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The Influence of Caldesmon on Papain Proteolysis of Monomeric Smooth Muscle Myosin

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The influence of caldesmon on papain digestion of chicken gizzard monomeric myosin in folded (10S) conformation depends on its phosphorylation. Caldesmon exposes the head/rod junction of myosin in phosphorylated form to proteolytic attack (particularly in the presence of Ca^{2+}) and slightly screens it in unphosphorylated form. In both folded forms RLCs are protected by caldesmon, more in unphosphorylated than in phosphorylated myosin. The results indicate that the conformations of folded unphosphorylated and phosphorylated myosin are distinct and suggest that caldesmon destabilizes the regulatory domain in folded conformation of phosphorylated myosin.

INTRODUCTION

Phosphorylation of myosin RLCs plays fundamental role in the actin activation of Mg^{2+}-ATPase of myosin, cross-bridge cycling and force development of smooth muscle (for review see [1,2]). Phosphorylation of myosin *in vitro* is correlated with conformational changes in its molecule, i.e. its transition from the compact, folded conformation (that sediments at 10S) to extended one (6S) that is able to produce thick filaments [3–10]. This transition, related to the alterations of the HC in the area of the head/rod (S1/S2) junction [11–13] and located in its vicinity N-terminal portion of the RLC [14,15], is somehow transmitted to distant (about 10 nm away) catalytic and actin binding sites on myosin heads through the C-terminal part of the RLC and the HC [16–18]. Similar process (or at least some elements of it) probably occurs *in vivo*, despite that irrespectively of phosphorylation the myosin predominantly exists in filamentous state [19,20]. It may be suggested that intramolecular interactions in folded 10S form of monomeric myosin are substituted for intermolecular interactions between adjoining molecules in filament and this way inactivate it. The conformational alterations of the myosin monomers in filaments can be also under control of proteins, like caldesmon, that bind to the head/neck region of smooth muscle myosin [21–23].

Caldesmon is a thin filament protein implicated in actin-linked regulation of smooth muscle contraction (for a review see [24,25]). While the regulatory activity is associated with the C-terminal actin-binding region of caldesmon molecule, the N-terminal region binds myosin. The functional importance of the caldesmon-myosin interaction is not fully elucidated. It is suggested that cross-linking of thin and thick filaments by caldesmon plays structural role and is particularly important for organization of contractile apparatus during myogenesis.

To detect conformational alterations in smooth muscle monomeric myosin at the head/rod junction upon binding of caldesmon we have applied the technique of limited proteolysis with papain which cleaves myosin HC predominantly at the S1/S2 junction [26]. The results show

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Abbreviations: HC, heavy chain; S1 and S2, myosin subfragments 1 and 2; RLC, regulatory light chain.

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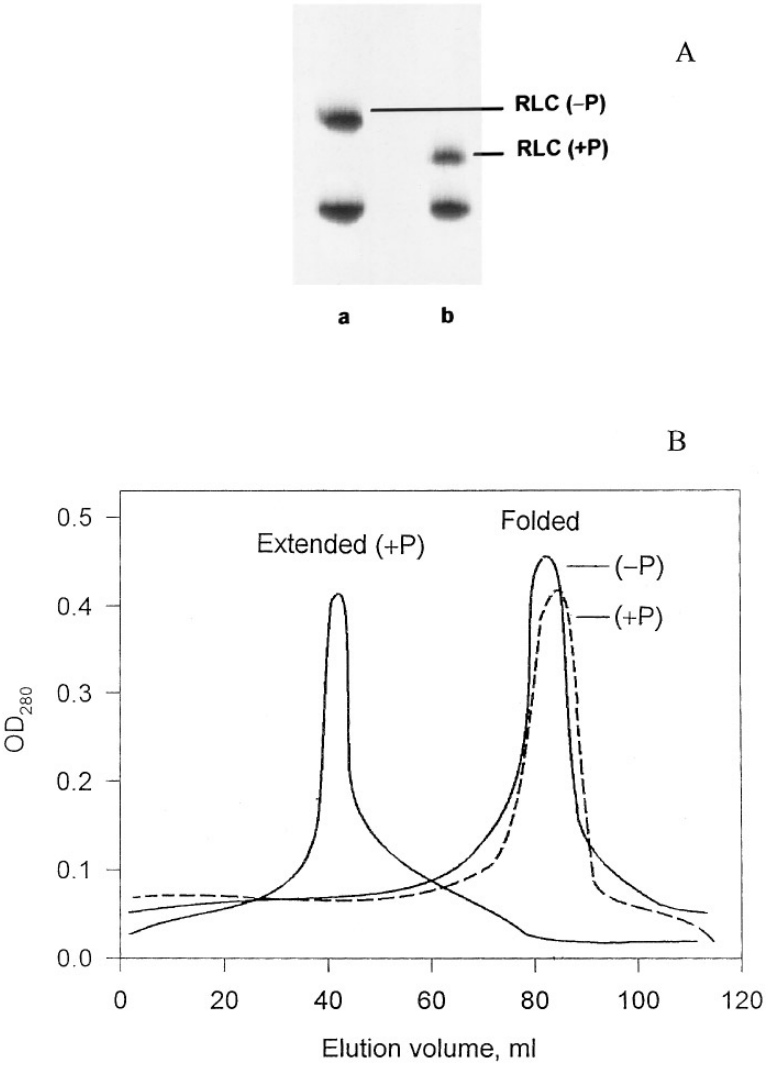

