Autophosphorylation of Smooth Muscle Myosin Light Chain Kinase at Its Regulatory Domain†

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Received September 15, 1994; Revised Manuscript Received January 17, 1995⊗

ABSTRACT: Autophosphorylation of smooth muscle myosin light chain kinase was initially reported by Foyt et al. [Foyt, H. L., & Means, A. R. (1985) *J. Cyclic Nucleotide Protein Phosphorylation Res. 260,* 8978−8983], however, the effects of autophosphorylation on the kinase activity as well as the location of the sites have not been elucidated. Here we demonstrate that MLCK is autophosphorylated at three sites, Thr 803, Ser 815, and Ser 823, and this phosphorylation alters MLCK activity. Two phosphorylation sites are located in the regulatory domain of the kinase, the threonine site toward the autoinhibitory region and the serine site (Ser 815) in close proximity to the calmodulin anchoring site. The autophosphorylation was significantly inhibited by the binding of calmodulin. The autophosphorylation at Thr 803 is an intramolecular process, and the alignment of the basic amino acid residues nearby Thr 803 was highly homologous to the phosphorylation site of myosin light chain, suggesting that the regulatory site is in close proximity to the catalytic site in the three-dimensional structure. The phosphorylation at the threonine site activated the calmodulin-independent activity while the phosphorylation at the serine site inhibited the calmodulin-dependent activity due to a decrease in the affinity for calmodulin. This finding shows another example of the activation of calmodulin-dependent kinases by autophosphorylation at its autoinhibitory region and provides a new clue for understanding the calmodulin/MLCK signalling pathway.

Myosin light chain kinase (MLCK),[1] a family of calmodulin-dependent protein kinases, widely distributed in many vertebrate cells, catalyzes the phosphorylation of the 20 000 dalton light chain of myosin. In smooth muscle and nonmuscle cells, this enzyme plays an important role in activating actomyosin-based contractility of the cells (Hartshorne, 1987; Sellers & Adelstein, 1987) and thus regulates smooth muscle cell contractility and cytokinesis (Warrick & Spudich, 1987; Tan & Spudich, 1992).

The complete amino acid sequence of MLCK has been determined from skeletal muscle (Takio et al., 1986; Roush et al., 1988; Herring et al., 1990), smooth muscle (Olson et al., 1990; Gallagher et al., 1991), and fibroblast (Shoemakar et al., 1990). While the primary structures at the catalytic core are homologous to each other, skeletal muscle MLCK is quite distinct in its entire primary structure from the other two MLCKs. On the other hand, smooth muscle and nonmuscle MLCKs share the same nucleotide sequence, and nonmuscle MLCK cDNA contains the entire open reading frame of smooth muscle MLCK with an additional unique 0.8 kb segment at the 5′-side (Shoemakar et al., 1990). Smooth muscle and nonmuscle MLCKs contain a catalytic core at the center of the molecule and a regulatory region residing toward the C-terminus of the catalytic core (Olson et al., 1990; Gallagher et al., 1991). The C-terminal domain of the molecule which is not present in skeletal MLCK is characterized by its acidic property, and it is found that this portion of molecule is independently expressed in situ, termed telokin (Ito et al., 1989; Gallagher et al., 1991; Collinge et al., 1992; Yoshikai & Ikebe, 1992) although its physiological function is not known.

Of interest is the mechanism by which calmodulin activates the kinase activity. The calmodulin binding region of smooth muscle MLCK was first identified by isolating the calmodulin binding peptide (Lukas et al., 1986), and it was revealed recently that two hydrophobic residues (Try 800 and Leu 813) are essential for calmodulin binding (Ikura et al., 1992; Meador et al., 1992). Toward the N-terminus of the calmodulin binding site, there exists an autoinhibitory region which inhibits the kinase activity in the absence of calmodulin (Kemp et al., 1987; Ikebe et al., 1987). Although the detailed mechanism by which the binding of calmodulin activates the kinase is still obscure, it is assumed that the binding of calmodulin at the calmodulin binding site attenuates the interaction between the autoinhibitory region and the catalytic core.

Smooth muscle MLCK can be phosphorylated by various protein kinases such as cAMP-dependent protein kinase (Adelstein et al., 1978), protein kinase C (Ikebe et al., 1985; Nishikawa et al., 1985), and calmodulin-dependent kinase II (Ikebe & Reardon, 1990; Hashimoto et al., 1990), and the phosphorylation decreases the kinase activity by reducing the affinity for calmodulin. It has also been shown that smooth muscle MLCK can be autophosphorylated although the sites and the function of the autophosphorylation are not elucidated (Foyt & Means, 1985). For other calmodulin-dependent protein kinases, the function of autophosphory-

† This work was supported by an American Heart Association grant-in-aid and also by National Institutes of Health Grants HL 37117, HL 47530, and AR 41653.

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⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1995.

