Physiol. Res. 54: 671-678, 2005

The Comparison of Ca^{2+}/CaM-Independent and Ca^{2+}/CaM-Dependent Phosphorylation of Myosin Light Chains by MLCK

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Received October 20, 2004
Accepted December 14, 2004
On-line available February 16, 2005

Summary

The main regulatory mechanism of smooth muscle contraction involves Ca^{2+}/calmodulin (CaM)-dependent phosphorylation of myosin (CDPM), by myosin light chain kinase (MLCK). It is also known that the increase in intracellular Ca^{2+} and phosphorylation of myosin occurs within a short time under physiological conditions, but the muscle tension may persist for a longer period of time. However, the mechanism of this phenomenon is still not clear. We hypothesize that MLCK also phosphorylates myosin in a Ca^{2+}/CaM-independent manner (CIPM). The difference between CIPM and CDPM are as follows. Firstly, the extent of CIPM by MLCK was temperature-independent, whereas CDPM by MLCK was apparently decreasing with increasing temperature. Secondly, in contrast to the decreased extent of CDPM, the prolongation of incubation time did not decrease the extent of CIPM. Thirdly, a high concentration of K^+ influences CIPM less than CDPM. Furthermore, the MLCK inhibitor ML-9 significantly inhibited CDPM by MLCK but not CIPM by MLCK. Lastly, arachidonic acid selectively increased CIPM by MLCK but not CDPM by MLCK. Finally, the activity of Mg^{2+}-ATPase of myosin followed the sequence as this: CDPM > CIPM > unphosphorylated myosin. Our results revealed some primary features of CIPM by MLCK.

Key words

Ca^{2+}/CaM-independent phosphorylation • Ca^{2+}/CaM-dependent phosphorylation • Myosin • MLCK

Introduction

Ca^{2+}/CaM-dependent phosphorylation of myosin light chains (CDPM) by MLCK is generally considered as the primary mechanism for regulating the contraction of smooth muscle. Phosphorylation can be simply described as an interaction of Ca^{2+} with calmodulin (CaM) that induces a conformational change of MLCK and activates MLCK. The activated MLCK catalyzes phosphorylation of myosin light chains. The phosphorylation of myosin light chains triggers the cycling of myosin cross-bridges along actin filaments and the development of force (Walsh 1991, Rembold 1992, Jiang and Stephens 1994). It is also known that increases in intracellular Ca^{2+} and smooth muscle CDPM occur within a short time under physiological conditions, but sustained muscle tension may remain stable for a longer period of time (Marston and Huber 1996, Gimona and Small 1996). However, the mechanism of this phenomenon remains to be investigated. It was proposed that two states of myosin, i.e. the phosphorylated and unphosphorylated myosins were present in the smooth

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muscle; and that the unphosphorylated myosin, which forms a special slow cycling crossbridges, termed latch-bridges, is also involved in sustained smooth muscle contractions (Haeberle 1999, Coirault and Blanc 2000).

An unexpected finding in our study that myosin light chains can also be phosphorylated by MLCK in a Ca^{2+}/CaM-independent manner attracted our attention. We hypothesize that three states of myosin are present in smooth muscle, i.e., CDPM by MLCK featured by initial burst of contraction with high energy consumption, CIPM by MLCK characterized by sustained tension with less energy consumption, and unphosphorylated myosin in the relaxation state with least energy consumption. The aim of our study was to reveal the action feature of CIPM by MLCK *in vitro*. We compared CIPM by MLCK and CDPM by MLCK under various assay conditions, i.e. the influence of different concentration of MLCK on CIPM and CDPM; the influence of different incubation temperature, different incubation time and the effect of ionic strength on MLCK-mediated CIPM and CDPM; the effects of MLCK inhibitor, ML-9((5-chloronaphthalene-1 -sulfonyl) 1H-hexahydro- 1,4-diazepine), and arachidonic acid (AA) on MLCK-mediated CIPM and CDPM; and the comparison of ATPase activities of unphosphorylated myosin, CIPM and CDPM.

Methods

Protein purification

The myosin and MLCK used in our study were purified from the chicken gizzard smooth muscle to homogeneity as described previously (Lin and Kohama 1993, 1998, Lin 2000).

Myosin phosphorylation

CIPM was carried out in a 20 mM Tris-HCl (pH 7.4) buffer containing 1 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 60 mM KCl, 2 mM ethylene glycol bis (beta-aminoethyl ether) N,N'-tetraacetic acid (EGTA), 4 μM myosin and 2 mM ATP. Various concentrations of MLCK, at different incubation times and incubation temperatures for myosin phosphorylation are described in detail in the corresponding figure legends. The assay conditions for CDPM by MLCK were the same as those for CIPM but with an addition of $CaCl_2$ and CaM to a final concentration of 0.1 mM and 5 μg ml^{-1}, respectively.

