Vol. 175, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1991 Pages 277-284

HETEROGENEITY OF SMOOTH MUSCLE MYOSIN LIGHT CHAIN KINASE IS REVEALED BY ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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Received January 15, 1991

When smooth muscle myosin light chain kinase, purified by standard procedures from chicken gizzard smooth muscle, was applied to an anion-exchange high-performance liquid chromatographic column, three well resolved peaks were obtained. Each peak contained a single protein whose electrophoretic mobility corresponded to that of MLCK. However each enzyme was characterized by a different specific activity. Peptide mapping experiments were unable to demonstrate different proteolytic patterns for the three proteins. Treatment of myosin light chain kinase with alkaline phosphatase, prior to ion chromatography, resulted in a change of elution profile. These experiments suggest that myosin light chain kinase could exist in three forms characterized by a different degree of phosphorylation.

The polymorphism of many contractile proteins is now well established in striated muscle. In smooth muscles, however, this heterogeneity has not been defined until quite recently (see ref.1 for a review). Myosin light chain kinase (MLCK) is an enzyme that has been suggested to play a fundamental role in regulating smooth muscle contraction, phosphorylating a particular myosin subunit. The effect of this phosphorylation is the stimulation of myosin ATPase activity and then the contraction (2).

In the past, the presence of MLCK isoforms was demonstrated only for rabbit skeletal muscle, on the basis of protein sequence (3). However, little is known about the existence of MLCK polymorphism in smooth muscle.

Recently we have proposed a method for rapid and selective purification of MLCK from avian smooth muscle using HPLC, employing a porous resin based weak anion (DEAE) exchange column (4). We noted, however, that the peak corresponding to MLCK was not symmetric, thus suggesting a possible

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0006-291X/91 $1.50

heterogeneity of the enzyme. By means of a different anion exchange column, (Mono Q, Pharmacia) we were able to obtain consistently three well separated peaks, each of them contained pure and active MLCK. Since the chromatographic pattern was affected by pretreatment with alkaline phosphatase of the protein, we suggest that the observed heterogeneity could be related to the existence of three forms of MLCK characterized by a different degree of phosphorylation.

MATERIALS AND METHODS

Preparation of MLCK

MLCK was purified essentially according to the procedures described by Ngai et al (5), with the modifications reported in (6). Briefly, 100 gr of chicken gizzard were homogenized in a buffer containing Triton X-100 and centrifuged at 17,000xg for 20 minutes. The resulting pellet was homogenized and centrifuged two other times with the same buffer but in the absence of Triton X-100. The pellet was extracted with 400 ml of a buffer which contained 25 mM $MgCl_2$. After centrifugation the clear supernatant was loaded on a DEAE-Sephacel column (2.6 x 33 cm) and MLCK was eluted by a linear gradient of NaCl. The fractions corresponding to MLCK were pooled, dialyzed and then loaded on a Blue Trisacryl column (1.6 x 20 cm). MLCK was eluted by NaCl gradient. The purified MLCK preparations,after dialysis against 40mM Tris/HCl pH=7.6, 20mM NaCl and 1mM DTT, were checked by gel electrophoresis and stored at -20°C.

Chemicals for HPLC

The water used for chromatography was deionized [Milli-Q (MIllipore)]. All other reagents were of analytical-reagent grade. The mobile phase solutions were passed through a 0.45-mm Millipore filter and degassed prior to chromatography.