[1] Abbreviations: MLCK, myosin light chain kinase; DTT, dithiothreithol; NMR, nuclear magnetic resonance.

0006-2960/95/0434-5173$09.00/0

lation is best understood with calmodulin-dependent protein kinase II in which autophosphorylation occurs at the autoinhibitory domain and this converts the kinase to a partially constitutively active form (Hanson & Schulman, 1992).

The aims of this study are to investigate the functional significance of autophosphorylation of MLCK and to understand the effects of autophosphorylation on the basis of its structure.

MATERIALS AND METHODS

Materials. Smooth muscle MLCK was purified from frozen turkey gizzards as previously described (Ikebe et al., 1987). Purified MLCK was subjected to additional column chromatography to eliminate the potentially contaminated kinases. Two distinct types of chromatography were performed. First, the pooled kinase fraction was dialyzed against 10 mM potassium phosphate (pH 6.8), 30 mM KCl, 10 μg/mL leupeptin, and 1 mM dithiothreitol (buffer A). The dialyzed fraction was applied to a hydroxylapatite column (1.0 × 13 cm) equilibrated with buffer A. The column was washed with 20 mL of the same solution. MLCK was eluted from the column by application of a 100-mL linear KP_i gradient (0−0.4 M) in buffer A at a flow rate of 20 mL/h. The peak fraction of MLCK activity was obtained at a KP_i concentration between 190 and 220 mM, and this was used for the autophosphorylation experiment. Second, MLCK was applied to a calmodulin Sepharose 4B column (2.0 × 15 cm) equilibrated with 25 mM Tris-HCl (pH 7.5), 30 mM NaCl, 0.5 mM $CaCl_2$, 10 μg/mL leupeptin, and 1 mM dithiothreitol (buffer B) after addition of 2 mM $CaCl_2$ and 3 mM $MgCl_2$. The column was washed with buffer B, and then washed with buffer B plus 0.5 M NaCl to remove nonspecific binding proteins. Finally, MLCK was eluted with buffer containing 1 mM EGTA, 0.5 M NaCl, 1 mM dithiothreitol, 10 μg/mL leupeptin, and 25 mM Tris-HCl (pH 7.5). The catalytic subunit of cAMP-dependent protein kinase was prepared from bovine heart (Reimann & Beham, 1983). The 20 000 dalton myosin light chain was obtained by employing recombinant protein expression technology (Kamisoyama et al., 1994). Calmodulin was prepared from bull testes (Walsh et al., 1983). The MLCK inhibitory peptide Sm-1 was synthesized according to the amino acid sequence of residues 480−501 of smooth muscle MLCK as described (Ikebe et al., 1987). SMP I and SMP IV were prepared according to Pato and Adelstein (1983) and Pato and Kerc (1985), respectively.

Assays. For autophosphorylation, MLCK (0.2 mg/mL) in a solution containing 20 mM NaCl, 1 mM $MgCl_2$, 100 nM microcystin, 10 μg/mL leupeptin, 1 mM dithiothreitol, and 30 mM Tris-HCl, pH 7.5 at 25 °C for a few minutes, and the autophosphorylation reaction was started by adding 0.75 mM [γ-^{32}P]ATP (50 000 cpm/nmol) (Amersham). An aliquot (50 μL) was removed at each time point and subjected to the phosphorylation determination assay (Walsh et al., 1983). The extent of MLCK phosphorylation was determined according to Walsh et al. (1983). MLCK was also pretreated with SMP I and SMP IV at 4 °C for 36 h prior to the autophosphorylation reaction (the added amount of each phosphatase dephosphorylated 10 nmol of myosin light chain within 10 min at 25 °C).

To determine the effects of autophosphorylation on smooth muscle MLCK activity, 0.6 mg/mL MLCK was first autophosphorylated at 25 °C for 6 h in the presence or absence of 0.17 mg/mL calmodulin under the same conditions as above; then MLCK activity was measured as described below. As a control, MLCK was also incubated under the same conditions but in the absence of $MgCl_2$ to avoid autophosphorylation. For autophosphorylation of MLCK by the catalytic subunit of cAMP-dependent protein kinase (A-kinase), MLCK was phosphorylated with A-kinase (2.5 μg/mL) at 25 °C for 6 h. The Ca^{2+}/calmodulin-dependent activity of MLCK was immediately determined in a solution containing 20 mM NaCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM DTT, 10 μg/mL leupeptin, 0.2 mM [γ-^{32}P]ATP, 0.25 mg/mL of the isolated 20 000-dalton light chain, and various concentrations of calmodulin at 25 °C. The Ca^{2+}/calmodulin-independent activity of MLCK (50 μg/mL) was also measured in a solution containing 20 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 μg/mL leupeptin, 1 mM DTT, 0.5 mM [γ-^{32}P]ATP, 30 mM Tris-HCl, pH 7.5, and 0.25 mg/mL of the isolated 20 000-dalton light chain at 25 °C.