Determination of myosin light chain phosphorylation

Glycerol polyacrylamide gel electrophoresis (Glycerol PAGE) was used to measure the extent of phosphorylation of myosin light chains. Glycerol PAGE was carried out using the following method (Lin *et al*. 1994): the separating gel contained 13.96 % acrylamide, 0.372 % bis-acrylamide, 40 % (v/v) glycerol, and 0.375 M Tris (pH 8.7) and the stacking gel contained 5.72 % acrylamide, 0.152 % bisacrylamide, 10 % (v/v) glycerol and 0.125 M Tris-HC1 (pH 6.7). Myosin samples which contained 7.5 M urea were added to the sample buffer containing 6 M urea, 20 % glycerol, 0.05 M Tris (pH 6.7), 14 mM β-ME, and a moderate amount of 0.01 % bromophenol blue (BPB). The reaction mixture was loaded onto the gel.

Measurement of CIPM or CDPM

The densitometry extent of CIPM or CDPM was measured with the Scion Image software (Scion Co. Ltd). The extent of diphosphorylation (DIP) of MLC20 was selected as the control (calculated as 100 %); the extent of monophosphorylation (MIP) of MLC_{20} was a relative value calculated from MIP/DIP.

Myosin Mg^{2+}-ATPase activity measurement

The method for measuring Mg^{2+}-ATPase activity of myosin was described previously (Lin and Kohama 1993, 1998, Lin 2000).

Other procedures

Protein concentrations were determined by the method of Bradford (1976). The graphs of CIPM, CDPM and myosin Mg^{2+}-ATPase activities were obtained with Microsoft Excel 2002.

The results of experiments are expressed as mean ± S.D. Student's t-test was used for evaluating the significance of the differences.

Results

The comparison between CDPM by MLCK and CIPM by MLCK of different concentrations

The high sensitivity and efficacy of CDPM by MLCK are known as those in the presence of Ca^{2+} and CaM. A very low concentration of MLCK is needed to phosphorylate myosin light chains (Tansey *et al.* 1994). For instance, myosin (1 μM) could be phosphorylated with (0.005-0.01 μM) MLCK. Considering that the characterization of CIPM by MLCK remains unknown, we chose different concentrations of MLCK to compare the possible difference between CDPM and CIPM.

