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CALDESMON: ANOMALOUS ELECTROPHORETIC BEHAVIOUR IN POLYACRYLAMIDE GEL

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In SDS gels caldesmon (Mr=140 kDa) and myosin light chain kinase (Mr=130 kDa) migrate as a closely separated doublet. When glycerol is added to the gel caldesmon is characterized by an anomalous migration. In fact under this latter condition, the distance between caldesmon and myosin light chain kinase is enhanced by two-three times. The nature of putative caldesmon and myosin light chain kinase was confirmed by physicochemical, enzymatic and immunological methods.

In smooth muscle the enzyme myosin light kinase (MLCK) catalyzes the phosporylation Ca and calmodulin dependent of a specific class of myosin light chain known as the phosporylatable or P-light chain (1). It is widely believed that P-light chain phosphorylation at Serine-19 is a prerequisite for the initiation of contraction (2). There is now increasing evidence that also the thin filament of smooth muscle contains a Ca-regulatory system (3). In fact it has been suggested that caldesmon, a protein associated to the actin thin filament may regulate the binding of myosin to actin. Notwhistanding caldesmon is a protein present in substantial quantities within the smooth muscle cells it is surprising that it has only recently been discovered (4). This may be due to its molecular weight which is similar to that of MLCK: 140,000 and 130,000 daltons respectively. In fact in SDS gel electrophoresis the two proteins migrate as a closely spaced doublet suggesting in the past

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that MLCK might be a dimer. It is now clear, however, that this 140,000 daltons protein is a distinct protein, since it lacks MLCK activity (5).
In this paper we present a simple electrophoretic method, basically carried out according to Laemmli (8), that allows to separate unambiguously caldesmon and MLCK from total extracts of smooth muscle. We have found that the adding of glycerol to both the stacking and the separating gel on the one hand greatly slows down the mobility of caldesmon and on the other hand does not affect significantly the mobility of MLCK. In this way the distance between the two electrophoretic bands is enhanced by about two-three times. We think that with our method, taking advantage of the anomalous migration of caldesmon, the evaluation of the content of both caldesmon and MLCK can be possible when their purification cannot be carried out with the classical methods on small amounts of tissue

MATERIALS AND METHODS

Purification of MLCK

MLCK was purified essentially according to the procedures described by Ngai et al (5). Briefly, 100 g of chicken gizzard were homogenized in a buffer containing Triton X-100 and centrifuged at 17,000xg for 15 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 Mg. After centrifugation the clear supernatant (high Magnesium extract) 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 Blue Trisacryl (IBF) column (1.6 x 20 cm). MLCK was eluted by NaCl gradient. The purified MLCK preparations were concentrated by inverted dialysis and stored at -20 C.

Caldesmon purification

Caldesmon was partially purified according to Bretscher (6). Briefly, fresh smooth muscle from chicken gizzard was minced, blended in extraction buffer and heated for 5 min in a boiling water bath. The heated material was chilled on ice and clarified by centrifugation. The supernatant contained mainly caldesmon, as judged by SDS-polyacrylamide gel electrophoresis.

Myosin purification

Myosin was purified according to the method reported by Ikebe and Hartshorne (7).

SDS gel electrophoresis

One dimensional gel electrophoresis was carried out essentially according to Laemmli (8) with 6% polyacrylamide in the separating gel.

The stacking gel was 4% polyacrylamide. When appropriate , all the gels, both the stacking and the separating, contained 37,5% glycerol.

Western Blot Analysis
The electrophoretic transfer of proteins from Laemmli slab gel on to nitrocellulose sheets was carried out under the general conditions of Towbin et al., (9). The nitrocellulose filter was first incubated with monoclonal antibodies against chicken gizzard caldesmon (generous gift of Prof. J.V. Small, Salzburg, Austria) and then with alkaline phosphatase-conjugated anti-mouse IgG as the second antibody. The sheets were stained according to the methods of Leary et al., (10).

Assay of myosin light chain kinase activity
The reaction mixture (1.0 ml) contains 50 mM Tris-HCl pH 7.5, 1.0 mM $MgSO_4$,125 mM KCl, 0,1 mM $CaCl_2$ (or 1.0 mM EGTA), about 2 µg/ml calmodulin, 5 µg/ml myosin ligth chain kinase, and 2.5 mg of myosin. Samples are preincubated at 25 C for 4'; the reaction is initiated by addition of 10 µl of 100 mM ATP solution (final concentration 1.0mM). After 10' of incubation at 25 C, an aliquot of each sample is withdrawn and treated with the same volume of glycerol-urea-PAGE sample solution to stop the reaction. The extent of phosphorylation is monitored using glycerol-urea-PAGE (11). This gel separates the phosphorylated (P-LC_{20}) and nonphosphorylated (LC_{20}) 20 KDa myosin light chains as well as the 17 KDa myosin light chain (LC_{17}) on the basis of charge while myosin heavy chain (MHC) does not enter into the gel.

