	−	+	−
18	IgG$_1$	+	−	−	−	+	−
19	IgG$_{2a}$	+	−	−	−	+	−
20	IgG$_1$	+	−	−	−	+	−
21	IgG$_1$	ND	−	−	−	+	−
22	IgG$_1$	+	−	−	−	+	−

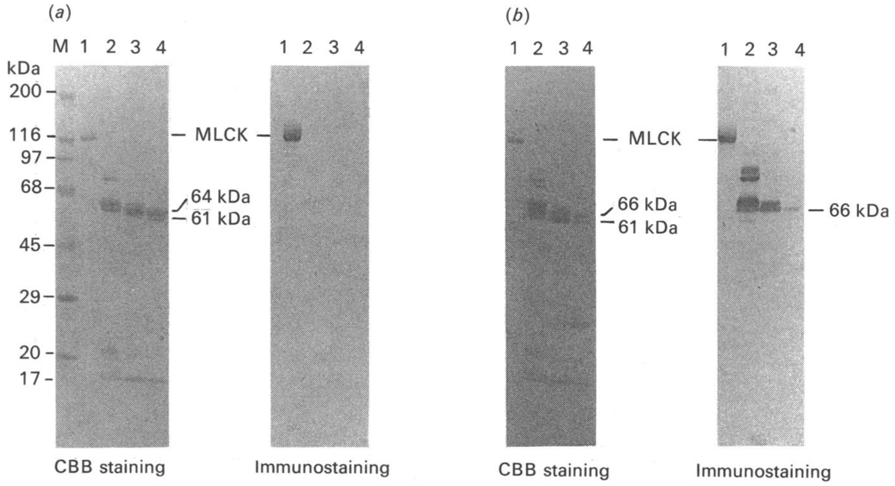

Fig. 1. Immunoblotting pattern of anti-MLCK monoclonal antibody (LKH-18)

(*a*) Coomassie Brilliant Blue (CBB) staining and immunostaining against a tryptic digest of MLCK in the absence of Ca^{2+}/calmodulin. Gizzard MLCK (0.7 mg/ml) was hydrolysed at 25 °C with trypsin (17.5 μg) in 30 mM-Tris/HCl (pH 7.5)/50 mM-KCl/1 mM-EGTA. The reaction was stopped by addition of soybean trypsin inhibitor (trypsin/inhibitor 2:3, w/w). A 10 μg portion of protein was applied to each lane of the SDS/PAGE gel. Key to lanes: M, molecular mass standards; 1, native MLCK; 2–4, MLCK after digestion for 30 s, 2 min and 20 min respectively. (*b*) CBB staining and immunostaining against a tryptic digest of MLCK in the presence of Ca^{2+}/calmodulin. Gizzard MLCK (0.7 mg/ml) was hydrolysed at 25 °C with trypsin (17.5 μg) in 30 mM-Tris/HCl (pH 7.5)/50 mM-KCl/calmodulin (0.11 mg/ml)/0.1 mM-CaCl$_2$. A 10 μg portion of protein was applied to each lane of the SDS/PAGE gel. Lanes are as in (*a*).

the 66 kDa (Fig. 1*b*) but not 64 kDa or 61 kDa fragments (Fig. 1*a*). Using isolated 66 kDa, 64 kDa and 61 kDa fragments prepared as described previously (Ikebe *et al.*, 1989), we confirmed that LKH-18 reacted only with the 66 kDa peptide (results not shown). We have previously shown that the *C*-terminal amino acids of the 66 kDa and 64 kDa peptides are Arg-825 and Lys-793 or Arg-797 respectively, and that the *N*-terminus of the 66 kDa peptide is longer by six amino acids (Ikebe *et al.*, 1989), corresponding to Lys-277–Lys-282 (Olson *et al.*, 1990). These results therefore suggest that the epitope of LKH-18 is either the amino acid sequence between residues 793 and 825 or that between Lys-277 and Lys-282. To differentiate between these possibilities, the immunological reactivity of LKH-18 against several synthetic peptide analogues of the calmodulin-binding