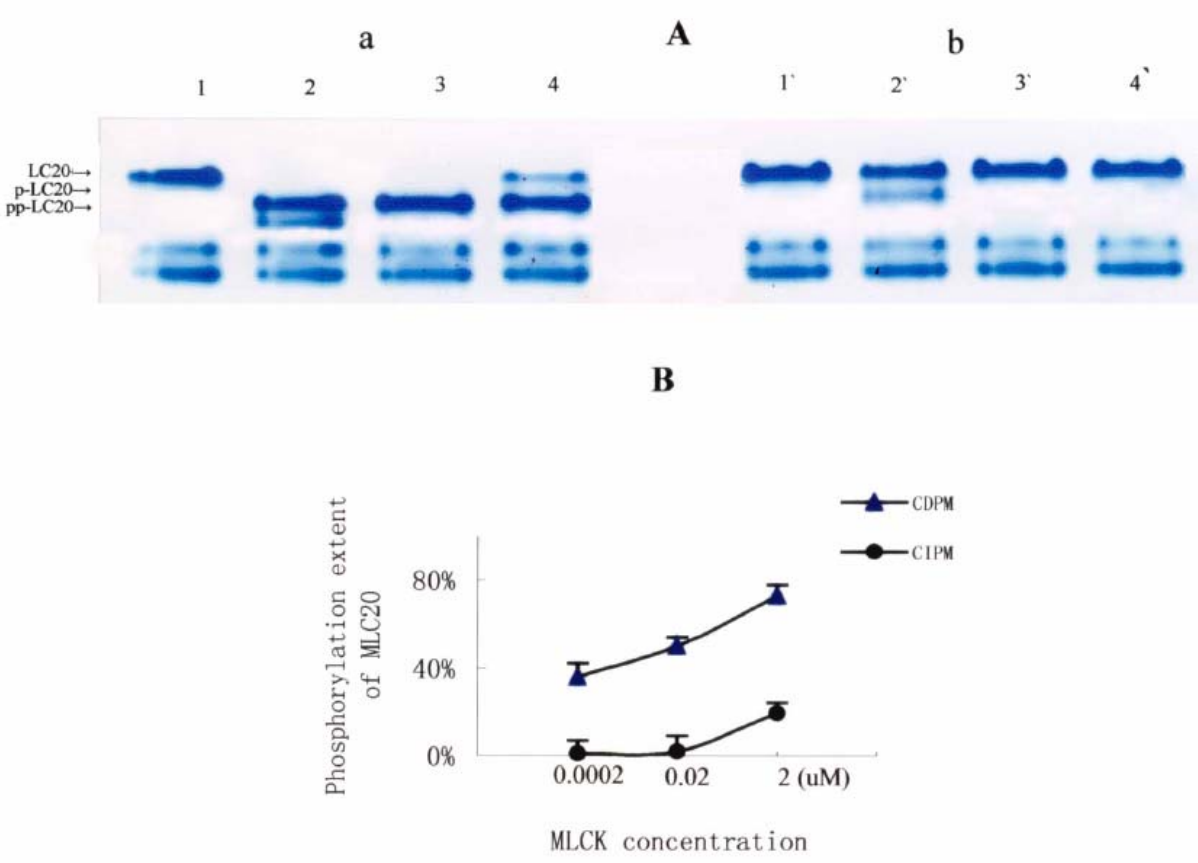

Fig. 1. The comparison between CDPM by MLCK and CIPM by MLCK in different concentrations. [Fig. 1 A(a), lanes 1-4] representing CDPM and [Fig. 1 A(b), lanes 1`-4`] representing CIPM are showed in Fig. 1. In both A(a) and A(b), lanes 1 and 1`, 2 and 2`, 3 and 3`, 4 and 4`, 0 μM, 2 μM (0.21 mg ml^{-1}), 0.02 μM (2.1 μg ml^{-1}) and 0.0002 μM (0.021 μg ml^{-1}) MLCK were added respectively for both CIPM and CDPM; 4 μM myosin (1.76 mg ml^{-1}) were used in both CIPM and CDPM at 25 °C for 20 min in the incubation. LC_{20} represents unphosphorylated 20 kDa myosin light chains; p-LC_{20} represents monophosphorylated 20 kDa myosin light chains; pp-LC_{20} represents diphosphorylated 20 kDa myosin regulatory light chains; LC_{17} represents 17 kDa myosin essential light chains. In B the relative numerical extent of phosphorylation of MLC_{20}. Dots (●): effect on CDPM by MLCK; triangles (▲): effect on CIPM by MLCK. The abscissa was different MLCK concentration, the ordinate was the relative extent of MLC phosphorylation. (mean ± S.D., n=6).

Three MLCK concentrations, i.e., 2, 0.02 and 0.0002 μM and 4 μM myosin were selected for both CDPM [Fig. 1 A(a), lanes 1-4] and CIPM [Fig. 1 A(b), lanes 1`-4`]. Figure 1 A(a) (lanes 1-4) shows that in the presence of Ca^{2+} and CaM (CDPM) myosin was phosphorylated at all the MLCK concentrations tested. However, in the presence of 2 mM EGTA instead of Ca^{2+} and CaM (CIPM) [Fig. 1 A(b), lanes 1`-4`] , MLCK was able to partially phosphorylate myosin light chains only at the highest concentration (2 μM).

The comparison of the influence of incubation temperature, incubation time and ionic strength (K^+) on CIPM and CDPM

To determine whether the incubation temperature influences CDPM [Fig. 2 A_1(a), lanes 1-5] and CIPM [Fig. 2 A_1(b), lanes 1`-5`], different temperatures, i.e. 20, 25, 30, and 35 °C (lanes 2 2`, 3 3`, 4 4` and 5 5`, respectively) were chosen to examine the phosphorylation of myosin light chains. The results showed that for CDPM [Fig. 2 A_1(a), lanes 1-5 B_1 ▲] phosphorylation of myosin light chains decreased with increasing incubation temperature ($P<0.01$); in contrast, CIPM by MLCK [Fig. 2 A_1(b), lanes 1`-5` B_1 ●] did not change.

To compare the difference of CIPM and CDPM, three different incubation times, i.e. 20, 40 and 60 min were selected. The results showed that whereas no apparent changes were observed in CIPM with increasing incubation time [Fig. 2 A_2(b), lanes 1`-4` ; B_2 ●], CDPM decreased [Fig. 2 A_2(a), lanes 1-4 ; B_2 ▲] ($P<0.001$ or $P<0.01$).

