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Myosin light chain kinase stimulates smooth muscle myosin ATPase activity by binding to the myosin heads without phosphorylating the myosin light chain

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Abstract

Myosin light chain kinase (MLCK) is a multifunctional regulatory protein of smooth muscle contraction [IUBMB Life 51 (2001) 337, for review]. The well-established mode for its regulation is to phosphorylate the 20 kDa myosin light chain (MLC 20) to activate myosin ATPase activity. MLCK exhibits myosin-binding activity in addition to this kinase activity. The myosin-binding activity also stimulates myosin ATPase activity without phosphorylating MLC 20 [Proc. Natl. Acad. Sci. USA 96 (1999) 6666]. We engineered an MLCK fragment containing the myosin-binding domain but devoid of a catalytic domain to explore how myosin is stimulated by this non-kinase pathway. The recombinant fragment thus obtained stimulated myosin ATPase activity by $V_{max} = 5.53 \pm 0.63$-fold with $K_m = 4.22 \pm 0.58\,\mu M$ ($n = 4$). Similar stimulation figures were obtained by measuring the ATPase activity of HMM and S1. Binding of the fragment to both HMM and S1 was also verified, indicating that the fragment exerts stimulation through the myosin heads. Since S1 is in an active form regardless of the phosphorylated state of MLC 20, we conclude that the non-kinase stimulation is independent of the phosphorylating mode for activation of myosin.
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Myosin light chain kinase (MLCK) is a regulatory protein for smooth muscle contraction ([1], for reviews). The intracellular concentration of Ca^{2+} is elevated upon stimulation by agonists, and Ca^{2+} forms a complex with calmodulin. The complex activates MLCK by binding of the regulatory domain of MLCK to a phosphorylate 20 kDa myosin light chain (MLC 20). The myosin thus phosphorylated is in an active form to interact with actin to induce contraction ([2], for review). The catalytic domain responsible for the phosphorylation is located at the central part of the MLCK molecule, and the domain that regulates the kinase activity neighbours the C-terminal of the catalytic domain [3, Fig. 1]. The C-terminal region of the MLCK molecule is know as the telokin domain and is expressed as an independent protein in a few smooth muscle tissues [4]. The C-terminal of an MLCK molecule is recognized to be the myosin domain of MLCK since telokin binds to myosin [5,6].

The myosin molecule exhibits a configuration of two globular heads with a long tail, its active sites are localized in the heads, which can be isolated from the myosin molecule by proteolysis ([1], for review). The proteolytic products are heavy mero-myosin (HMM), with two heads but lacking the C-terminal 2/3 of the tail, and subfragment-1 (S1), containing a single head and no tail. The mode of phosphorylating MLC 20 is exerted through the heads, where the MLC 20's are located. In addition, it has been verified that assembly of myosin through the tail modifies the myosin ATPase activity [7].

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We mixed MLCK with unphosphorylated myosin in the absence of Ca^{2+} and calmodulin to explore the regulatory role of the myosin-binding activity of MLCK [8]. Although MLCK did not phosphorylate the myosin, the ATPase activity increased with an increase in the MLCK concentration, suggesting non-kinase stimulation by MLCK. We then obtained various MLCK fragments devoid of the catalytic domain as recombinant proteins. Stimulation of the myosin ATPase activity was undetectable with an MLCK fragment consisting of only a telokin domain. However, stimulation by the fragment became detectable when the recombinant fragment was further extended in the N-terminal direction of MLCK. These data enabled us to identify the domain of MLCK responsible for non-kinase stimulation ([9], for review). This paper is an advanced study that describes which part of the myosin molecule accepts the stimulation.

Materials and methods

Plasmid construction. C-terminal bovine smooth muscle MLCK containing residues from 860 to 1176 [10] was prepared using polymerase chain reaction (PCR) with an expanded high-fidelity system (Roche Molecular Biochemicals) using primers of 5′-CGCGGAACCA GGATCAAGCTCATCGACTTC-3′ and 5′-CGGGATCCTCACTCT TCCTCCTCCTCTTCTCC-3′. The amino acid at 860 (Thr) was located at the central part of the kinase domain, and that at 1176 (Glu) was at the C terminal (Fig. 1). The PCR reaction was performed for 35 cycles of denaturing at 94 °C for 1 min, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The last extension cycle was at 72 °C for 10 min. This PCR product, referred to as the 860/1176 fragment, was digested with *Bam*HI and *Eco*RI and subcloned into pGEX-6P-1 furnished with a glutathione-*S*-transferase (GST) tag (Amersham Pharmacia Biotech).