High-performance liquid chromatography

A Perkin-Elmer high-performance liquid chromatographic system was used. This consisted of a Series 3B liquid chromatograph equipped with a Model LC75 variable-wavelenght UV detector monitoring at 280 nm and a Reodyne Model 7105 sample injector with a 1-ml sample loop.
Anion-exchange separations were performed on a Mono Q column from Pharmacia. All chromatographic runs were carried out at room temperature at a flow-rate of 1.0 ml/min. Solvent A contained 20 mM Tris/HCl pH=8.0, while solvent B contained 20 mM Tris/HCl pH=8.0 and 0.5 M NaCl. A linear gradient composed by the following steps was begun immediatly upon injection of the sample : from 0 to 60 % solvent B in 6 min, from 60 % B to 75 % B in 20 min, from 75 % B to 100 % B in 2 min. A 2-min reverse linear gradient and a short re-equilibration period were necessary for satisfactory reproducibility. The peaks were collected manually and analyzed by SDS-gel electrophoresis.

Treatment with alkaline phosphatase

200 μg of MLCK purified by standard techniques were incubated for 30 min at 37°C with 50 μg of alkaline phosphatase (Calf intestine, grade II, Boehringer) in a buffer containing 40 mM Tris-HCl pH 7.6, 20 mM NaCl, 1 mM DTT. After the incubation MLCK was immediately injected in the anion exchange column.

SDS-gel electrophoresis

10% polyacrylamide SDS-gel electrophoresis was carried out according to Laemmli (7). The 0.75-mm gels were run at room temperature. The gels were stained with Coomassie blue or silver technique.

Assay of myosin light chain kinase activity
Myosin light chain kinase activity was assayed in a volume of 0.1 ml of 20 mM Tris/HCl pH=7.3, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 50 μg/ml BSA, 10 μg calmodulin/ml, 185 μg/ml myosin light chains, 10^{-9} M MLCK and 0.1 mM ATP (0.2 Ci/mmol), at 24° C for 4 minutes. The assay was terminated by addition of 0.1 ml of a solution containing 20% trichloroacetic acid and 4% sodium pyrophosphate. After Millipore filtration and washing with 50 ml of 10%trichloroacetic acid and 2% sodium pyrophosphate the filters were counted.

One-dimensional peptide mapping
MLCK (33 μg/ml) present in the chromatographic peaks was incubated at 24° C with S.aureus V8 protease (8 μg/ml) in a buffer containing 1 mM Tris-HCl pH 6.8, 1 M NaCl, 0.1 mM $MgCl_2$, 1% SDS, 20 % glycerol. Aliquots (100μl) of reaction mixtures were withdrawn at 10, 60, 120 min and 10 μl each of 10 %SDS and 2-mercaptoethanol were immediately added. The mixtures were immersed in a boiling water bath for 10 min. Then they were analyzed on a 10% polyacrilamide gel, along with undigested MLCK.

RESULTS

When chicken MLCK purified by standard procedures is applied to a Mono Q column and eluted with a gradient of NaCl, the pattern shown in Fig. 1,A is obtained. The chromatogram is composed mainly by three peaks termed α,β and γ in order of increasing retention time. The second component (β) is the more abundant, while α and γ are present in less amount. Very similar results were achieved with a different preparation of chicken MLCK as demonstrated in Fig. 1,B. Interestingly, the same basic chromatographic pattern is obtained with turkey MLCK (Fig.1,C), but in this latter case the relative proportion of the three peaks is different as compared to chicken MLCK, in that α and β are present in about the same amount.

The electrophoretic analysis of the chromatographic effluents, stained with the high sensitive silver technique is shown in Fig. 2, panel A. Each peak derived from chicken MLCK contained only protein corresponding to MLCK and no smaller peptide is present, thus negating the possibility that the chromatographic heterogeneity observed was due to proteolysis. Identical results were obtained for turkey MLCK (Fig.2, panel B).

The protein present in each of the three peaks was tested for enzymatic activity (see Table I). The values are the mean of two experiments carried out on two different MLCK preparations. All of the peaks retain kinase activity but the specific activity of β is about twice that of α and γ. These findings support the evidence that proteolysis did not cause the chromatographic heterogeneity.