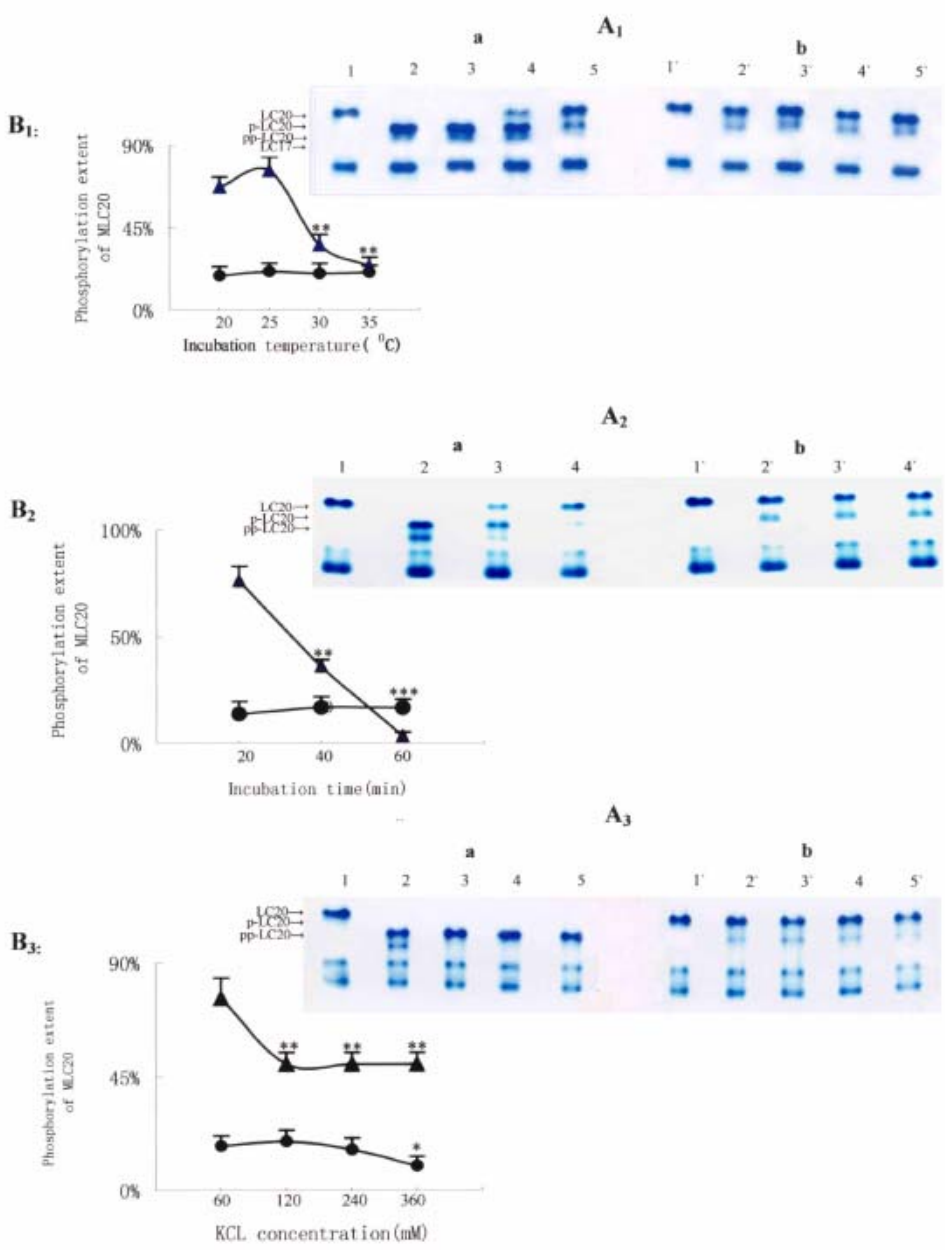

Fig. 2. A_1 and B_1 The comparison between CDPM by MLCK and CIPM by MLCK at different incubation-temperature. [Fig. 2 A_1(a), lanes 1-5] representing CDPM and [Fig. 2 A_1(b), lanes 1`-5`] representing CIPM. a_0 and b_0 are blank controls without incubation. In both a and b, four different incubation temperatures, i.e. lanes 1 and 1` = 20 °C; 2 and 2` = 25 °C; 3 and 3` = 30 °C; 4 and 4` = 35 °C were selected for CDPM and CIPM; Lanes 1 and 1` were controls for comparison from lanes 2 to 5 and 2` to 5`, respectively. Samples were incubated for 20 min with 4 μM myosin and 2 μM MLCK. In B the relative numerical extent of phosphorylation of MLC_{20}. Dots (●): effect on CDPM by MLCK; triangles (▲): effect on CIPM by MLCK. The abscissa was incubation temperature, the ordinate was the relative extent of MLC phosphorylation. Significant effects of different incubation temperature on CDPM and CIPM: **p<0.01 compared to the corresponding controls. **Fig. 2 A_2 and B_2** The comparison between CDPM by MLCK and CIPM by MLCK at different incubation time. [Fig. 2 A_2(a), lanes 1-4] represents CDPM and [Fig. 2 A_2(b) lanes 1`-4`] represents CIPM. Among a and b, different incubation times, i.e. lanes 2 and 2`= 20 min, lanes 3 and 3` = 40 min, lanes 4 and 4` = 60 min were chosen for CDPM and CIPM. Lanes 1 and 1` were blank controls without incubation for CDPM and CIPM, respectively. The samples were incubated with 4 μM myosin and 2 μM MLCK at 25 °C. Lanes 1 and 1` were controls for comparison from lanes 2 to 4 and 2` to 4`, respectively. In B the relative numerical extent of phosphorylation of MLC_{20}. Dots (●): effect on CDPM by MLCK; triangles (▲): effect on CIPM by MLCK. The abscissa was incubation time, the ordinate was the relative extent of MLC phosphorylation. Significant effects of different incubation time on CDPM and CIPM: *** p<0.001, ** p<0.01 compared to the corresponding controls. **Fig. 2 A_3 and B_3** The effects of different ionic strength of KCl on CDPM