RESULTS

When the high-Mg extract of gizzard myofibrils is analyzed by SDS-polyacrylamide gel electrophoresis four major bands are visible: actin (42 kDa), filamin (240 kDa) and two closely migrating proteins which molecular weight is 140 kDa (protein a) and 130 kDa (protein b), respectively (Fig.1, lane 2 and inset). We have found that the adding of glycerol to both the stacking and the separating gel affected to different extent the electrophoretic mobilities of these proteins. We like to point out that the use of these conditions on the one hand greatly slows down the mobility of protein a and on the other hand slightly modifies the mobility of protein b. In this way the distance between the two bands is enhanced by about two-three times (Fig. 2 lane b). On the basis of the data reported in literature the slower migrating protein characterized by an apparent mass of 140 kDa could be caldesmon, while the faster migrating protein characterizes by an apparent mass of 130 kDa could be MLCK.

In order to identify unambiguously these two polypeptides we have further purified caldesmon and MLCK from high-Mg extract of smooth

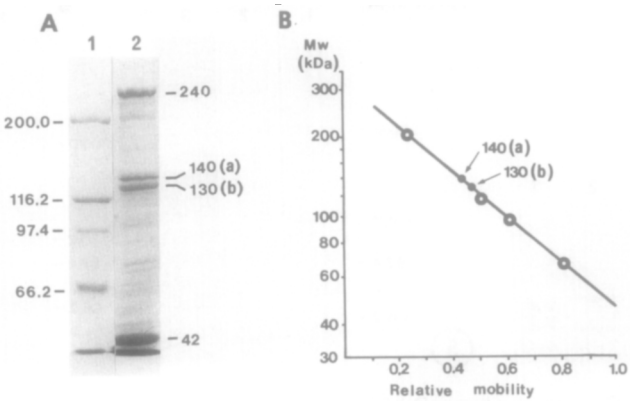

Figure 1_ SDS 6% polyacrylamide gel electrophoresis carried out in the absence of glycerol of proteins present in high-Mg extract of chicken gizzard.
Panel A. Coomassie blue staining. Key: 1) molecular weight standards in kilodalton (BioRad); 2) high-Mg extract of chicken gizzard. The position of filamin (240 kDa) and actin (42 kDa) is indicated. Letters a and b refer to protein of Mr = 140 kDa and 130 kDa, respectively.
Panel B. Molecular weight determinations.

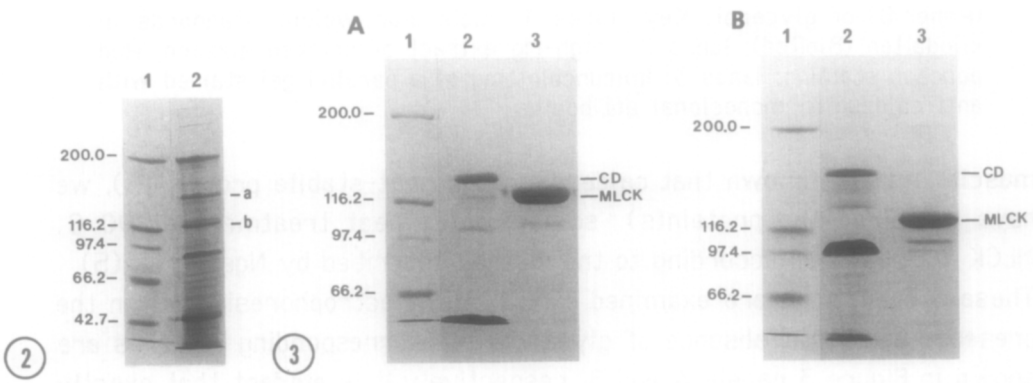

Figure 2_ SDS 6% polyacrylamide gel electrophoresis carried out in the presence of glycerol of proteins present in high-Mg extract of chicken gizzard.
Key: 1) molecular weight standards in kilodalton (BioRad); 2) high-Mg extract of chicken gizzard. Letters a and b refer to protein of Mr = 140 kDa and 130 kDa, respectively present in Figure 1 panel A.

Figure 3_ SDS 6% polyacrylamide gel electrophoresis of partially purified caldesmon and purified myosin light chain kinase.
Panel A: the electrophoresis was carried out in the absence of glycerol.
Panel B; the electrophoresis was carried out in the presence of glycerol as detailed in Materials and Methods section.
Key: 1) molecular weight standards in kilodalton (BioRad); 2) heat-soluble -extracted proteins derived from chicken gizzard homogenate; 3) purified myosin light chain kinase.
The position of Caldesmon (CD) and myosin light chain kinase (MLCK) is indicated.