If the proteins contained in the three peaks were the product of different genes, i.e. were isoforms of MLCK, one could expected that their peptide mapping was indeed different. To test this possibility we have performed the proteolytic

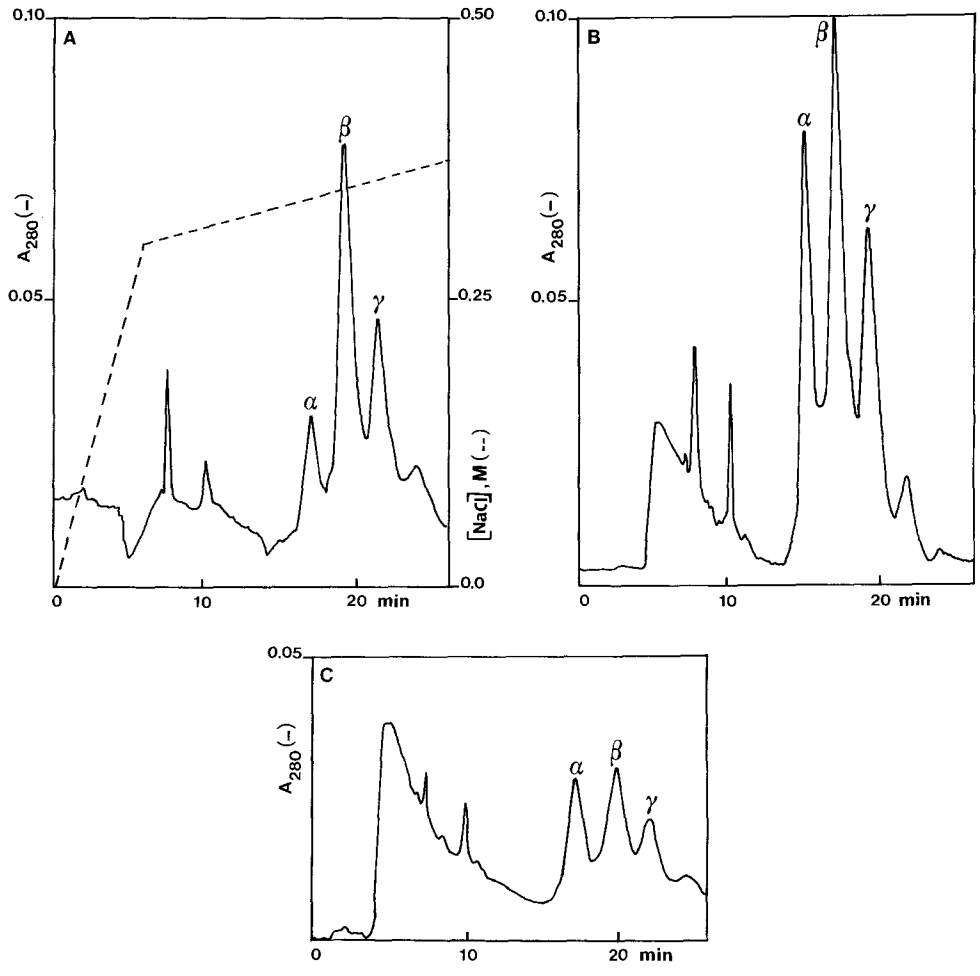

Figure 1. Anion-exchange HPLC of smooth muscle MLCK.
MLCK purified by standard procedures was chromatographed at 1.0 ml/min on a Mono Q column equilibrated with 100% solvent A. A linear gradient composed by the following steps was begun immediately upon injection of the sample : from 0 to 60 % solvent B in 6 min, from 60 % B to 75 % B in 20 min, from 75 % B to 100 % B in 2 min. Solvent A contained 20 mM Tris/HCl pH 8.0, while solvent B contained 20 mM Tris/HCl pH 8.0 and 0.5 M NaCl.
Key: (A) chicken MLCK (0.64 mg), preparation #1; (B) chicken MLCK (1.0 mg), preparation #2; (C) turkey MLCK (0.2 mg). Peaks labelled α,β,γ were collected.

digestion of the proteins contained in α, β, γ. The results are illustrated in Fig. 3. One-dimensional peptide map revealed that the three peaks gave origin to very similar profiles at all times of digestion. However from these experiments we cannot conclude with certainty that the three proteins are identical, since that with these methods it is very difficult to detect single aminoacidic substitution in the polypeptide chain.

