Myosin Light Chain Kinase as a Marker for Smooth Muscle: a Polyclonal Antibody Study

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Abstract

We performed immunological studies with affinity-purified antibodies raised in rabbit against chicken gizzard smooth muscle myosin light chain kinase (MLCK).

In Western blotting experiments on crude homogenates from different rat smooth muscle tissues our antibody recognizes one protein band only (135 kDa), that has a slightly higher molecular mass than chicken MLCK (130 kDa). No reactivity was found with the crude homogenate of cardiac and skeletal muscle.

The antibody reactivity was confirmed by using smooth muscle preparations in which a partial purification of MLCK was carried out by high-performance liquid chromatography (HPLC).

Immunofluorescence experiments performed on different rat tissues showed that our antibody specifically stained only smooth muscle components.

Based on these data, we conclude that MLCK, as detected by our antibody, can be used as a specific marker for smooth muscle cells.

Key words: Myosin light chain kinase, vascular smooth muscle, myosin light chain kinase antibody.

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The contractile state of smooth muscle is regulated primarily by cytosolic free Ca^{2+}-concentration. A variety of stimuli that induce smooth muscle contraction trigger an increase in cytosolic free Ca^{2+} from resting levels of 120-270 to 500-700 nM. Myosin Light Chain Kinase (MLCK) is the most important Calcium-Calmodulin-dependent enzyme which regulates actin-myosin interaction in smooth muscle by phosphorylating the 20 kDa subunit of myosin [for review see 1-8-11-13]. This event leads to force generation and/or muscle shortening. Thus the kinase, being completely inactive in the absence of Ca^{2+} and Calmodulin (CaM), plays a mayor role in regulating smooth muscle contraction, although it is suggested by some Authors [14] that, especially with respect to force development, caldesmon, and perhaps calponin, have some importance.

Several biochemical, immunochemical and immunocytochemical studies have demonstrated that cytoskeletal and cytocontractile proteins are valuable markers of smooth muscle cells [see ref. 7 for review]. In spite of the pivotal role played by MLCK in the regulation of smooth muscle contraction, few studies have been presented on the use of this protein as a specific smooth muscle marker. Only recent studies have used polyclonal antibodies to identify by means of Western blot analysis MLCK in the myometrium of pregnant sheep [12]. A comparison between different MLCKs purified from turkey gizzard, bovine tracheal smooth muscles and human platelets, has been carried out by deLanerolle et al [5]. They also used a polyclonal antibody. However the distribution and reactivity of MLCK in vascular smooth muscle remains rather ill defined.

In the present study we used a polyclonal antibody prepared and characterized in our laboratory [3], aiming to the following objectives: [1] evaluate the specificity of our