Determination of Autophosphorylation Sites of MLCK. Five milliliters of 0.2 mg/mL MLCK was incubated for 12 h with [γ-^{32}P]ATP under the same conditions described above except no calmodulin and Sm-1 were added. The phosphorylated MLCK was precipitated with 10% trichloroacetic acid, and the pellets were collected by centrifugation for 30 min at 10000g at 4 °C. The precipitated MLCK was dissolved in 8 M urea and 100 mM Tris-HCl, pH 8.5. The urea concentration was then reduced to 2 M and hydrolyzed with 0.15 mg/mL TPCK-treated trypsin (Worthington) at 37 °C for 12 h. The sample was applied to an iminodiacetic acid−Sepharose 6B column (Sigma) equilibrated as described (Muszynska et al., 1986; Michel & Benett, 1987). The phosphorylated peptides were eluted with 1% ammonium acetate, pH 8.3, at a flow rate of 20 mL/h. Elution of the phosphopeptides was monitored by measuring the radioactivity of each fraction (0.5 mL). More than 90% of the radioactivity was recovered. For further purification, the radioactive fractions were pooled. The sample was lyophilized, dissolved in 2 mL of 0.1% trifluoroacetic acid, and applied to a Spheri 5 ODS C-18 reverse-phase column (Brownlee Labs) attached to a Perkin-Elmer Series 410 HPLC system. The peptides were eluted with a linear gradient of CH_3CN from 0 to 45% for 5 h at a flow rate of 0.8 mL/min. Elution of the phosphopeptides was monitored by the absorbance at 217 nm and by the radioactivity of each fraction (0.8 mL). Phosphopeptides were treated with ethanethiol in alkaline conditions and subjected to gas-phase sequence analysis, as described by Ando et al. (1989). The amino acid sequence of the isolated peptides was determined using an Applied Biosystems 470A gas-phase sequencer equipped with a 120A on-line PTH amino acid analyzer, using the 03R PTH program.

Identification of Phosphoamino Acids. Each radioactive phosphopeptide was subjected to acid hydrolysis in 6 N HCl for 3 h at 110 °C. After drying, the samples were subjected to horizontal electrophoresis in the buffer (acetic acid/formic acid/H_2O = 78:25:897) at pH 2.6 with a cellulose thin layer plate (Conti & Adelstein, 1981) on a cooling plate (0 °C). The electrophoresis was carried out at 700 V for 2 h.

Determination of Protein Concentration. Protein concentrations were estimated by spectrophotometric measurements