It is well known that MLCK can be phosphorylated by a variety of protein kinases. Autophosphorylation of MLCK has also been reported (see ref.8 for a

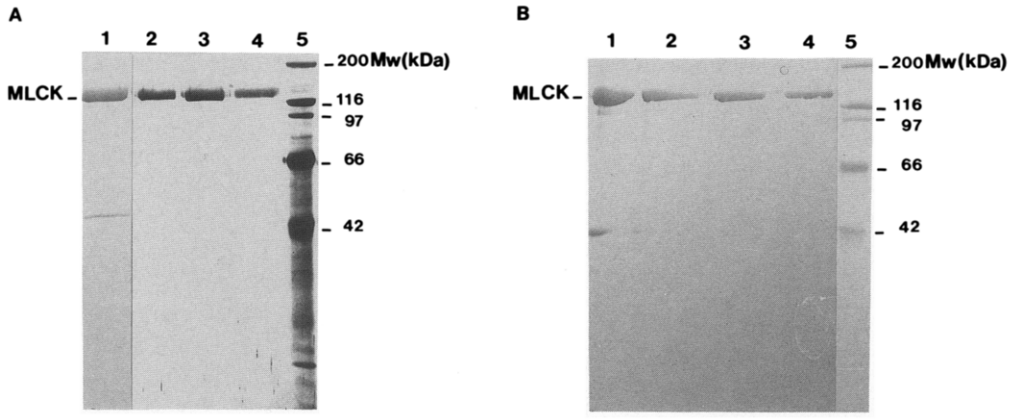

Figure 2. 10% polyacrylamide SDS-gel electrophoretic analysis of the peaks obtained after anion-exchange chromatography.
Panel A: chicken MLCK. Key: (1) Starting material: MLCK purified by standard procedures; (2) peak α; (3) peak β; (4) peak γ; (5) molecular weight standards. Lane (1) was stained with Coomassie blue; lanes (2)-(5) were stained with silver technique.
Panel B: turkey MLCK. Key: (1) Starting material: MLCK purified by standard procedures; (2) peak α; (3) peak β; (4) peak γ; (5) molecular weight standards. All lanes were stained with Coomassie blue.

general review on these topics). Therefore there is the possibility that the heterogeneity of MLCK preparations observed by anion-exchange chromatography is due to different degree of phosphorylation of the protein. To test this possibility we have treated with alkaline phosphatase MLCK prior to chromatography. The relative results are depicted in Fig. 4. The analysis of the chromatographic patterns revealed that the treatment with alkaline

TABLE I

CHICKEN MLCK SPECIFIC ACTIVITY BEFORE AND AFTER ANION-EXCHANGE HPLC

Fraction	Activity (μmol Pi / min / mg Pr)
MLCK (before HPLC)	3.0
MLCK (after HPLC)	
Peak α	0.8
Peak β	1.8
Peak γ	0.8