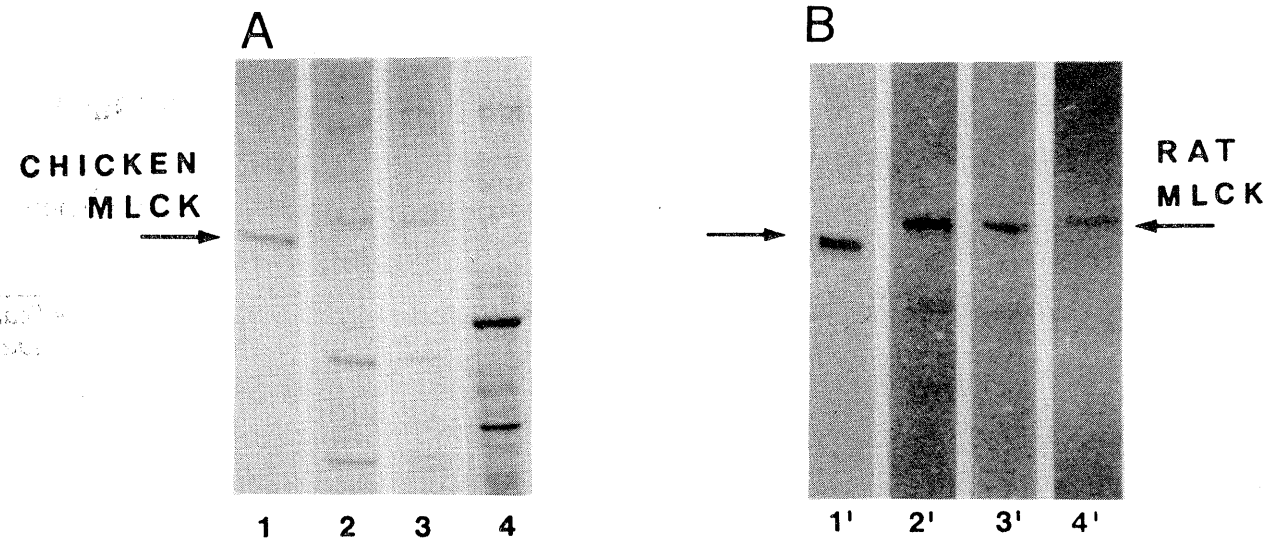

Figure 1. Characterization of MLCK from rat tissue extracts. A) Coomassie Blue stained, SDS-polyacrylamide gel electrophoresis (6% acrylamide gel) and B) Western Blot analysis using a polyclonal antibody against MLCK, of extracts from different rat tissues. Key: Lane 1-1', MLCK from chicken gizzard (0.06 μg); Lane 2-2', uterus; Lane 3-3', bladder; Lane 4-4', aorta. Approximatively 20 μg of protein were loaded per lane. In panel A the arrow points to chicken gizzard MLCK. In panel B the leftward arrow points to rat smooth muscle MLCK, the rightward one, pointing to chicken MLCK, shows that the electrophoretic mobility of the latter is slightly higher.

antibody for smooth muscle tissue; [2] analyse MLCK distribution in different smooth muscle districts.

Materials and Methods

Preparation of Tissue Extracts

Tissues used in this study were taken from adult rats after sacrifice and immediately frozen in liquid nitrogen. Frozen tissues (1 gram or less) were homogenized in 3 ml of a buffer solution of the following composition: 62.5mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol. The homogenates were boiled for 5 minutes and centrifuged for 10 minutes at 2000 rpm at 4°C temperature. The supernatant fraction was stored at -80°C in various aliquots until electrophoresis was carried out. Protein concentration was measured by using the bicinchoninic acid protein assay method (BCA Protein Assay; Pierce Chemical Co.) according to the manufacturer's directions.

Preparation of Mg^{2+} Extracts from Tissues

The Mg^{2+} extracts were prepared following the protocol described by Sobieszek and Barylko [15]. Briefly, a small amount of tissue (1 to 7 grams) was homogenized in a buffer solution (4 ml for 1g of tissue) containing 0.05% (v/v) Triton x-100. The homogenate was then centrifuged at 13000 rpm for 20 minutes. The resulting pellet was homogenized and centrifuged two more times with the same buffer but in absence of Triton x-100. The pellet was extracted with a buffer solution containing 40 mM Tris-HCl pH = 7.5; 60 mM NaCl; 25 mM $MgCl_2$; 1 mM DTT; 1 mM EGTA (4 ml for 1g of tissue). After centrifugation the supernatant fraction was stored at -80°C in various aliquots until electrophoresis or HPLC analysis were performed.

Preparation of Polyclonal Antibodies

Specific IgG to purified chicken gizzard MLCK were raised in adult rabbits as described by Dalla Libera [3].

Western blotting

The electrophoretic transfer of proteins from slab gel [9] onto a nitrocellulose sheet was carried out under the general conditions of Towbin et al. [16], as described in detail by Biral et al. [2]. The sheets were stained according to the methods of Learly et al. [10].