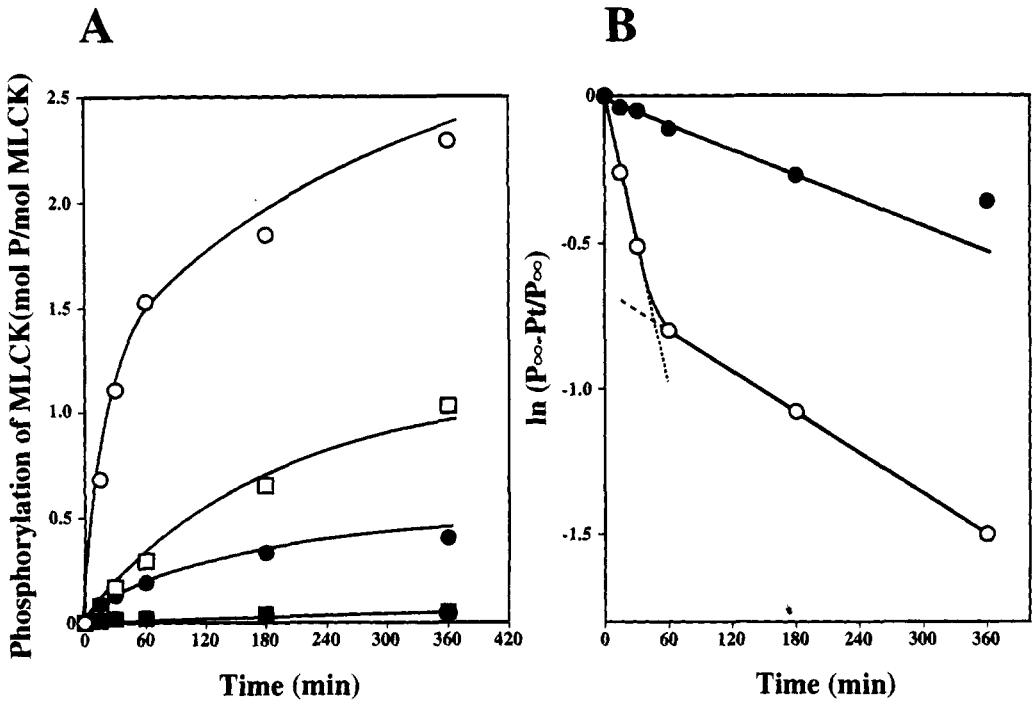

FIGURE 1: Autophosphorylation of smooth muscle MLCK. (A) Time course of autophosphorylation. (○) No calmodulin and no inhibitor peptide (Sm-1); (●) 0.17 mg/mL calmodulin without inhibitor peptide; (□) 30 μg/mL Sm-1; (■) 0.17 mg/mL calmodulin and 30 μg/mL Sm-1. (B) Semilogarithmic plot of the time course of autophosphorylation. P_∞ (the maximum extent of phosphorylation) was taken to be 3.0 and 1.0 mol of P/mol of MLCK in the absence and presence of calmodulin, respectively. (○) No calmodulin and no inhibitor peptide (Sm-1); (●) 0.17 mg/mL calmodulin without inhibitor peptide.

for calmodulin [$A_{277}^{1\%} = 1.9$ (Watterson et al., 1976)] and for MLCK [$A_{280}^{1\%} = 11.4$ (Adelstein & Klee, 1981)].

RESULTS

The autophosphorylation kinetics of purified chicken gizzard smooth muscle MLCK were first examined (Figure 1). The reaction was carried out in the presence of 100 nM microcystine to prevent potential dephosphorylation of MLCK during the reaction. The extent of phosphorylation reached 2.29 ± 0.13 mol of P_i/mol of MLCK, suggesting that three sites were phosphorylated. The extent of phosphorylation was significantly reduced in the presence of Ca^{2+}/calmodulin to 0.35 mol of P_i/mol of MLCK (Figure 1A). These results suggest that Ca^{2+}/calmodulin is not required for autophosphorylation, but rather that the sites are blocked by the binding of calmodulin. The semilog plot of the time course revealed that the kinetics in the absence of Ca^{2+}/calmodulin are dual-phase while those in the presence of Ca^{2+}/calmodulin are single-phase (Figure 1B). The rate constant of the initial phase of autophosphorylation in the absence of Ca^{2+}/calmodulin was approximately 10 times larger than that of the second phase. Phosphoamino acid analysis revealed that the autophosphorylation occurred on both serine and threonine residues (Figure 2A); however, in the early time course (less than 30 min of phosphorylation), serine sites were prodominantly phosphorylated (data not shown). As shown in Figure 2, the phosphorylation on the serine residues was completely blocked by the binding of calmodulin. Although the effect was less extreme, the threonine phosphorylation was also significantly decreased (Figure 2A). Therefore, it was concluded that (1) the autophosphorylation occurred at more than two sites, serine residues and threonine residues; (2) the rate constant of serine phosphorylation was approximately 10 times larger than that for the threonine sites, and (3) both serine and threonine phosphorylation was blocked by calmodulin binding although the phosphorylation of the serine was more dramatically inhibited by calmodulin binding than the threonine sites.

The phosphorylation observed in this study is unlikely to be due to potentially contaminated protein kinases because of the following reasons: (1) A specific cAMP-dependent protein kinase inhibitor peptide (Cheng et al., 1986) did not influence the phosphorylation of MLCK at all (data not shown), indicating that the phosphorylation is not catalyzed by cAMP-dependent protein kinase. (2) Phosphatidylserine/phorbol ester (PMA) did not affect the autophosphorylation (data not shown), indicating that protein kinase C is not involved in the observed phosphorylation. (3) Ca^{2+}/calmodulin is not required, suggesting that exogenous Ca^{2+}/calmodulin-dependent protein kinases are not responsible for the observed phosphorylation. (4) The addition of cGMP did not increase the rate of phosphorylation, which excludes the possibility of cGMP-dependent protein kinase-induced phosphorylation. (5) A specific peptide inhibitor of MLCK (Sm-1) (Ikebe et al., 1987) markedly inhibited the autophosphorylation (Figure 1A). While the MLCK preparation has been shown to be pure and free from contaminating proteins as judged from SDS−PAGE [data not shown; see Ikebe et al. (1987)], we performed additional chromatography steps to eliminate the possibility that a potential contaminating trace amount of kinase might be responsible for the phosphorylation of MLCK. The MLCK preparation was subjected to either hydroxylapatite or calmodulin−Sepharose 4B chromatography (see Materials and Methods), and the further purified MLCK was analyzed for autophosphorylation kinetics. The autophosphorylation was carried out both in the presence and in the absence of Ca^{2+}/calmodulin as shown in Figure 1 for the conventional MLCK preparation. The