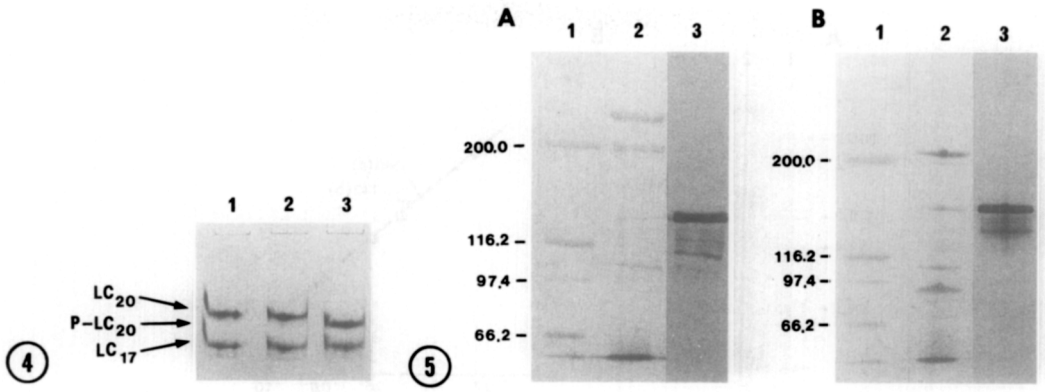

Figure 4_ Glycerol-Urea-PAGE of myosin 20kDa light chain (LC_{20}) phosphorylation experiments.
Myosin was phosphorylated with purified MLCK preparations as described in Materials and methods section. Key: 1) non phosphorylated control myosin; 2) control myosin + MLCK in the absence of Calcium; 3) control myosin + MLCK in the presence of Calcium.
LC_{20}: unphosphorylated 20 kDa light chain; P-LC_{20}: phosphorylated 20 kDa light chain; LC_{17}: 17 kDa light chain.

Figure 5_ Identification of chicken gizzard caldesmon by immunoblotting with anti-caldesmon monoclonal antibodies.
Electrophoresis of high-Mg extract from chicken gizzard was performed on 6% polyacrylamide gels in the absence (Panel A) and in the presence (Panel B) of glycerol. Key: lanes 1: molecular weight standards in kilodalton (BioRad); lanes 2: high-Mg extract of chicken gizzard, Red ponceau staining; lanes 3: immunoblotting of a parallel gel stained with anti-caldesmon monoclonal antibodies.

muscle. As it is known that caldesmon is a heat-stabile protein (6), we have examined the protein(s) soluble after heat treatment at 90 C. MLCK was purified according to the method described by Ngai et al. (5). These preparations are examined by SDS gel electrophoresis both in the presence and in the absence of glycerol. The corresponding patterns are shown in Figure 3 panels A and B, respectively.It is evident that exactly the protein present in major amount in the heat-treated extract is characterized by an anomalous mobility when the electrophoresis is carried out in the presence of glycerol, while the mobility of purified MLCK is virtually unaffected.

The identity of MLCK was confirmed by the fact that the protein prepared according to Ngai et al. (5) was able to catalyze the phosphorylation of LC20 of smooth muscle myosin in a Ca and calmodulin dependent manner (Fig.4).

The nature of the anomalous migrating protein was further investigated by immunoblotting using monoclonal antibody anti chicken caldesmon

(generous gift of Prof.Small) . By means of these experiments we are able to identify the 140,000 dalton protein as caldesmon (see Figure 5).

DISCUSSION

The results presented in this paper indicate that smooth muscle caldesmon is characterized by a slower mobility in SDS polyacrylamide gel when the electrophoretic run is carried out in the presence of glycerol. Our confidence about the nature of the protein we identified as caldesmon is based on three different experimental approaches. Firstly, in agreement with previous observations (see ref. 12) the apparent molecular mass in SDS gels, in the absence of glycerol, was 140 kDa. Secondly, the protein is heat-stable and is the main component of a heat-stable extract derived from total smooth muscle homogenate. Thirdly, it reacts with monoclonal antibodies against chicken gizzard caldesmon.

In the past it was observed that the mobility in SDS gels of myosin heavy chains, a class of polypeptides characterized by high molecular weight (about 200 kDa), was modified by the presence of glycerol (13). Furthermore it was reported that also tropomyosin displayed an altered electrophoretic mobility in urea/SDS gels (14-16). Recently a paper appeared in which, by means of sedimentation equilibrium experiments in the analytical ultracentrifuge, it has been found that caldesmon is characterized by a molecular mass of 93 kDa (12). This method depends neither on the shape nor on the molecule's ability to bind SDS. Therefore it could be possible that the observed higher molecular weight of caldesmon obtained by gel electrophoresis may be the result of anomalous migration in the presence of SDS/glycerol, like in the case of rabbit calpastatin (17). The physiochemical properties of caldesmon responsible for such behaviour are yet to be defined, but they must reflect rather unique amino acid composition.

Anyway we like to stress that in the experimental conditions we describe in this paper caldesmon migrates much more slowly than MLCK, the mobility of which is virtually unaffected by the presence of glycerol. Thus this rapid method allowing the unambiguous separation from complex mixtures of caldesmon and MLCK, two proteins that control smooth muscle contraction, may have a worthwhile value in the study of smooth muscle biology.

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