and CIPM. [Fig. 2 A_3(a), lanes 1-5] represents CDPM and [Fig. 2 A_3(b), lanes 1`-5`] represents CIPM. In both a and b, lanes 2 and 2`= 60 mM KCl, 3 and 3` = 120 mM KCl, 4 and 4` = 240 mM KCl, 5 and 5` = 360 mM KCl were used in both CDPM and CIPM. Lanes 1 and 1` were controls without incubation for CDPM and CIPM, respectively. The assay was carried out at 25 °C for 20 min with 4 μM myosin and 2 μM MLCK. Lanes 1 and 1` were controls for comparison with lanes 2 to 5 and 2` to 5`, respectively. In B the relative numerical extent of phosphorylation of MLC_{20}. Dots (●): effect on CDPM by MLCK; triangles (▲): effect on CIPM by MLCK. The abscissa was KCl concentration, the ordinate was the relative extent of MLC phosphorylation. Significant effects of different ionic strength of KCl on CDPM and CIPM by MLCK: *p<0.05, **p<0.01 compared to the corresponding controls. All data are means ± S.D. (n=6).

To investigate the influence of ionic strength of KCl on CIPM and CDPM, myosin light chain phosphorylation by MLCK was determined in the presence of 60, 120, 240 and 360 mM KCl. The results indicated that for CDPM, diphosphorylation was observed only at 60 mM KCl [Fig. 2 A_3(a), lanes 1-5; B_3 ▲], and that when KCl was over 120 mM, only monophosphorylation of myosin was observed ($P<0.01$). Furthermore, no apparent changes on CIPM were observed in the range of 60 to 240 mM KCl [Fig. 2 A_3 (b), lanes 1`-4`; B_3 ●] with the exception of 360 mM KCl where a significant decrease was observed compared to the control ($P<0.05$).

The effects of ML-9 on MLCK-mediated CIPM and CDPM in different incubations

ML-9 is widely used as a potent inhibitor of MLCK on CDPM. We wanted to know whether ML-9 exerts the same effect on CIPM by MLCK. We studied the effect of ML-9 on CIPM and CDPM at 10, 20, 40 and 80 min. The results indicated that ML-9 significantly inhibited the effect of MLCK on CDPM but not on CIPM in our assay conditions ($P<0.01$) [Fig. 3 A(a), lanes 1-6 ; B ▲ and Fig. 3 A(b), lanes 1`-6` ; B ●, respectively].