Protein expression. Recombinant plasmid of the 860/1176 fragment fused with the GST described above was transfected to BL21(DE3) cells. The cells were incubated with shaking at 37 °C until the optical density at 600 nm became 0.5. Protein induction was achieved by incubating the cells for an additional 6 h at 28 °C in the presence of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. The cells were harvested by centrifugation at 6000*g* for 10 min and resuspended in 20 ml lysis buffer of 20 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, and 5 μg/ml leupeptin. The cells were then disrupted by sonication at 4 °C. This cell lysate was centrifuged at 40,000*g* for 30 min at 4 °C. The expressed GST fusion protein, referred to as the 860/1176 fragment-(GST), was purified from the lysate by affinity column chromatography with glutathione–Sepharose 4B according to the manufacturer's instructions (Amersham Pharmacia Biotech), followed by dialysis against a dialysis buffer of 50 mM NaCl, 20 mM Tris–HCl (pH 7.5), and 1 mM dithiothreitol (DTT). The dialysate was stored at −80 °C. The GST tag was removed by proteolysis of the 860/1176 fragment-(GST) with PreScission Protease (Amersham Pharmacia Biotech), followed by subjecting the proteolytic product to affinity column chromatography with glutathione–Sepharose 4B. The resultant 860/1176 fragment was stored at −80 °C after dialysis against the dialysis buffer. The effects of the 860/1176 fragment-(GST) are not altered regardless of whether GST was removed (Table 1), as will be described in the text. Therefore, reference to the "860/1176 fragment" or "expressed fragment" in this paper includes the 860/1176 fragments both with and without GST.

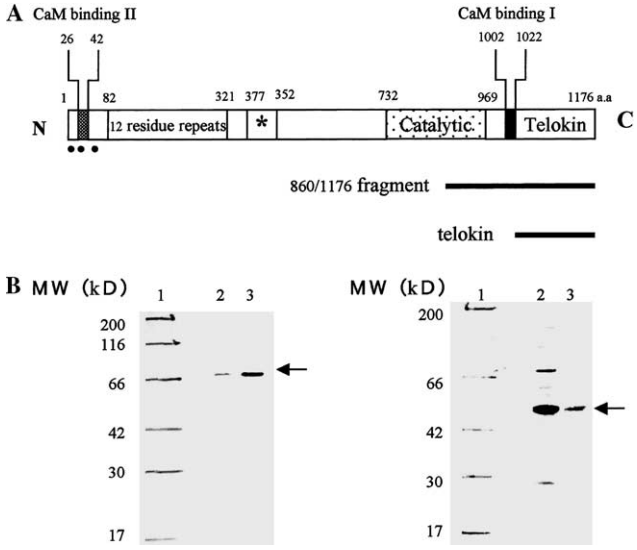

Fig. 1. Location of the recombinant fragments in the MLCK molecule. The number corresponds to the amino acid number of the primary structure on translation of the full-length cDNA of bovine stomach MLCK [11]. (A) Domain structure of MLCK. The location of the recombinant proteins used in this study, i.e., the 860/1176 fragment and telokin, are indicated by bars. CaM binding I is calmodulin-binding site for regulating kinase activity of MLCK [5]; CaM binding II is the calmodulin-binding site for regulating actin-binding activity of MLCK [20]; the dots (●) indicate actin-binding, DFRXXL motifs [28]; the asterisk (*) indicates an additional actin-binding site that is not regulated by the regulatory II domain [20,29]. (B) SDS–PAGE of the 860/1176 fragment. Left, the 860/1176 fragment purified as a fusion with GST (lanes 2 and 3) was electrophoresed with molecule weight markers (lane 1). Right, the fusion protein was digested by the PreScission Protease, and the 860/1176 fragment was purified from the digest. The purified fragment (lane 3) was electrophoresed with molecule weight markers (lane 1) and the digest (lane 2).

Table 1
Effect of MLCK and the 860/1176 fragments with and without GST on the actin-activated ATPase activity

	V_{max} (-fold)	K_m (μM)
MLCK	6.52 ± 0.74 (3)	4.16 ± 0.59 (3)
Recombinant fragment with GST	5.53 ± 0.63 (4)	4.22 ± 0.58 (4)
Recombinant fragment without GST	6.56 ± 0.63 (3)	3.18 ± 0.48 (3)

The numbers in parentheses refer to the number of experiments.