High-Performance Liquid Chromatography (HPLC)

A Bio-Rad high-performance liquid chromatographic system was used, equipped with a LC75 variable-wavelenght UV detector (Perkin Elmer) and a Reodyne 7105 sample injector with a 1-ml sample loop. HPLC anion-exchange separations were performed on an analytical (75 x 7.5 mm I.D.) Bio-Gel DEAE-5-PW column from Bio-Rad, as described in Dalla Libera et al. [4]. Peaks were collected manually and analyzed for MLCK reactivity.

Immunofluorescence

Immunofluorescence experiments were performed on cryosections from normal adult rat tissues. Since MLCK is rapidly degratated at room temperature, tissues were frozen within few seconds after trimming. Five mm thick nonfixed sections of rat tissues were stained with the antibody against chicken gizzard MLCK for 20 minutes at 37°C in a humidified chamber. The antibody solution was

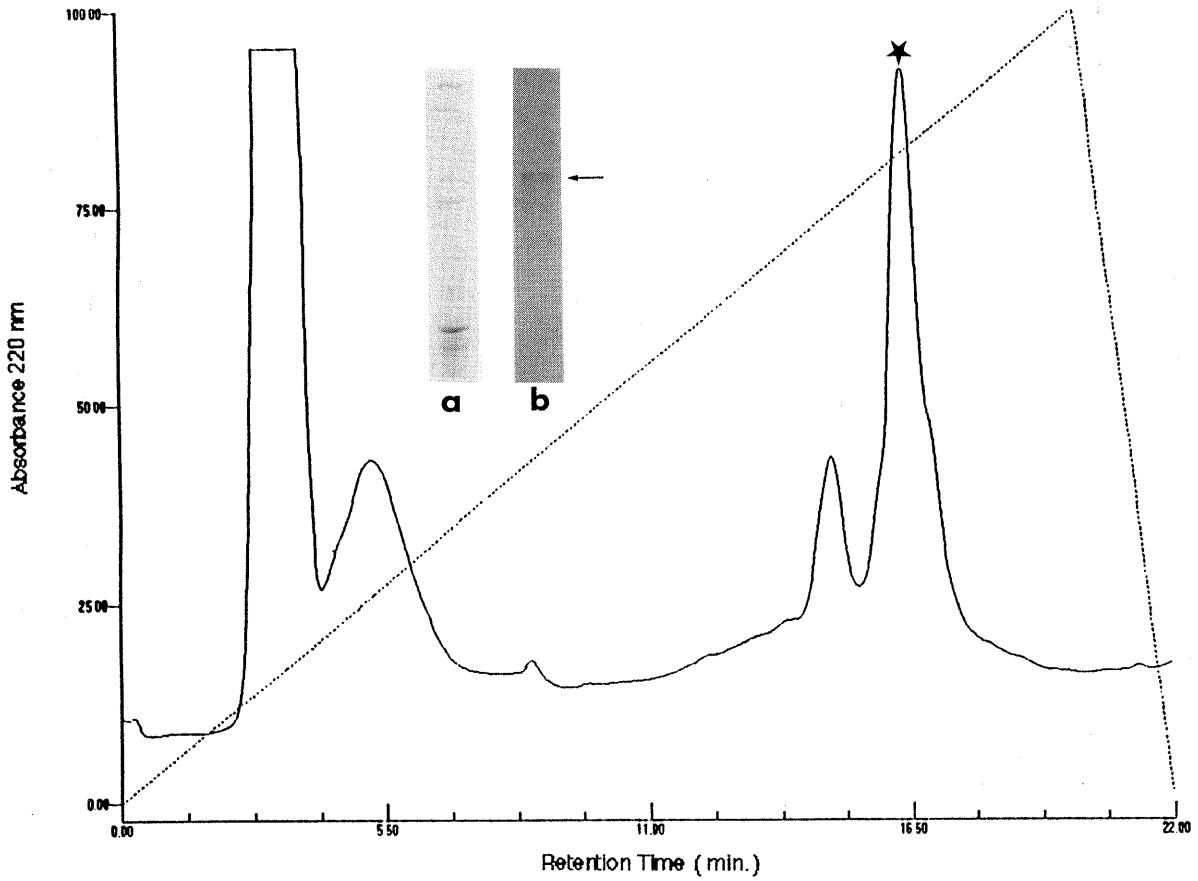

Figure 2. Anion-exchange Chromatography on Bio-Gel DEAE-5-PW Column of rat aorta Mg^{2+} extract. Protein was monitored by Absorbance at 220nm and bound proteins were eluted with a NaCl gradient. Inset: analysis of the effluents corresponding to the star-marked peak: a) SDS-polyacrylamide gel electrophoresis (6% acrylamide gel) Coomassie Blue stained. b) Western Blot analysis with anti-MLCK polyclonal antibodies.

diluted 40 times. They were then washed twice with PBS (for 5 minutes and for 10 minutes, respectively). The bound antibody was revealed by rhodamine isothiocianate-labelled (RITC) polyclonal antirabbit-IgG (Dako) under the same conditions described above. After being washed twice with PBS, sections were mounted