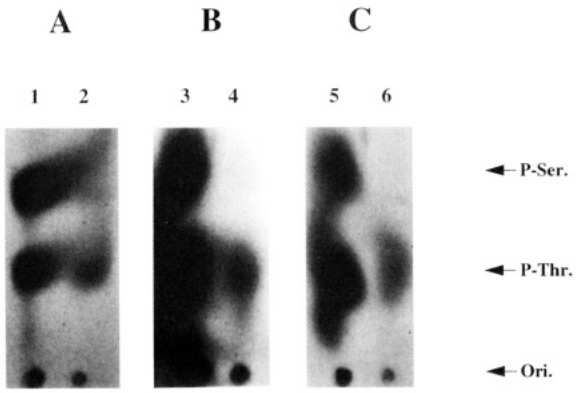

FIGURE 2: Phosphoamino acid analysis. (A) Conventional MLCK preparation. (B) MLCK purified by hydroxylapatite chromatography. (C) MLCK purified by calmodulin Sepharose 4B chromatography. MLCK was autophosphorylated at 25 °C for 6 h under the conditions described in Figure 1 in the presence (lanes 2, 4, and 6) and absence (lanes 1, 3, and 5) of 0.17 mg/mL calmodulin. Other conditions are as described under Materials and Methods. The positions of the origin (Ori.), phosphothreonine (P-Thr.), and phosphoserine (P-Ser.) are indicated.

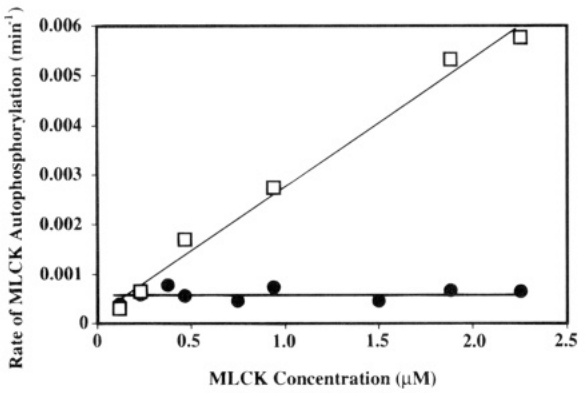

FIGURE 3: Rate of autophosphorylation as a function of MLCK concentration. Autophosphorylation was performed at various MLCK concentrations in the solvent conditions as described in Figure 1, except Sm-1 was omitted. (□) In the absence of calmodulin; (●) in the presence of 0.17 mg/mL calmodulin. The rate constants were estimated from the initial time course of the reaction. Therefore, the rate constants in the absence of calmodulin represent the rate of phosphorylation at serine sites because the rate of serine sites was significantly faster than that of threonine site (Figure 1B). In the presence of calmodulin, the serine phosphorylation was completely blocked as shown in Figure 2, and thus the rate constants represent the rate of threonine phosphorylation.

time course of the phosphorylation was analyzed by a semilog plot to estimate the rate constants, and these were compared with those obtained for the conventional MLCK preparation. The rate constant of the initial rapid phase in the absence of calmodulin and that in the presence of Ca^{2+}/calmodulin were estimated. Neither autophosphorylation in the absence of calmodulin nor autophosphorylation in the presence of calmodulin was affected by a further purification step (data not shown). Phosphoamino acid analysis revealed that phosphorylation at both serine and threonine sites was unchanged by these additional purification steps (Figure 2). These results further support that the observed autophosphorylation is not due to a contaminating kinase.

Figure 3 shows the rate constant of autophosphorylation as a function of MLCK concentration. The rate constant was measured in two conditions, i.e., in the presence and absence of Ca^{2+}/calmodulin. The initial phase of the time

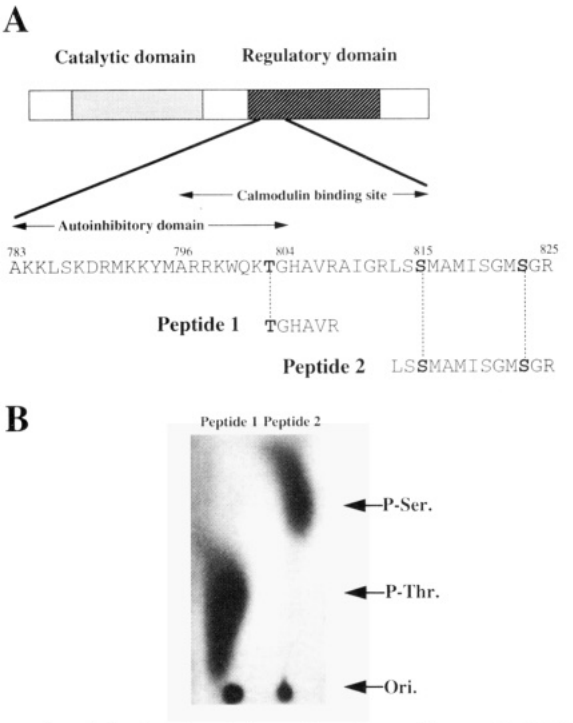

FIGURE 4: Autophosphorylation sites of smooth muscle MLCK. (A) Location of the autophosphorylation sites. The amino acid sequences of the isolated phosphopeptides (Peptides 1 and 2) are aligned according to the published sequence of chicken gizzard MLCK (Olson et al., 1990). Amino acid residues are represented by the single-letter code. Phosphorylated serine and threonine residues are indicated by boldface letters. (B) Phosphoamino acid analysis of the isolated phosphopeptides (Peptides 1 and 2). The positions of the origin (Ori.), phosphothreonine (P-Thr.), and phosphoserine (P-Ser.) are indicated.