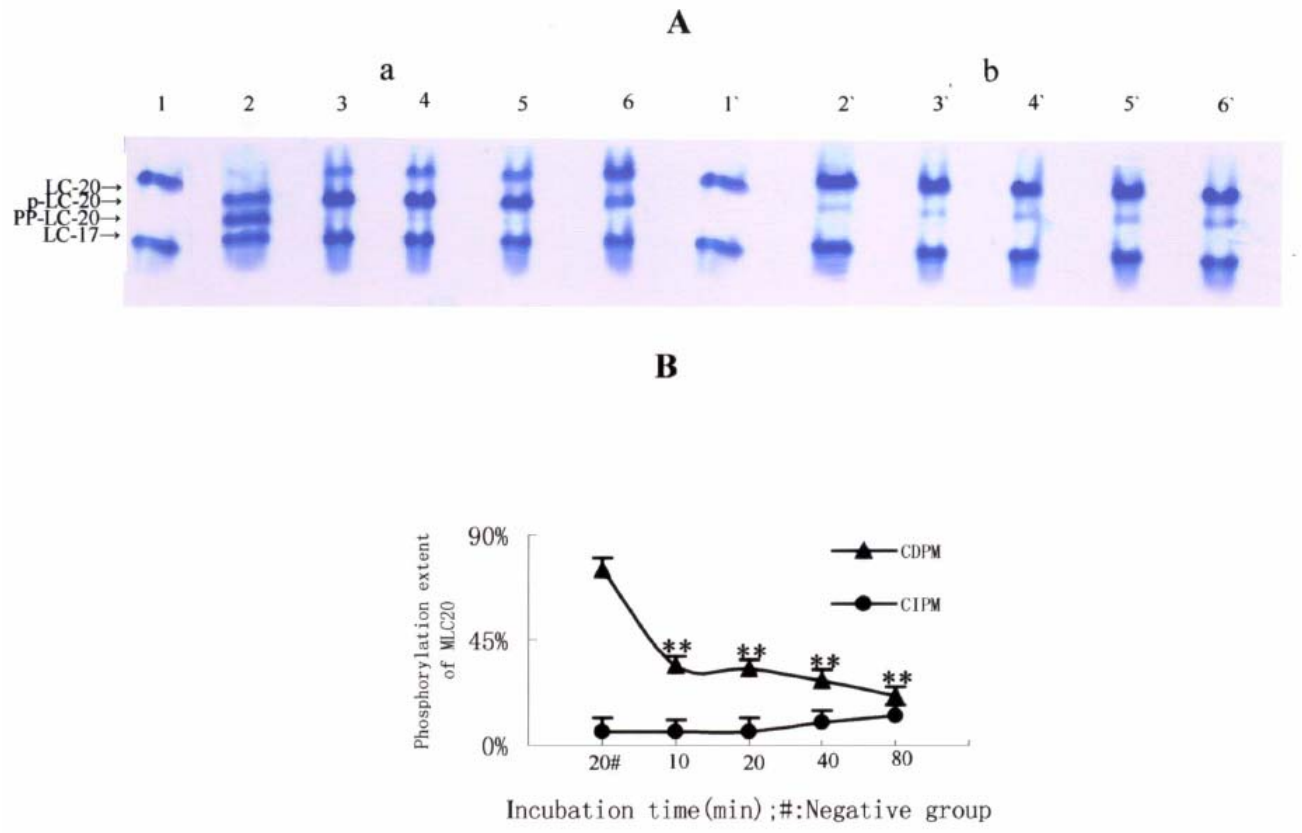

Fig. 3. The effects of 0.1 mM ML-9 on CDPM and CIPM by MLCK at different incubation time. [Fig. 3 A (a) lanes 1-6] representing CDPM and [Fig. 3. A (b) lanes 1`-6`] representing CIPM are shown in Fig. 3 A. Lanes 1 and 1` were blank controls without incubation for CDPM and CIPM, respectively. Lanes 2 and 2` were assay controls incubated for 10 min without adding ML-9; In both a and b, different incubation times, i.e. lanes 2 and 2`= 10 min, 3 and 3` = 20 min, 4 and 4` = 40 min, 5 and 5` = 80 min, were chosen for CDPM and CIPM. The samples were incubated with 4 μM myosin and 2 μM MLCK at 25 °C. Lane 1 is the control for lane 2 to 6; lane 1` is the control for 2` to 6`. In B the relative numerical extent of phosphorylation of MLC_{20}. Dots (●): effect on CDPM by MLCK; triangles (▲): effect on CIPM by MLCK. The abscissa was ML-9 concentration, the ordinate was the relative extent of MLC phosphorylation. Significant effects of 0.1 mM ML-9 on CDPM and CIPM by MLCK at different incubation time: **p<0.01 compared to the corresponding controls, (mean ± S.D., n=6).

The effects of arachidonic acid (AA) on CIPM and CDPM

AA is a multifunctional regulatory factor which is reported to enhance Ca^{2+}-dependent phosphorylation of myosin light chains by inhibiting phosphatase activity (Gong *et al.* 1992). We tried to reveal the effects of AA on CIPM taking CDPM as a control. We did not observe any effects of AA on myosin light chain phosphorylation

when they were fully phosphorylated (CDPM) by MLCK (data not shown), In order to make the comparison available, approximately the same extent of phosphorylation for both CIPM and CDPM was selected, i.e. MLCK for CDPM was 0.0001 μM and MLCK for CIPM was 2 μM. Our result [Fig. 4 A(b), lanes 1`-6`; B ●] indicated that AA selectively increased the extent of CIPM in a concentration-dependent manner, whereas AA exerted no effect on CDPM [Fig. 4 A(a), lanes 1-6; B ▲]

Comparison of myosin Mg^{2+}-ATPase activities between CDPM by MLCK, CIPM by MLCK and unphosphorylated myosin

Hydrolyzing ATP by myosin is directly related to contractile function and structural changes in the head region of myosin during a contraction. It is likely that changes in myosin enzymatic activity accompany changes in myosin structure and function (Thomas *et al.* 1995).