Protein preparation. Myosin was extracted from chicken gizzards and purified as described [11]. Actin was purified from the acetone powder of chicken skeletal muscle using the method of Spudich and Watt [12] with slight modifications [13] and was used as actin after polymerization. HMM was prepared by digesting myosin with chymotrypsin as described elsewhere [14]. HMM was also obtained as a recombinant protein as described elsewhere [15]. In short, Sf-9 cells were infected by baculovirus constructs of gizzard HMM heavy chain and the two light chains of gizzard myosin. The expressed HMM was precipitated by forming a complex with actin. The HMM was dissociated from the complex in the presence of ATP and was then purified with ion-exchange column chromatography by Mono Q HR

(Amersham Bioscience) followed by gel filtration column chromatography by Superose 6HR (Amersham Pharmacia Biotech). S1 was produced by digesting myosin with V8 protease [16]. The digestion was terminated by 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, immediately after which the digest was subjected to Superose 6HR column chromatography. MLCK was purified from chicken gizzard smooth muscle by the method of Adelstein and Klee [17] with slight modifications [18]. Telokin was obtained as a recombinant protein as described elsewhere [8].

ATPase assay. The Mg-ATPase activity and actin-activated ATPase activity of myosin in the assay buffer of 60 mM KCl, 5 mM $MgCl_2$, 0.2 mM EGTA, 1 mM dithiothreitol (DTT), 20 mM Tris–HCl (pH 7.5), and 0.5 mM ATP were assayed at 25 °C for 10 min by the colorimetric method using malachite green, as previously described [19]. Mg-ATPase activities of HMM and S1 were determined in the assay buffer by the same method. The extent of stimulation was expressed relative to the ATPase activity in the absence of the expressed fragments [8,20,21]. V_{max} and K_m were determined by double-reciprocal plots. Myosin, HMM, and S1 were used for the activity without phosphorylating their light chains, unless otherwise specified.

Interaction of the expressed fragments and telokin with HMM and S1. About 10 mg of HMM produced by proteolysis was conjugated to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's instructions. This HMM–Sepharose was then packed in a small column. An 80 μg aliquot of the expressed fragment was applied to the affinity column equilibrated with a low-salt buffer of 60 mM KCl, 4.5 mM $MgCl_2$, 0.2 mM EGTA, 1 mM DTT, and 20 mM Tris–HCl (pH 7.5). The column was washed with the low-salt buffer. We confirmed that no protein was eluted and applied a high-salt buffer of 500 mM KCl and 100 mM Tris–HCl (pH 7.5) to the column to elute the bound expressed fragment. The protein concentrations of the eluates were determined as described below.

The interaction was also confirmed by the following centrifugation method. HMM–Sepharose was suspended in 2 ml of low-salt buffer. Aliquots of 200 μl were removed from the suspension to four Eppendorf tubes, to which 11 μg of the expressed fragment was added. After incubating at room temperature for 30 min, the tubes were briefly centrifuged. The protein concentrations of the supernatant were then determined. Control experiments were conducted at the same time with the four tubes containing 200 μl of the low-salt buffer mixed with 11 μg of the expressed fragment. The differences in the protein amounts of the supernatant was considered to be those of the expressed fragment bound to HMM. The same experiment was conducted with telokin.

The third method adopted for the binding interaction between HMM and the expressed fragment was measurement of the surface plasmon resonance (SPR) with a BIACORE apparatus (Biacore). HMM (about 8 ng) was immobilized in the sensor chip according to the manufacturer's protocol. Various concentrations of the expressed fragment dissolved in the low-salt buffer were applied to the chip. The SPR, as expressed by the resonance unit (RU), was then recorded at 25 °C.

The binding interaction between S1 and the expressed fragment was examined in the low-salt buffer with the sensor chip conjugating 9.6 ng S1 by the BIACORE method, as described for HMM. Telokin was bound to HMM and S1 in the low-salt buffer by the BIACORE method described for the recombinant fragment.

Other methods. Protein concentrations were determined by Protein Assay (Bio-Rad) using bovine serum albumin as the standard [22]. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli [23] with slight modification [24] to confirm the purity ($\geqslant$95%) of the proteins. The SDS–PAGE of the 860/1176 fragments with and without GST is shown in Fig. 1 as an example of the purity. Phosphorylation of MLC 20 was monitored by urea–glycerol PAGE [27].