with Elvanol. Controls were performed on samples of different tissue sections. The reactivity of non immune IgG and of the fluorescent antibody alone was also tested. Specimens were examined with a Zeiss Axioplan microscope equipped with an epi-illumination apparatus and an HBO 100 W high-pressure mercury light , with Neofluar 10 X N.A. 0.30, 20 X N.A. 0.50, 40 X N.A. 0.75 objective lenses. Micrographs were taken with Kodak Technical Pan film developed in HC-110 developer.

Results

Immunoblot analysis of smooth tissues

Crude extracts of rat smooth muscle tissues were tested for the presence of MLCK by means of immunoreactivity with polyclonal antibodies raised against adult chicken MLCK (Fig. 1). It is clear that in all the rat tissues analyzed (uterus, bladder and aorta) the antibody reacted with a protein band present in the whole homogenate, the electrophoretic mobility of which is slightly lower than that peculiar to adult chicken MLCK. Molecular weight determinations indicate that rat MLCK is characterized by a molecular mass of 135 kDa. However, the rat ileum extracts showed only a tiny immunoreactive band with the same electrophoretic mobility (result not shown). A more intense staining could have been expected since rat ileum is a "fast " muscle among smooth muscles. It is likely that the tiny reactivity is due to the presence of proteolytic enzymes, rapidly degradating MLCK, despite the tissue was immediately frozen in liquid nitrogen and homogenized in boiling SDS solution just before electrophoretic analysis.

Both skeletal and cardiac muscles did not react with anti-MLCK antibody (results not shown).