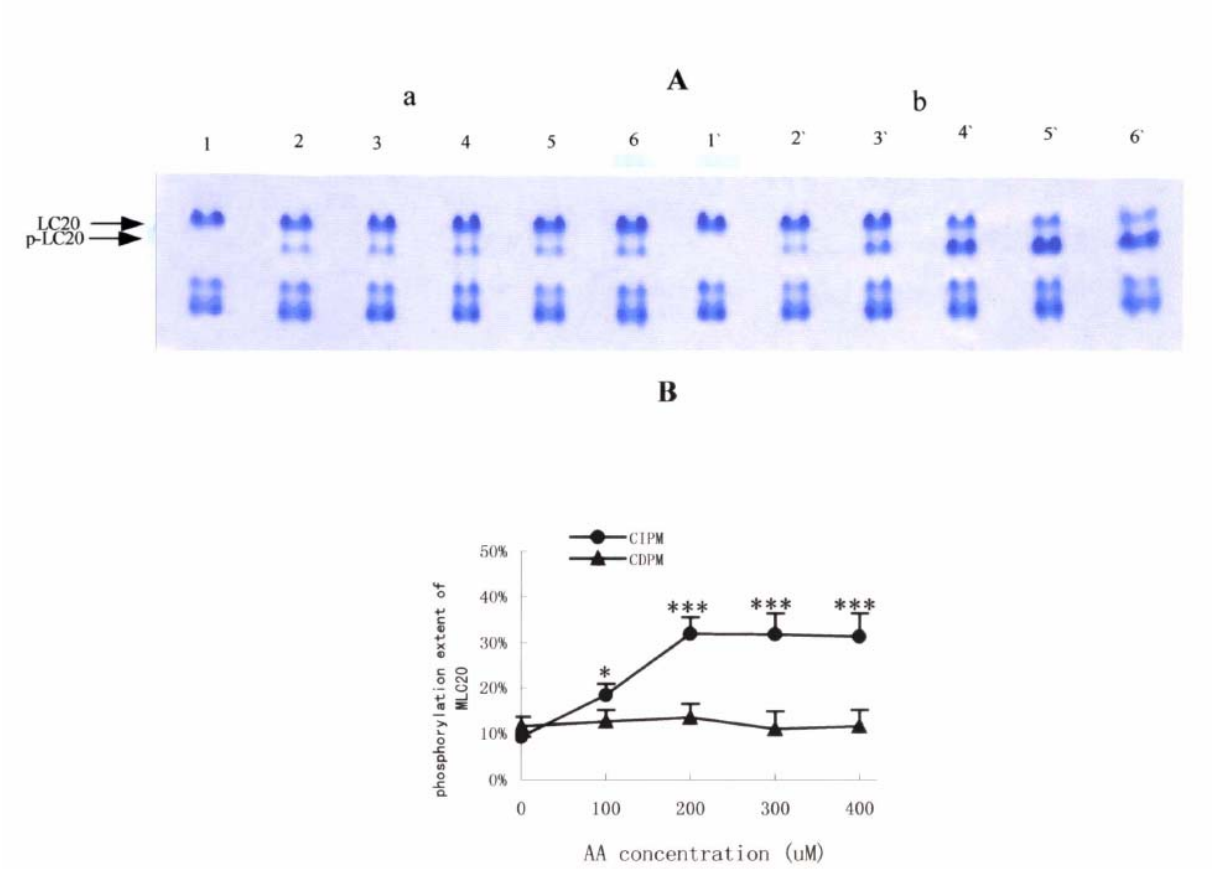

Fig. 4. The effects of AA on CIPM and CDPM. Fig. 4 A(a): glycerol electrophoresis results of CDPM; Fig. 4 A(b): glycerol electrophoresis results of CIPM. Lanes 1, 1`: unphosphorylation control; lanes 2, 2`: 0 μM AA; lanes 3, 3`: 100 μM AA; lanes 4, 4`: 200 μM AA; lanes 5, 5`: 300 μM AA; lanes 6, 6`: 400 μM AA. In B the relative numerical extent of phosphorylation of MLC_{20}. Dots (●): effect on CDPM by MLCK; triangles (▲): effect on CIPM by MLCK. The abscissa was AA concentration, the ordinate was the relative extent of MLC_{20}

Our results (Fig. 5) indicated that different incubation time, i.e. 5, 10, 20, 40 and 60 min were chosen to examine myosin Mg^{2+}-ATPase activities. It showed that Mg^{2+}-ATPase activities of the three states of myosin were all enhanced by prolonging the incubation time. The results indicated that at different incubation times, the highest Mg^{2+}-ATPase activity was observed when myosin was in the state of Ca^{2+}-dependent phosphorylation, the second one was Ca^{2+}-independent-phosphorylated myosin and the lowest was the unphosphorylated myosin.