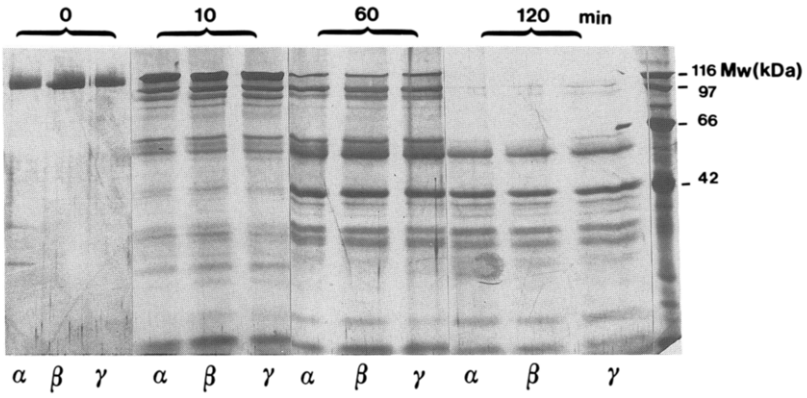

Figure 3. V8 protease digests of the peaks obtained after anion-exchange chromatography.
Silver-stained gel of V8 protease digestion performed as described under "Materials and Methods". Final concentrations in the reaction mixtures were 33 µg/ml MLCK and 8 µg/ml V8 protease. The digest mixtures were subjected to electrophoresis on a 10% SDS-polyacrylamide gel (3µg of MLCK/lane).

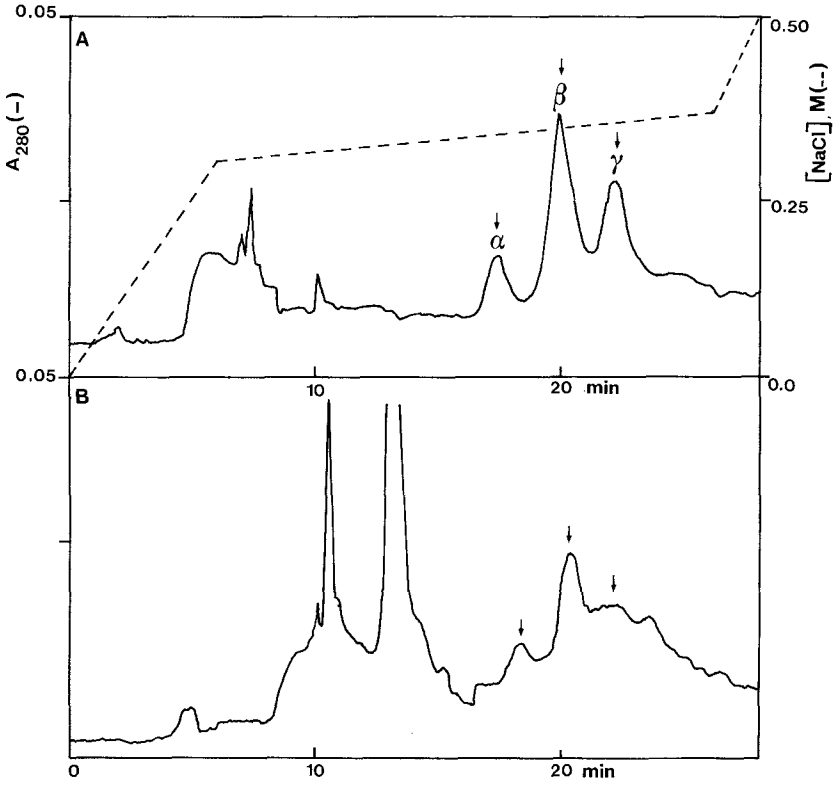

Figure 4. Anion-exchange HPLC of MLCK after treatment with alkaline phosphatase.
MLCK purified by standard procedures was incubated with alkaline phosphatase as described under "Materials and Methods". After the appropriate incubation time MLCK was injected in a Mono Q column and chromatographed as indicated in the legend of Figure 1.
Key: (a) MLCK incubated in the absence of alkaline phosphatase; (b) MLCK incubated in the presence of alkaline phosphatase. Note the dramatic reduction of peak γ in treated MLCK.

phosphatase results in a marked reduction of the γ component (compare in Fig. 4 panel A with panel B). The electrophoresis of control MLCK, i.e. MLCK incubated for 30 min at 37 C in the absence of alkaline phosphatase, revealed that no significant proteolysis occurred (result not shown).