Table 2. Inhibition of MLCK–mAb (LKH-18) binding by synthetic peptides

The peptide sequences are as follows: [1] Leu-Ser-Lys-Asp-Arg-Met-Lys-Lys-Tyr-Met-Ala-Arg-Arg-Lys-Trp-Gln; [2] Ala-Arg-Arg-Lys-Trp-Gln-Lys-Thr-Gly-His-Ala-Val; [3] Ala-Arg-Arg-Lys-Trp-Gln-Lys-Thr-Gly-His-Ala-Val-Arg-Ala-Ile-Gly-Arg-Leu-Ser-Ser; [4] Arg-Ala-Ile-Gly-Arg-Leu-Ser-Ser.

Synthetic peptide	IC_{50} (μM)
MLCK-(786–801)[1]	> 500
MLCK-(796–807)[2]	> 500
MLCK-(796–815)[3]	0.31
MLCK-(808–815)[4]	0.50

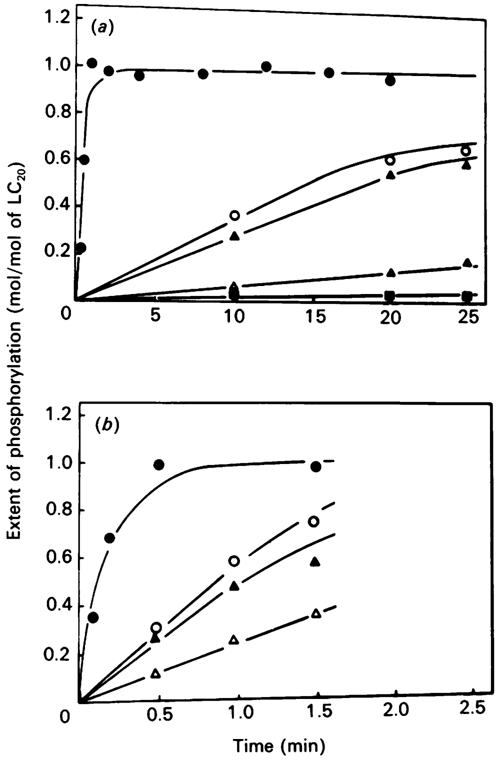

Fig. 2. Activation of MLCK activity by LKH-18 in the absence of Ca^{2+}/calmodulin

(*a*) Intact LKH-18. LKH-18 concentrations were 400 μg/ml (○), 100 μg/ml (▲), 10 μg/ml (△) and 0 (■). ●, $CaCl_2$ (0.1 mM) and calmodulin (80 nM) were used instead of EGTA in the absence of LKH-18. (*b*) Fab fragment of LKH-18. Fab concentrations were 360 μg/ml (○), 180 μg/ml (▲) and 90 μg/ml (△). ●, $CaCl_2$ (0.1 mM) and calmodulin (80 nM) in the absence of Fab fragment were used instead of EGTA. In both (*a*) and (*b*) assays were performed in 1 mM-$MgCl_2$/30 mM-KCl/30 mM-Tris/HCl (pH 7.5)/ 1 mM-EGTA/MLCK (10 μg/ml)/LC_{20} (0.1 mg/ml)/200 μM-ATP. Before MLCK assay, MLCK and LKH-18 (or its Fab fragment) were incubated for 30 min at 25 °C. The kinase activity was determined as described in the Experimental section.