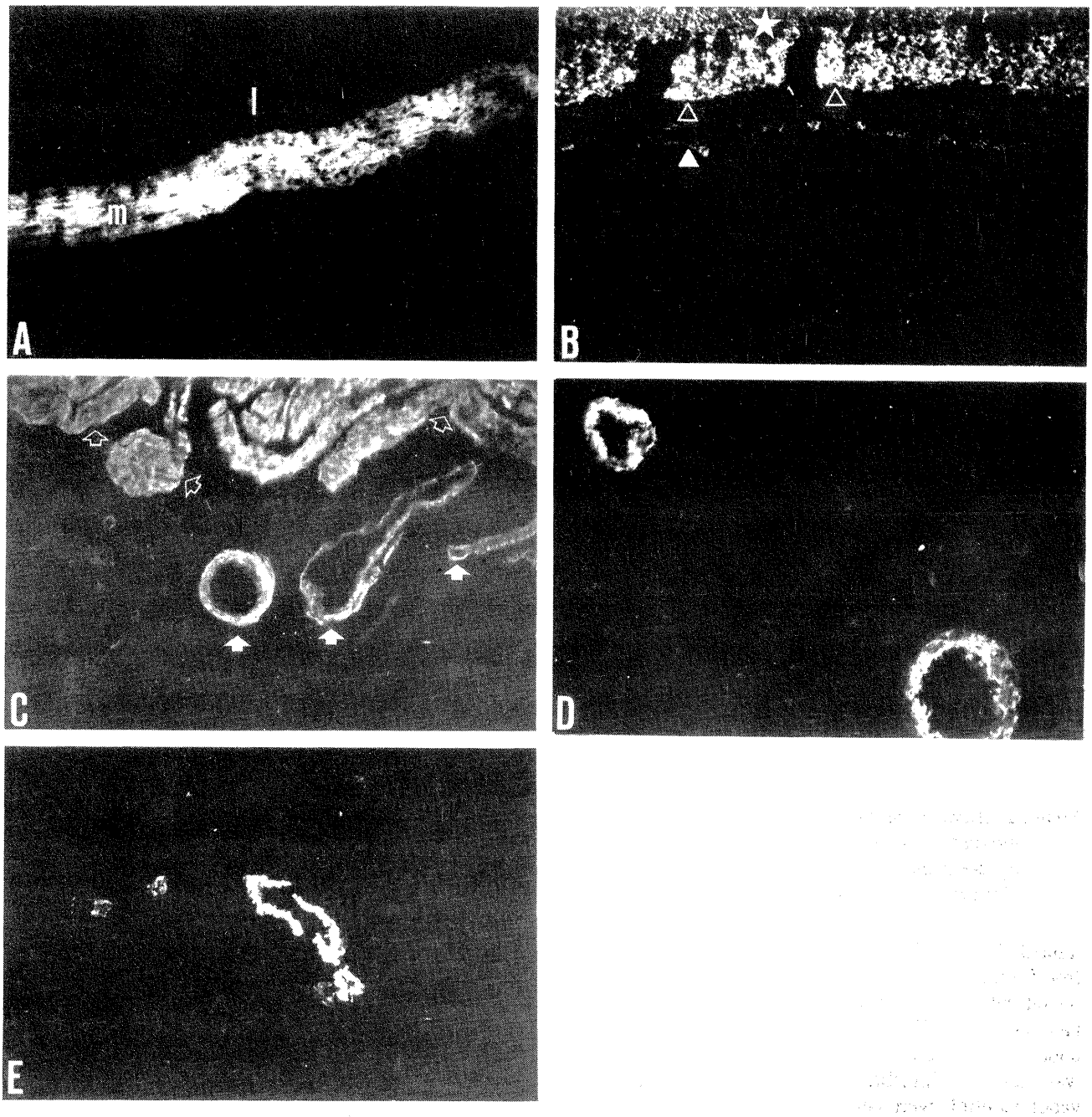

Figure 3. Immunofluorescence staining of rat tissues. (A) Aorta: (l) lumen, (m) media. (B) Ileum: Full stars: longitudinal muscle layer. Open triangles: circular muscle layer. Full triangles: muscularis mucosae. (C) Urinary bladder: Open arrows: smooth muscle bundles. Filled arrows: arteriolae. (D) Kidney. (E) Skeletal muscle.

High-Performance Liquid Chromatography of MLCK

Since we previously reported [4] that HPLC can be successfully used to purify MLCK from complex mixtures, such as the magnesium extracts of turkey and chicken gizzard, we applied this method to different smooth muscle tissues in the rat. We collected all the effluents corresponding to major chromatographic peaks and we performed an immunoblot against MLCK antibodies. The only peak which always showed a reactivity with our antibody was that eluting after 17 minutes (peak flagged by a star in Fig. 2 which shows a typical rat aorta chromatogram). Although different chromatographic profiles were obtained for other rat tissues, the peak reacting with the anti-MLCK antibody was eluted at the same retention time (results not shown). The observed retention time is very similar to that reported for chicken gizzard MLCK [4].