Discussion

For revealing the changes of CIPM by MLCK, selection of suitable indexes is of great importance. It was reported that the temperature-dependence of steady state force, which reflected the effects of temperature on myosin ATPase activity, was present in smooth muscles. The parameters of contraction velocity in vascular smooth muscle depend on the temperature, For instance, the maximum speed of contraction and relaxation decreased by lowering the temperature in smooth muscle

(Jaworowski and Arner 1998, Sunano and Miyazaki 1981). It is also reported that phosphorylation of the regulatory light chain of myosin catalyzed by myosin light chain kinase (MLCK) is determined, and mono- and diphosphorylated MLC_{20} were detected in different durations of CDPM action (Filenko and Sobieszek 1998). Electrostatic interactions involved in cross-bridge attachment to thin filaments are found to be dependent on ionic strength by Andrews *et al.* (1991).

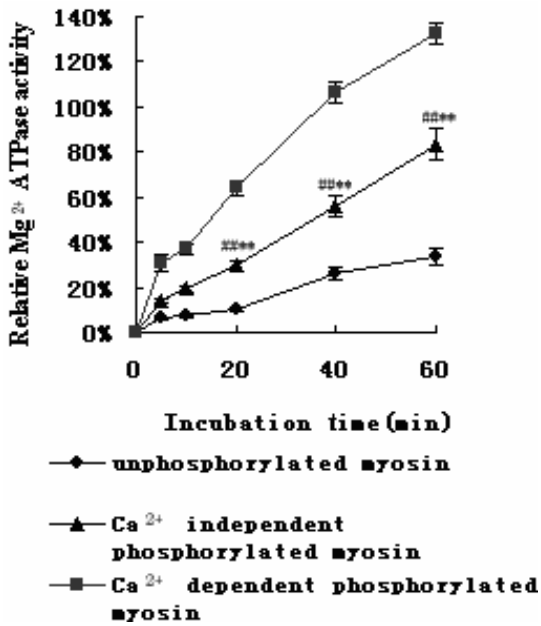

Fig. 5. The comparison of myosin Mg^{2+}-ATPase activities among CDPM by MLCK, CIPM by MLCK and unphosphorylated myosin during different incubation time. The curves ▲, ● and ■ represent the Mg^{2+}-ATPase activities of unphosphorylated myosin, CIPM and CDPM, respectively. 4 μM myosin and 2 μM MLCK were used in the assay at different incubation time, i.e. 5, 10, 20, 40 and 60 min. Significant differences: **p<0.01 compared with unphosphorylated myosin, ##p<0.01 compared with CDPM. (mean ± S.D., n=6)

Since it is known that incubation temperature, incubation time, and ionic strength affect the function of myosin, we selected these indexes to reveal MLCK-mediated CIPM taking CDPM as the control. Our results indicated that compared to CDPM by MLCK, high concentrations of MLCK are needed for CIPM. CIPM is less influenced by increased incubation temperature, by prolonged incubation time, and by increased ionic strength. ATPase activities of CIPM are lower than those of CDPM but higher than those of unphosphorylated myosin. These data suggested MLCK-mediated CIPM are involved in sustained-tension of the smooth muscle, which is characterized by less energy consumption and long duration.

We select ML-9 known as a MLCK inhibitor and arachidonic acid which inhibits phosphatase activity to observe the feature of MLCK-mediated CIPM as compared to CDPM. The results showed that ML-9 exerted less influence on CIPM as compared with the action of MLCK on CDPM. AA selectively increased CIPM by MLCK which suggests the presence of a different mechanism for MLCK-mediated CIPM and CDPM. However, the exact mechanism of MLCK-mediated CIPM and the aspect of CIPM *in vivo* in maintaining sustained tension of smooth muscles remains to be investigated.

Acknowledgements

This program was supported by the National Natural Scientific Foundation of China, No. 30070203. We thank Professor Norma Adragna, Professor Kazuhiro Kohama, Professor Lin Mao-zhang and Professor Wang Li-Wen, and Professor Jiang Xin for their valuable comments.

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