course of the former condition predominantly measures the serine phosphorylation while the latter condition measures the threonine phosphorylation. The rate constant of threonine phosphorylation was independent of the MLCK concentration while that of serine phosphorylation was increased proportionally to the MLCK concentration (Figure 3). The results suggest that the phosphorylation at the threonine site is an intramolecular process while that at the serine site is an intermolecular process.

To determine the precise location of autophosphorylation sites, MLCK was autophosphorylated in the absence of Ca^{2+}/calmodulin and subjected to complete tryptic proteolysis. The phosphopeptides were then isolated by chelating Sepharose chromatography and C-18 reverse-phase chromatography as described under Materials and Methods. Two major phosphopeptides, peptide 1 and peptide 2, which eluted at 3.5% and 17.5% CH_3CN, respectively, were isolated. The amino acid sequence of the two phosphopeptides aligned with the known smooth muscle MLCK amino acid sequence is shown in Figure 4. Peptide 1 contained phosphothreonine as judged by phosphoamino acid analysis (Figure 4B) and contained a single threonine residue in its sequence; therefore, threonine 803 was identified to be the autophosphorylation site. Phosphoamino acid analysis of peptide 2 revealed that it contained phosphoserine (Figure 4B). Four serine residues are found in the sequence of peptide 2. To determine the sites, peptide 2 was treated with ethanethiol in alkaline conditions in which phosphoserine residues are specifically converted to *S*-ethylcysteine (Meyer et al., 1986). *S*-Ethylcysteine was observed at the third and eleventh cycles

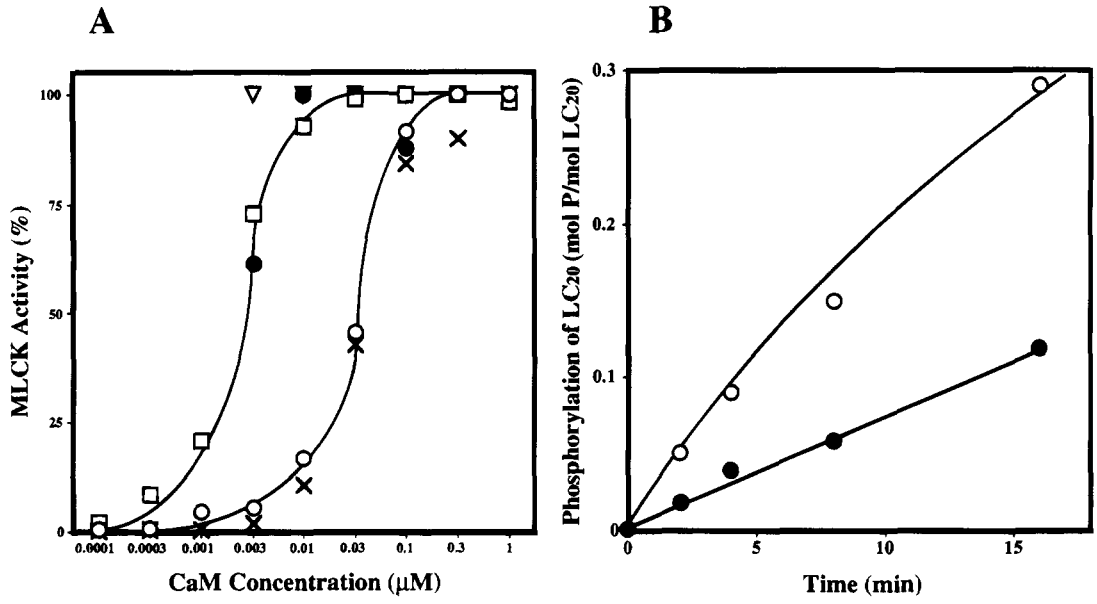

FIGURE 5: Effects of autophosphorylation on smooth muscle MLCK activity. (A) Decrease in the affinity of MLCK for calmodulin by autophosphorylation at Ser 815. The MLCK activity was measured at various calmodulin concentrations in the conditions described under Materials and Methods. (○) MLCK autophosphorylated at serine 815, serine 823, and threonine 803 (2.31 mol of P/mol of MLCK); (●) MLCK autophosphorylated at only threonine 803 (0.44 mol of P/mol of MLCK); (□) unphosphorylated MLCK; (▽) unphosphorylated MLCK incubated with calmodulin prior to MLCK activity assay; (×) MLCK phosphorylated by cAMP-dependent protein kinase (1.85 mol of P/mol of MLCK). (B) Activation of the Ca^{2+}/calmodulin-independent activity of MLCK by autophosphorylation. (○) Phosphorylated at threonine 803 (0.44 mol of P/mol of MLCK); (●) unphosphorylated control MLCK (MLCK incubated in the absence of Mg^{2+}).