Result

Stimulatory effect of MLCK and 860/1176 fragments with and without GST on myosin ATPase activities

Actin-activated ATPase activity of myosin was determined in the presence of various concentrations of MLCK. MLCK stimulated the activity by $V_{max} = 6.52 \pm 0.74$-fold with $K_m = 4.16 \pm 0.59$ μM ($n = 3$), as illustrated in Table 1. Notably, stimulation was detected in the absence of calmodulin, an MLCK activator of kinase activity. Thus, the absence of MLC 20 phosphorylation was confirmed by urea–glycerol PAGE (data not shown). The extent of the stimulation was comparable with a figure published previously [8, Fig. 2], confirming non-kinase activation of myosin by MLCK. We obtained an 860/1176 fragment, a C-terminal MLCK fragment, as a fusion protein of GST. This 860/1176 fragment-(GST) stimulated ATPase activity by $V_{max} = 5.53 \pm 0.63$-fold with $K_m = 4.22 \pm 0.58$ μM ($n = 4$). Similar to the non-kinase effect of MLCK, stimulation by the 860/1176 fragment-(GST) was not attributable for MLC 20 to be phosphorylated, since the 860/1176 fragment-(GST) was designed to avoid the catalytic domain responsible for MLCK kinase activity.

The extent of stimulation was not altered when the GST tag was removed from the 860/1176 fragment-(GST), i.e., $V_{max} = 6.56 \pm 0.63$-fold with $K_m = 3.18 \pm 0.48$ μM ($n = 4$). Therefore, we combined the data of the 860/1176 fragment-(GST) with the 860/1176 fragment without GST in subsequent experiments.

Telokin, whether native or recombinant, scarcely alters ATPase activity [5,8]. We confirmed this by measuring actin-activated ATPase activity under the experimental conditions in Table 1 (data not shown).

The 860/1176 fragment also stimulated Mg-ATPase activity of myosin in the absence of actin (Table 2) by $V_{max} = 5.91 \pm 0.64$-fold with $K_m = 4.10 \pm 0.27$ μM ($n = 4$). The extent of stimulation was similar to that in the presence of actin, indicating that the action sites of the fragment were in myosin rather than in actin. This raise the question of whether the fragment exerts its stimulatory effect through myosin heads. We examined the effect through use of HMM to answer this question. The extent of Mg-ATPase activity stimulation by the fragment was $V_{max} = 5.14 \pm 0.30$-fold with $K_m = 3.54 \pm 0.42$ μM ($n = 4$) for HMM produced by proteolysis and $V_{max} = 5.42 \pm 0.89$-fold with $K_m = 4.56 \pm 1.80$ μM ($n = 3$) for recombinant HMM, as indicated in Table 2. The stimulatory effect on S1 ATPase activity by the fragment was similar in extent, i.e., $V_{max} = 6.27 \pm 1.82$-fold with $K_m = 3.06 \pm 0.98$ μM ($n = 4$). The data for HMM and S1 verify that the fragment site of action is in the myosin heads.

Table 2
Effect of 860/1176 fragment on ATPase activity of myosin in the absence of actin

	V_{max} (-fold)	K_m (μM)
Myosin	5.91 ± 0.64 (3)	4.10 ± 0.27 (3)
HMM (digestion)	5.14 ± 0.30 (4)	3.54 ± 0.42 (4)
HMM (expression)	5.42 ± 0.89 (3)	4.56 ± 1.81 (3)
S1	6.27 ± 1.82 (4)	3.06 ± 0.98 (4)

The numbers in parentheses refer to the number of experiments.

Interaction of a recombinant fragment with myosin heads

HMM produced by proteolysis was cross-liked to Sepharose 4B, constructing an affinity column of HMM. The fragment was then applied to the column. The bound fragment was eluted by increasing salt concentration after removal of the excess unbound fragment. Its binding to HMM was clarified, as indicated by the arrow in Fig. 2.

The binding interaction of the fragment to HMM was confirmed by the centrifugation method. Aliquots of Sepharose 4B cross-linking HMM were put into the Eppendorf tubes, and the fragments were mixed with HMM–Sepharose 4B. The fragment was allowed to form a complex with the HMM–Sepharose, and the complex was then precipitation by centrifugation. The protein in the supernatant after centrifugation was reduced to 0.53±0.63 μg ($n = 4$). The control supernatant in the tubes without HMM–Sepharose contained 10.68 ± 1.84 μg ($n = 4$). The difference of the protein in the supernatant confirms the binding of the fragment to HMM. The interaction was further confirmed by measuring the SPR with the BIACORE apparatus, as described in Materials and methods. The SPR signal, as the expressed RU from the sensor chip conjugating the HMM, increased with an increase in the fragment applied to the chip (Fig. 3A), indicating a binding interaction between them.