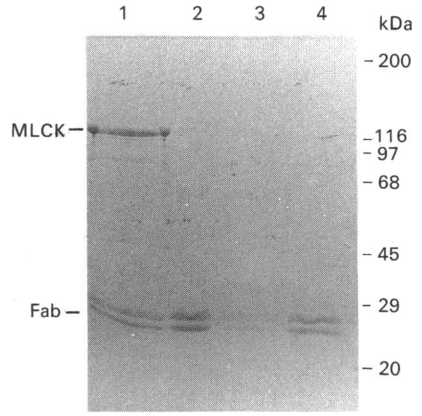

Fig. 3. Ternary complex formation between MLCK, calmodulin and the Fab fragment of LKH-18

Key to lanes: alternating pellet (ppt) and supernatants (sup) for the following additional conditions: 1 (ppt) and 2 (sup): MLCK, 33 μg/ml, Fab of LKH-18, 100 μg/ml; 3 (ppt) and 4 (sup): Fab of LKH-18, 100 μg/ml. The same volume (30 μl) of sample was applied to each lane of the SDS/PAGE gel.

region and inhibitory region was examined. The binding activity of three synthetic peptides to LKH-18 was estimated by measuring the competition between the peptides and MLCK for antibody binding (see the Experimental section). The peptides MLCK-(796–815) and MLCK-(808–815) showed competition with MLCK and inhibited the binding of LKH-18 to MLCK by 50% at concentrations of 312.5 nM and 500 nM respectively, whereas two other peptides [MLCK-(786–801) and MLCK-(796-807)] did not inhibit binding, even at a concentration of 500 μM (Table 2). Therefore we concluded that the LKH-18-binding site is the sequence between Arg-808 and Ser-815.

Activation of MLCK by LKH-18

The effects of LKH-18 on MLCK activity in the absence of Ca^{2+}/calmodulin were studied. As shown in Fig. 2(*a*), LKH-18 activated MLCK in the absence of Ca^{2+}/calmodulin, although the rate of phosphorylation of LC_{20} (MLCK activity) was less than 5% of the maximal rate of phosphorylation of LC_{20} in the presence of Ca^{2+}/calmodulin. Maximum activation was obtained at 0.4 mg of LKH-18/ml, and higher antibody concentrations (up to 1 mg/ml) did not further stimulate MLCK activity (results not shown). The activation of MLCK by the Fab fragment of LKH-18 (0.36 mg/ml) in the absence of Ca^{2+}/calmodulin was much more pronounced. The rate of phosphorylation of LC_{20} by MLCK in the presence of EGTA was up to approx. 30% of the rate observed in the presence of Ca^{2+}/calmodulin by the addition of the Fab fragment (Fig. 2*b*). Similar activation by LKH-18 was also observed when the 66 kDa calmodulin-dependent active fragment was used; however, the antibody did not activate the 64 kDa inactive fragment of MLCK (results not shown). It should be noted that the activation of MLCK activity by antibody in the presence of EGTA was only observed using LKH-18, and no other antibodies at similar concentrations affected MLCK activity. Since MLCK can express the Ca^{2+}/calmodulin-independent activity on proteolysis (Ikebe *et al.*, 1987), we examined whether or not the production of Ca^{2+}/calmodulin-independent activity induced by LKH-18 was due to the proteolysis of MLCK. However, no proteolysis of MLCK was observed on an SDS/PAGE gel after the incubation of MLCK with LKH-18 (or its Fab fragment) for 1 h at 37 °C (results not shown).

As shown in Fig. 3, the Fab fragment of LKH-18 did not inhibit calmodulin–MLCK binding, and it was found that both the Fab fragment and calmodulin bound simultaneously to MLCK (in Fig. 3, calmodulin cannot be seen in the gel since calmodulin is covalently attached to the resin). It was also found that the Fab fragment up to a concentration of 400 μg/ml did not inhibit the kinase activity in the presence of Ca^{2+}/calmodulin (results not shown). Since calmodulin can bind to MLCK even in the presence of LKH-18, this result suggests that the binding