during gas-phase sequencing analysis, indicating that both Ser 815 and Ser 823 are phosphorylated. The phosphorylation at Thr 803 and Ser 823 has not been found as phosphorylation sites induced by other protein kinases which are known to phosphorylate MLCK. The sequence results also indicated that phosphorylation at these sites was not catalyzed by proline-directed protein kinases such as mitogen-activated protein kinase, cdc 2 kinase, casein kinase 2, etc. The results, therefore, further support that the observed phosphorylation is not due to a contaminated kinase.

The effects of autophosphorylation on MLCK activity were studied, and the results are shown in Figure 5. To investigate the effect of autophosphorylation on a threonine site and serine sites separately, MLCK was autophosphorylated in the presence of Ca^{2+}/calmodulin which incorporated P_i only on the threonine site (Figure 1). As shown in Figure 5, the Ca^{2+}/calmodulin-independent activity of MLCK was significantly activated when the kinase was phosphorylated only at the threonine residue (0.44 mol/mol of MLCK). Approximately 3-fold activation was achieved with phosphorylation of 0.44 mol/mol of MLCK (Figure 5B), and a higher extent of autophosphorylation anticipates even higher activation. It should be noted that the incubation of MLCK without Mg^{2+} to prevent autophosphorylation did not activate the basal activity of MLCK; therefore, the activation is not due to the potential proteolysis of MLCK during autophosphorylation. On the other hand, when MLCK was autophosphorylated in the absence of Ca^{2+}/calmodulin, therefore phosphorylated predominantly at the serine residues (Figure 1), the concentration of calmodulin required for the activation of MLCK was significantly increased without changing the maximum activity (Figure 5A), suggesting that the affinity of MLCK for calmodulin is decreased. A similar change in the calmodulin dependence of MLCK was also observed for MLCK phosphorylated by cAMP-dependent protein kinase (Figure 5A). The phosphorylation sites responsible for the calmodulin dependence are the serine sites because MLCK autophosphorylated at only the threonine site did not alter its calmodulin dependence on the kinase activity (Figure 5A). To examine whether or not MLCK preparation was significantly preautophosphorylated, MLCK was incubated with both SMP I (type IIA phosphatase) and SMP IV (type I phosphatase) prior to autophosphorylation. This treatment altered neither the MLCK activity nor the autophosphorylation stoichiometry.

DISCUSSION

Three phosphorylation sites were identified, and these were all located in the regulatory domain of the kinase, i.e., threonine 803, serine 815, and serine 823. The autophosphorylation affected the kinase activity in two respects. First, the phosphorylation at threonine 803 activated MLCK activity in the absence of Ca^{2+}/calmodulin. It is well-known (Lou & Schulmann, 1989) that calmodulin-dependent protein kinase II is autophosphorylated at a threonine residue (Thr 286 in a subunit) located in the autoinhibitory region and this phosphorylation significantly activates calmodulin-independent kinase activity, thus converting the enzyme to a partially constitutively active enzyme. Although the activation of MLCK by autophosphorylation observed for MLCK is not as extreme as that for calmodulin-dependent protein kinase II (the activity of the Ca^{2+}/calmodulin-independent activity of autophosphorylated MLCK is still much less than the Ca^{2+}/calmodulin-dependent activity), since threonine 803 resides in the autoinhibitory region of the kinase, the results suggest that a common mechanism may be operating for the activation of the calmodulin-dependent kinase by autophosphorylation at the inhibitory domain of the kinase. It is plausible since the alignment of the basic amino acid residues around the threonine site, which is important as substrate recognition, is similar to that of the myosin light chain (substrate of MLCK); the activation

Table 1: Sequence Alignment of MLCKs and Smooth Muscle Myosin Regulatory Light Chain[a]

Protein	Amino acid sequence
sm MLCK	^{783}AKKLSKDRMKKYMARRKWQK**T**GHAVRAIGRLS**S**MAMISGM**S**GR
sk MLCK	^{328}RRLKSQILLKKYLMKRRWKKNFIAVSAANRFKKISSSGALMAL
LC20	^{1}SSKRAKAKTTKKRPQRA**TS**NVF

[a] The amino acid sequence of chicken gizzard smooth muscle MLCK is aligned with the sequences of skeletal muscle MLCK (Takio et al., 1985) and smooth muscle 20 000-Da myosin light chain (Pearson et al., 1984). Amino acid residues are represented by the single-letter code. Phosphorylated serine and threonine residues are indicated by boldface letters. The conserved basic amino acid residues which are important for substrate recognition are underlined.

of MLCK by threonine autophosphorylation observed here may be achieved by the partial disruption of the pseudosubstrate inhibition mechanism (Kemp et al., 1987).