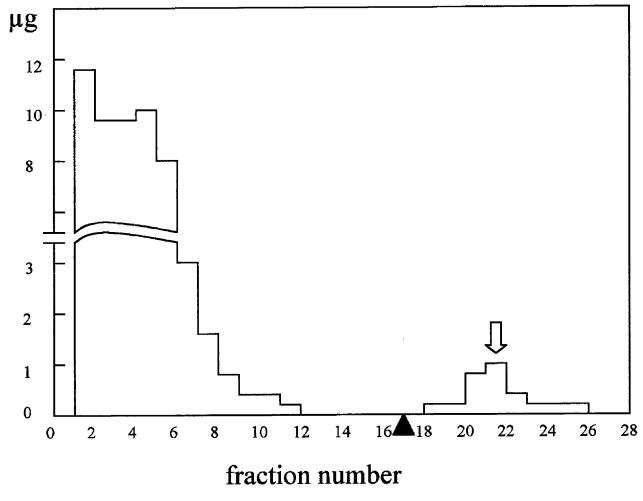

Fig. 2. Binding of the 860/1176 fragment to HMM as examined by affinity column chromatography. The 860/1176 fragment was applied to the column of Sepharose 4B conjugating HMM in the low-salt buffer. A high-salt buffer was applied (fraction 16) after removal of unbound protein (fractions 2–12), as indicated by a triangle, eluting the 860/1176 fragment bound to HMM (fractions 18–26), as indicated by an arrow.

HMM is composed of myosin heads associated with a short myosin tail. It is possible that the fragment interacts with the short tail region. Therefore, we produced S1, the interaction of which was detected by the BIACORE method. The SPR increased with an increase in the fragment applied to the S1-conjugating chip, as shown in Fig. 3B, which indicates a binding interaction between them.

The binding site of telokin in the myosin molecule is reportedly within the neck region, since telokin binds to HMM but not to S1 [5]. We measured the changes in SPR from the sensor chips conjugating HMM (Fig. 3A) and S1 (Fig. 3B) upon application of telokin. We confirmed its binding to HMM and its failure to bind to S1.

Telokin does not stimulate the myosin ATPase activity, as described above, although it binds to myosin. However, the recombinant fragment, which was constructed to extend its length toward the N-terminal beyond the telokin domain (Fig. 1), bound to the myosin head and effectively stimulated activity (Table 2). Therefore, we conclude that MLCK binds to the head of the myosin molecule at its extended region to stimulate non-kinase activation of myosin. The region should be

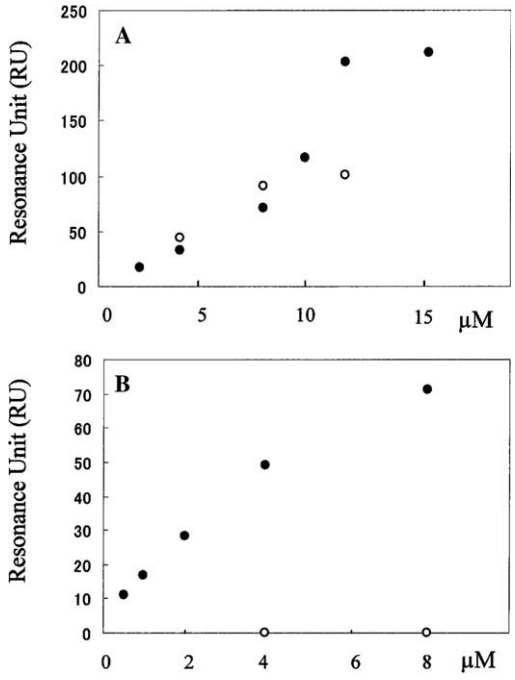

Fig. 3. Binding of the 860/1176 fragment and telokin to HMM and S1. (A) HMM was immobilized in the sensor chip. SPR, expressed as a resonance unit (RU), was recorded by applying the recombinant fragment (dots) or telokin (circles) to the chip in the low-salt buffer. (B) SPR was recorded in a way similar to HMM with the chip conjugating S1.

at Ala1002–Ser1031 of MLCK, which will be discussed later.