DISCUSSION

Recent investigations have revealed polymorphism of many contractile proteins in striated muscles. Several isoforms, both tissue and species specific, have been described for the costituents of thick and thin filaments. Also MLCK exists as several distinct molecular species. In fact separate forms are present in skeletal muscle, heart, and smooth muscle or non-muscle tissue. However less is known about the presence of isoforms within the same tissue. Only for rabbit skeletal muscle MLCK sequence analysis indicates the presence of approximately equal quantities of two isoforms differing in a single amino acid replacement (3). As far as protein kinase family is concerned, isoforms were found for cAMP-dependent protein kinase (cAPK) and protein kinase C (PKC) (see ref.9 for a review).

In this paper we present some evidences about the possibility of the existence of different isoforms of MLCK in chicken gizzard smooth muscle. The heterogeneity was consistently observed by anion exchange high-performance liquid chromatography in different preparations not only of chicken but also turkey MLCK. Also Garone reported a similar chromatographic pattern for turkey MLCK (8), in which the first two peaks were present in about the same amount. Therefore it is unlikely that we are dealing with artifacts originating during the protein purification. Furthermore the electrophoretic analysis of the protein present in the three peaks always revealed the presence of one component only with a mobility correspondent to that of unfractionated MLCK. Even though the slab gels were stained with the high sensitive silver technique (whose lowest limit of detection is about 10 ng) no peptide is present below MLCK, thus suggesting that proteolysis is not at the origin of the three peaks. A further evidence that each peak contained MLCK is that the proteins are characterized by a Calcium-calmodulin dependent kinasic activity, phosphorylating the 20,000-dalton light chain of myosin. These tentative isoforms should be ascribed to different levels of phosphorylation of the protein rather than to differences in primary structure. Even though our experiments with alkaline phosphatase are not definitive, they clearly suggest that the heterogeneity we observed by anion exchange chromatography, is linked to the presence of phosphate bound to the protein. If there are indeed three distinct forms of MLCK, elucidation of the physiological significance will lead to a fuller understanding of the regulation of smooth muscle contraction.

ACKNOWLEDGMENTS

We are indebted to Prof. F. Meggio for providing us with alkaline phosphatase and to Prof. R. Sabbadini for helpful discussions. We also thank the slaughter-house "SIVAL - CARNI" for providing chicken and turkey gizzards and Mr. S. Belluco for skillful technical assistance. This research was supported in part by a grant from GLAXO S.p.A, Verona, Italy.

REFERENCES

1 Zanellato, A.M.C., Borrione, A.C., Giuriato, L., Tonello, M.M., Scannapieco, G., Pauletto, P. and Sartore, S. (1990) Dev. Biol. 141, 431-446.
2 Walsh, M.P., Bridenbaugh, R., Kerrik, W.G.L. and Hartshorne, D.J. (1983) Fed. Proc. FASEB 42, 45-50.
3 Takio, K., Blumenthal, D.K., Walsh, K.A., Titani, K. and Krebs, E.G. (1986) Biochemistry 25,8049-8057.
4 Dalla Libera, L., Cavallini, P., Fasolo, M., Cavanni, P., Ratti, E. and Gaviraghi, G. (1990) Biochem.Biophys. Res.Commun. 167, 1249-1255.
5 Ngai, P.K., Carruithers, C.A. and Walsh, M.P. (1984) Biochem.J. 218, 863-870.
6 Cavallini, P., Cavanni, P., Ratti, E., Gaviraghi, G. and Dalla Libera, L. (1988) in Sarcomeric and non-sarcomeric Muscles: basic and applied research prospects for the 90's (Carraro, U. ed.) Unipress Padova, Italy, pp 677-685.
7 Laemmli, U.K. (1970) Nature 227, 680-685.
8 Garone, M.L. in Smooth Muscle Regulation (Moreland, R.S. editor), in press.
9 Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) Science 241, 42-52.