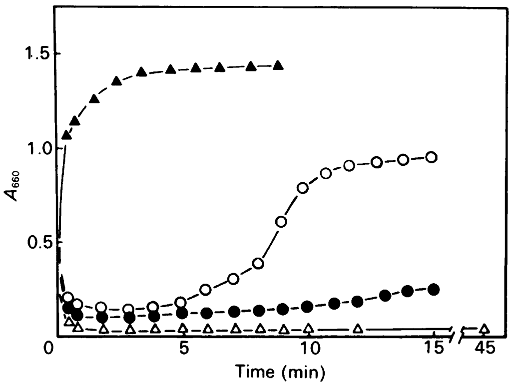

Fig. 4. Superprecipitation of actomyosin in the presence of LKH-18 and its Fab fragment

Conditions: 30 mM-Tris/HCl (pH 7.5), 50 mM-KCl, 1 mg of myosin/ml, 1 mg of F-actin/ml, 8 mM-$MgCl_2$, 1 mM-EGTA, 50 μg of MLCK/ml and 500 μM-ATP, plus 400 μg of Fab fragment/ml (○), 400 μg of LKH-18/ml (●), no antibody (△) or 0.1 mM-$CaCl_2$/calmodulin (10 μg/ml) instead of EGTA (▲). Before the superprecipitation assay, myosin, F-actin, MLCK and LKH-18 (or its Fab fragment) were incubated for 30 min at 25 °C. The time course of the superprecipitation was started by addition of ATP at room temperature, and the turbidity was monitored at 660 nm.

of LKH-18 changes the MLCK conformation to a partially active form and that the subsequent binding of calmodulin further changes the conformation to the fully active form.

It is well known that the smooth muscle contractile apparatus is activated by the phosphorylation of myosin by MLCK. To test whether or not LKH-18 can activate the smooth muscle contractile apparatus, the effects of LKH-18 on the actomyosin ATPase activity and the superprecipitation of actomyosin were examined. Fig. 4 shows the time course of superprecipitation of smooth muscle actomyosin. Even in the absence of Ca^{2+}/calmodulin, the superprecipitation of actomyosin was enhanced by LKH-18, and the activation was more pronounced when the Fab fragment was used. Similar activation of the ATPase activity of actomyosin was also observed (results not shown).

DISCUSSION

The structure–function relationship of MLCK has been studied using monoclonal antibodies. Nunnally *et al.* (1987) reported that a monoclonal antibody which bound to the calmodulin-binding site of rabbit skeletal muscle MLCK inhibited the kinase activity competitively with respect to calmodulin. However, this antibody did not exhibit cross-reactivity to other calmodulin-binding proteins, including smooth muscle MLCK, suggesting that the calmodulin-binding domains of different calmodulin-regulated proteins have distinct structures. For smooth muscle MLCK, Hagiwara *et al.* (1989) generated several monoclonal antibodies and reported that none of these antibodies cross-reacted with skeletal muscle MLCK. They also showed that one of these antibodies inhibited kinase activity competitively with respect to ATP. In the present study, 22 monoclonal antibodies against smooth muscle MLCK were generated and one of these (designated LKH-18) recognized the MLCK sequence comprising amino acids 808–815, which is thought to be a part of the calmodulin-binding site (Lukas *et al.*, 1986; Ikebe *et al.*, 1989; see Fig. 5).

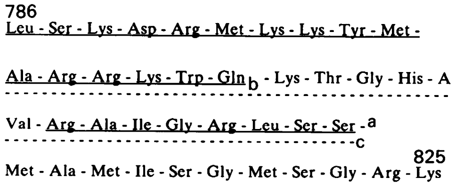

Fig. 5. Amino acid sequence of the regulatory site of gizzard smooth muscle MLCK

The end of the LKH-18-binding site (underlined) is indicated by a; b indicates the end of the peptide sequence that exhibits the potent inhibitory activity against Ca^{2+}/calmodulin-independent MLCK (Ikebe *et al.*, 1987) (underlined), and c is the end of the peptide sequence that exhibits the strong affinity to calmodulin (Lukas *et al.*, 1986) (broken line).