Discussion

In this study, we obtained the C-terminal half of MLCK as a recombinant protein, that exhibits myosin-binding activity devoid of myosin-phosphorylating activity. This recombinant fragment stimulated ATPase activities of not only myosin but also of HMM and S1. We took into account the binding ability of the recombinant 860/1176 fragment to HMM and S1 and concluded that stimulation was exerted by binding to myosin heads.

Does parent MLCK stimulate HMM and S1 in the same way as myosin? To answer this question, we measured the ATPase activity of HMM in the presence of various concentrations under conditions in which MLCK could not phosphorylate MLC 20, i.e., in the absence of Ca^{2+} and calmodulin (cirles in Fig. 4), and in the presence of Wortmannin, an inhibitor of MLCK (squares in Fig. 4) [30]. The ATPase activities increased in both cases with an increase in the MLCK concentration. Further, the extent was as great as that of the ATPase activity of HMM stimulated by the 860/1176 fragment (dots in Fig. 4). These data agree with the proposal that ATPase activity of HMM can be stimulated by non-kinase activation of myosin. Stimulation was observed when S1 was mixed with parent MLCK (crosses in Fig. 4) and with the 860/1176 fragment (open triangles in Fig. 4). We presume that S1 would be stimulated in a similar way.

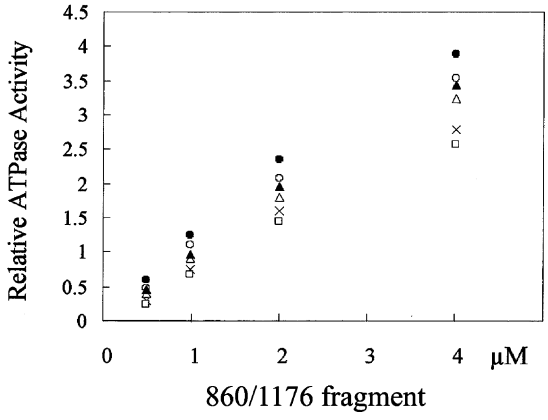

Fig. 4. Effect of MLCK and the 860/1176 fragment on the ATPase activities of HMM and S1. MLCK and the 860/1176 fragment were mixed with HMM and S1 in the absence of actin and their ATPase activities were examined. Typical data of the ATPase activities with myosin + the recombinant fragment and those with HMM + the recombinant fragment were incorporated into the figure for comparison with the data in Table 2. (○) Myosin + the recombinant fragment; (▲) HMM + the recombinant fragment; (△) HMM + MLCK; (□) HMM + MLCK + 1μM Wortmannin; (●) S1 + the recombinant fragment; (×) S1 + MLCK.

We previously proposed that the Ala1002–Ser1031 sequence, which corresponds to Ala796–Ser815 of chicken MLCK, is of primary importance in non-kinase activation of myosin [8]. We synthesized a peptide of this sequence and examined the effect of the peptide on the binding interaction between S1 and the 860–1176 fragment using the BIACORE method. We then measured the SPR from the sensor chip conjugating S1 upon application of 25 μM of the peptide, 4 μM of the recombinant fragment, and their mixture. We noted that the SPR profile of the recombinant fragment alone was greatly altered by coexistence with the peptide. We interpreted these data to indicate that the binding interaction was modified and that the binding of MLCK at Ala1002–Ser1031 to myosin heads may play an important role in non-kinase activation of myosin.

Another question is whether the non-kinase effect of MLCK on HMM is a result of proteolysis. This can be ruled out by the data indicating that the stimulatory effect of the fragment on recombinant HMM was as great as that of the 860/1176 fragment on HMM produced by proteolysis (Table 2).

It must be noted that S1 is in an active form regardless of whether the myosin light chain is phosphorylated [16,25]. The stimulation of ATPase activity of S1 by the 860/1176 fragment (Fig. 4) raises a question as to whether the fragment stimulates phosphorylated myosin. The answer is affirmative; the fragment stimulated ATPase activity by $V_{max} = 4.17$-fold with $K_m = 2.63$ μM in a preliminary study. This observation agrees with our reports that ATP-dependent movement of actin on a glass surface coated with phosphorylated myosin can be enhanced under limited conditions [26, Fig. 1A]). Thus, the fragment stimulated ATPase activities of both phosphorylated and unphosphorylated myosin. We speculate that stimulation of myosin by non-kinase activity of MLCK is regulatory in a way completely independent of the way MLC 20 is phosphorylated by the kinase activity of MLCK.

Acknowledgments

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