Immunofluorescence analysis

Immunofluorescence localization studies, illustrated in Figure 3, demonstrated that rat smooth muscles are selectively stained by the rabbit antibody against chicken MLCK. In the case of thoracic aorta (Fig. 3A) the whole vascular wall is stained. In the ileum (Fig. 3B) the positivity is localized to the circular and longitudinal muscle layers and to the muscularis mucosae, whilst in the urinary bladder (Fig. 3C) the positivity is confined to the arteriole and to the smooth muscle bundles of the wall. In the kidney (Fig. 3D), although a weak background fluorescence was present, the arteries are more intensely stained. Studies on sections prepared from rat skeletal muscle (Fig. 3E) showed that the anti-MLCK antibodies do not bind to antigenic determinants found in striated cells, but do bind to vascular smooth muscle. Skeletal muscle vessels were in fact found to be markedly positive. The positivity was confined to muscular tissue since the endothelium was not stained. Cardiac muscle showed the same reactivity pattern of the skeletal one.

Discussion

MLCK is a Ca^{2+}-CaM dependent enzyme responsible for the regulation of actin-myosin interaction in smooth muscle. In the presence of calcium the enzyme phosphorylates the regulatory light chain of myosin, permitting a cyclic interaction of myosin heads with actin filaments. The kinase, being completely inactive in the absence of CaM and calcium, represents the primary molecular switch leading to contraction of this muscle type. In view of its significance there have been numerous studies aiming to determine the molecular mechanisms by which the Ca^{2+}-CaM complex activates this target enzyme. However it is known that the contractile state of smooth muscle varies in different districts. These differences might be sustained by a different tissue distribution of MLCK. Unfortunately, only few studies concerning the distribution of this enzyme are available.

In the present report we show that the polyclonal antibody anti-MLCK can be used as a specific marker for smooth muscle cells, since it selectively recognizes these structures.

Our Western blotting experiments revealed that in all rat smooth muscle tissues MLCK molecular weights were fairly similar, regardless of muscle type. The same protein, characterized by a molecular weight of 135 kDa, is present both in phasic smooth muscle, such as uterus, and in tonic smooth muscle, such as aorta. This value is in agreement with the molecular weight reported by Gallagher et al. [6] for rat smooth muscle MLCK. In this paper we confirm that MLCK is a species- and not tissue-specific protein [6], therefore extending to rat what is known from the literature for other species. The antibody we used is highly specific for MLCK present in smooth muscles and gave no reactivity with skeletal and cardiac muscle cells.

The antibody reactivity was confirmed by using smooth muscle preparations in which a partial HPLC purification of MLCK was carried out. In these experiments we observed a reactivity with a peak eluting with the same retention time observed for chicken gizzard MLCK [4]. This finding confirms the structural homology between the MLCK isoforms obtained from chicken and rat. This homology has been already suggested by the antibody cross-reactivity.

Immunochemistry is a sensitive method for detecting the tissue presence of a particular antigen. Our antibody specifically stained smooth muscle cells in the different tissues examined. No reactivity was observed with tissues different from smooth muscle such as endothelial and epithelial cells, skeletal and cardiac myocytes. Therefore immunocytochemical experiments confirmed the immunological variability of smooth- and striated-muscle MLCKs. The evidence for that comes from the skeletal muscle sections in which our antibody stained selectively small blood vessels but not skeletal and cardiac fibers.

The high selectivity demonstrated by this antibody for vascular smooth muscle cells gives the potential for studying pathological conditions in which the contractile state of the arterial wall is altered, for instance hypertension.

Moreover, a lot of effort has been put in finding reliable markers of differentiation in the atherosclerotic process [17]. MLCK- antibodies, since highly specific for smooth muscle cells, could help to distinguish, within the atherosclerotic plaque, different smooth muscle cells subpopulations or different phenotypes of intimal-medial smooth muscle cells. This objective maybe deserves consideration.

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