Activation of MLCK by calmodulin binding may arise from changes in the conformation of the kinase to generate an active conformation. In this regard, it has been suggested (Kemp *et al.*, 1987; Ikebe *et al.*, 1987, 1989; Pearson *et al.*, 1988) that smooth muscle MLCK contains the intramolecular inhibitor sequence which lies right next to the *N*-terminal end of the calmodulin-binding site, and that the activation of MLCK by calmodulin is achieved by abolishing the interaction between the inhibitory region and the catalytic region which is induced by the binding of calmodulin to the calmodulin-binding site.

The present work indicates that LKH-18 is capable of activating MLCK in the absence of Ca^{2+} and calmodulin and, moreover, that this effect is more pronounced with the Fab fragment. The difference in the extent of activation may arise from the difference in molecular size between the native antibody and its Fab fragment (i.e. steric effects), although other possibilities cannot be ruled out. It is conceivable from the above discussion that the binding of the antibody to MLCK may also interfere with the interaction between the putative inhibitory region and the catalytic site. Support for this view comes from the finding that LKH-18 binds to a peptide (residues 808–815 of MLCK) implicated to be important for calmodulin binding (Lukas *et al.*, 1986; Bagchi *et al.*, 1989).

Bagchi *et al.* (1989) reported that the removal of amino acid residues 811–815 of smooth muscle MLCK abolishes calmodulin binding to MLCK and results in an inactive kinase, indicating that this region (residues 811–815) is important for calmodulin binding. It has also been reported that an inhibitory region exists in the amino acid sequence adjacent to the *N*-terminal side of the calmodulin-binding region (Pearson *et al.*, 1988; Ikebe *et al.*, 1989). This raised the idea that, in native molecules, the binding of calmodulin at the calmodulin-binding site reverses the effect of the inhibitory region. The possible location of the inhibitory region was reported by Pearson *et al.* (1988) to be at the *C*-terminus of the inactive MLCK fragment (produced by tryptic proteolysis), which is Arg-808, whereas Ikebe *et al.* (1989) indicated that it was either Lys-793 or Arg-797. Since the regulatory region of MLCK (calmodulin-binding region and inhibitory region) contains a number of basic amino acids which are susceptible to tryptic proteolysis, the difference in the *C*-terminus of the inactive fragment of MLCK may arise from a difference in the extent of MLCK digestion by trypsin to produce the 64 kDa inactive fragment. If this is accepted, then it is possible that proteolysis at Arg-808 abolishes calmodulin binding and that the inhibitory activity is predominantly derived from the amino acid residues on the *N*-terminal side of Lys-793. Binding of LKH-18 to residues 808–815 probably performs a similar function to calmodulin by abolishing the effects of the inhibitory region in MLCK. We have previously shown (Ikebe *et al.*, 1987; Ikebe, 1990) that a 61 kDa Ca^{2+}/calmodulin-independent active fragment is inhibited by a synthetic peptide analogue of the inhibitory region of MLCK, suggesting a direct interaction of the inhibitory region and the kinase active site which results in inactivation of the enzyme. Therefore we suggest that the binding of LKH-18 induces the dissociation between the active site and the inhibitory region.

Although activation of MLCK by LKH-18 is less than with Ca^{2+}/calmodulin, it is sufficient to activate the contractile machinery of the cell. In fact, the superprecipitation of smooth muscle actomyosin and actomyosin ATPase activity were significantly activated by LKH-18 even in the absence of calmodulin (Fig. 4). In future studies LKH-18 could be used to investigate the role of MLCK in cell function, since this antibody specifically binds to MLCK and markedly activates the activity in the absence of Ca^{2+}/calmodulin.

We thank Dr. J. Morita and Dr. G. Scott-Woo for their technical support and helpful discussion. We also thank Miss K. Toll for secretarial work. This work was supported by National Institutes of Health grant no. AR38888. M.I. is an Established Investigator of the American Heart Association and is selected as a Syntex Scholar.