Second, the phosphorylation at the serine residues decreased the affinity of MLCK for calmodulin. We think that the site responsible for the change in affinity for calmodulin is Ser 815 and not Ser 823 because of the following reasons. (1) It has been known that the phosphorylation site for cAMP-dependent protein kinase (Adelstein et al., 1978) and calmodulin-dependent protein kinase II (Ikebe and Reardon, 1990) which decreases the affinity of MLCK for calmodulin is serine 815. (2) It was shown recently by multidimensional NMR analysis of the complex of the calmodulin/calmodulin binding peptide of MLCK that two hydrophobic residues (Trp 800 and Leu 813) in the calmodulin binding site of MLCK are essential for anchoring the calmodulin binding peptide to calmodulin (Ikura et al., 1992; Meador et al., 1992). Serine 815 is in close proximity to one of the calmodulin anchoring sites (Leu 813), and according to multidimensional NMR analysis (Ikura et al., 1992; Meador et al., 1992), the serine residue is proximal to the cluster of glutamic acid residues localized in the central part of the calmodulin molecule. Therefore, it is likely that the phosphorylation destabilizes the interaction between MLCK and calmodulin due to electrostatic repulsion.

On the other hand, threonine 803 localizes more toward the N-terminal side of the regulatory domain of MLCK where an autoinhibitory sequence exists. The threonine site is also within the span of the calmodulin binding region, but structural analysis of the calmodulin/calmodulin binding peptide complex suggests that this threonine residue is proximal to the hydrophobic environment of calmodulin when MLCK binds to calmodulin and the incorporation of the negative charge may not completely attenuate the electrostatic interaction between MLCK and calmodulin. The fact that threonine autophosphorylation is not completely protected by calmodulin binding is consistent with this notion.

An interesting finding is the structural similarity between the threonine autophosphorylation site and the myosin light chain substrate site (Table 1). A number of the basic residues at the N-terminal side of Ser 19 of LC_{20} are conserved in the smooth muscle MLCK sequence near the threonine site. Since threonine autophosphorylation is an intramolecular event, the results suggests that threonine 803 is in close proximity to the catalytic pocket of MLCK. This supports the hypothesis that the autoinhibitory region is in direct contact with the catalytic site which inhibits the kinase reaction rather than achieving inhibition indirectly via a change in the conformation.

Autophosphorylation of skeletal muscle MLCK was also reported recently (Gao et al., 1992), and the autophosphorylation is Ca^{2+}/calmodulin-dependent. Two sites are phosphorylated, both of which are serine residues located toward the amino-terminal side of the catalytic domain. The autophosphorylation of skeletal muscle MLCK has no significant effects on MLCK activity, probably because of the lack of phosphorylation at the regulatory domain. It is reasonable that skeletal muscle MLCK does not autophosphorylate at the regulatory domain because neither threonine nor serine sites found in this study are conserved in the skeletal MLCK sequence (Table 1).

The physiological significance of the autophosphorylation requires further study; however, several problems in the regulation of smooth muscle contraction may be related. It was shown previously that the phosphorylation level of smooth muscle fiber in Ca^{2+}-free solution is much higher than that expected from in vitro MLCK activity in Ca^{2+}-free conditions (Kitazawa et al., 1991). It has also been shown that okadaic acid induces Ca^{2+}-free contraction in α-toxin skinned smooth muscle fiber and the rate of contraction is significantly faster than the expected rate according to MLCK activity in Ca^{2+}-free conditions (Gong et al., 1992). Although there are many alternative possibilities, the relatively high myosin light chain kinase activity in smooth muscle fiber in Ca^{2+}-free conditions may be attributed to the activation of MLCK by threonine autophosphorylation. Since the phosphorylation at threonine 803 occurs in Ca^{2+}-free conditions, the phosphorylation can be achieved regardless of the muscle activation; therefore, despite its slow phosphorylation kinetics, autophosphorylation at Thr 803 may have a physiological relevance.

The modulation of kinase activity by autophosphorylation of MLCK provides new insight on the regulatory mechanisms of physiological events which MLCK is involved in such as smooth muscle contraction (Hartshorne, 1987) and cytokinesis (Satterwhele & Pollard, 1992). The results also show another example of the activation of calmodulin-dependent kinase activity by autophosphorylation which to date has only been known for calmodulin-dependent protein kinases II and IV, although the extent of activation is not as dramatic as those for calmodulin-dependent protein kinases II and IV. The phosphorylation sites for both enzymes are localized in the autoinhibitory domain and therefore may possess similar mechanisms for activation.

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BI942184Y