REFERENCES

Adelstein, R. S. & Eisenberg, E. (1980) Annu. Rev. Biochem. **49**, 921–956

Araki, Y., Kurata, S., Oikawa, T., Yamashita, T., Hiroi, M., Naiki, M. & Sendo, F. (1987) J. Reprod. Immunol. **11**, 193–208

Bagchi, I. C., Kemp, B. E. & Means, A. R. (1989) J. Biol. Chem. **264**, 15843–15849

Driska, S. & Hartshorne, D. J. (1975) Arch. Biochem. Biophys. **167**, 203–212

Edelman, A. M., Takio, K., Blumenthal, D. K., Hansen, R. S., Walsh, K. A., Titani, K. & Krebs, E. G. (1985) J. Biol. Chem. **260**, 11275–11285

Hagiwara, M., Tokumitsu, H., Onoda, K., Tanaka, T., Ito, M., Kato, N. & Hidaka, H. (1989) J. Biochem. (Tokyo) **106**, 71–75

Hartshorne, D. J. (1987) in Physiology of the Gastrointestinal Tract, 2nd edn. (Johnson, L. R., ed.), vol. 1, pp. 423–482, Raven Press, New York

Hathaway, D. R. & Haeberle, J. R. (1983) Anal. Biochem. **135**, 37–43

Higashihara, M., Young-Frado, L. L., Craig, R. & Ikebe, M. (1989) J. Biol. Chem. **264**, 5218–5225

Ikebe, M. (1990) Biochem. Biophys. Res. Commun. **168**, 714–720

Ikebe, M. & Hartshorne, D. J. (1985*a*) J. Biol. Chem. **260**, 13146–13153

Ikebe, M. & Hartshorne, D. J. (1985*b*) Biochemistry **24**, 2380–2387

Ikebe, M., Stepinska, M., Kemp, B. E., Means, A. R. & Hartshorne, D. J. (1987) J. Biol. Chem. **262**, 13828–13834

Ikebe, M., Maruta, S. & Reardon, S. (1989) J. Biol. Chem. **264**, 6967–6971

Kemp, B. E., Pearson, R. B., Guerriero, V., Jr., Bagchi, I. C. & Means, A. R. (1987) J. Biol. Chem. **263**, 2542–2548

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Lukas, T. J., Burgass, W. H., Prendergast, F. G., Lau, W. & Watterson, D. M. (1986) Biochemistry **25**, 1458–1464

Nunnally, M. H., Blumenthal, D. K., Krebs, E. G. & Stull, J. T. (1987) Biochemistry **26**, 5885–5890

Oi, V. T. & Herzenberg, L. A. (1980) in Selected Methods in Cellular Immunology (Mishell, B. B. & Shiigi, S. M., eds.), pp. 351–372, W. H. Freeman Co., San Francisco

Olson, N. J., Pearson, R. J., Needleman, D. S., Hurwitz, M. Y., Kemp, B. E. & Means, A. R. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 2284–2288

Pearson, R. B., Wettenhall, R. E. H., Means, A. R., Hartshorne, D. J. & Kemp, B. E. (1988) Science **241**, 970–973

Roush, C. L., Kennelly, P. J., Glaccum, M. B., Helfman, D. M., Scott, J. D. & Krebs, E. G. (1988) J. Biol. Chem. **263**, 10510–10516

Schulman, M., Wilde, C. D. & Kohler, G. (1978) Nature (London) **276**, 269–270

Takio, K., Blumenthal, D. K., Walsh, K. A., Titani, K. & Krebs, E. G. (1986) Biochemistry **25**, 8049–8057

Towbin, H., Staehelin, T. & Gordon, J. (1976) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354

Walsh, M. P., Hinkins, S., Dabrowska, R. & Hartshorne, D. J. (1983) Methods Enzymol. **99**, 279–288

Weber, K., Pringle, J. R. & Osborn, M. (1972) Methods Enzymol. **26C**, 3–27

Received 3 December 1990; accepted 13 December 1990