FIG. 1. (A) Urea-glycerol-PAGE of unphosphorylated and thiophosphorylated chicken gizzard myosin. Lane a, unphosphorylated; lane b, thiophosphorylated myosin. 20 mg of myosin was applied to each lane. (B) Elution profiles of myosin samples from a Sepharose CL-6B column (80×1 cm) equilibrated with buffer A containing 0.1 mM $CaCl_2$, 1 mM ATP, and either 40 or 150 mM KCl to maintain the corresponding myosin conformation.

that the effect of caldesmon on papain sensitivity of monomeric myosin depends on its phosphorylation, ionic strength and the presence of Ca^{2+}.

MATERIAL AND METHODS

Preparation of proteins. Caldesmon was prepared from frozen chicken gizzards according to the method of Bretscher [27]. Unphosphorylated myosin was isolated from fresh chicken gizzards as described by Sobieszek [28]. This myosin (although electrophoretically pure) contained endogenous myosin RLC kinase and calmodulin at the amounts sufficient for phosphorylation. Unphosphorylated myosin in 40 mM KCl, 1 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.5), 1 mM DTT (buffer A) and 1 mM EGTA was thiophosphorylated by addition of 1 mM ATPγS (Sigma) and 1.5 mM $CaCl_2$. Phosphorylation was complete in 15 min at 25 °C. Excess of nucleotide and calcium was removed by dialysis against buffer A containing 0.1 mM $CaCl_2$ or 1mM EGTA. Only fully thiophosphorylated and fully unphosphorylated preparations of myosin (Fig. 1A) were used in experiments. Prior to digestion, myosin in corresponding buffer was

depolymerized by addition of 1 mM ATP and remaining filamentous material (about 5% and 60% in the case of unphosphorylated and phosphorylated myosin, respectively) was removed by centrifugation (120000 × g, 30 min) [14]. This way were obtained folded unphosphorylated and phosphorylated forms of myosin. Phosphorylated monomers in extended conformation were obtained by rising KCl concentration in buffer A (containing either 0.1 mM $CaCl_2$ or 1 mM EGTA) up to 150 mM. Homogeneity of folded and extended forms of monomeric myosin was analysed by gel filtration chromatography on Sepharose CL-6B column [14] (Fig. 1B). Centrifugation (120000 × g, 15 min) of myosin after addition of caldesmon has shown that amount of pelleted material was less than 5%.

Papain digestion. Papain (1 mg/ml) (type III, purchased from Sigma) was activated before use by incubating for 30–60 min at 35 °C in 50 mM Tris-HCl (pH 7.0), 0.5 mM EDTA, and 20 mM DTT. The reaction of proteolysis of smooth muscle myosin alone (1 mg/ml) or complexed with caldesmon (0.3 mg/ml) in buffer A containing either 0.1 mM $CaCl_2$ or 1 mM EGTA was started by the addition of activated enzyme. Aliquots (80 μl) of the digest were withdrawn at certain time intervals and added to the solution containing monoiodoacetic acid at a final concentration of 2 mM to stop digestion. Then 20 μl sample buffer containing 10% SDS, 0.3 M Tris-HCl (pH 6.8), 25% β-mercaptoethanol and 0.02% bromophenol blue was added and the samples were heated for 3 min in boiling water.

Caldesmon affinity column. Affinity column was prepared by coupling caldesmon to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology Inc.) as described by the manufacturer. The column was equilibrated with buffer A containing 0.5 mM ATP and 1 mM EGTA. Thiophosphorylated 10S or unphosphorylated 10S myosin prepared as described above was applied on the column, allowed to react for 20 min and then eluted with KCl gradient (50–500 mM) in the same buffer.

Electrophoresis. SDS-PAGE of papain digestion products was carried out on 7–25% polyacrylamide gradient mini-slab gels using the discontinuous Tris-glycine buffer system of Laemmli [30]. For calculation of molecular mass, the following marker proteins were used: β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), β-lactoglobulin (18 kDa) and lysozyme (14.3 kDa).

Urea-glycerol-PAGE performed in 10% polyacrylamide, 40% glycerol and, in stacking gel, 8.5 M urea [31] was used to control the extent of myosin phosphorylation.

Gels were stained in 0.05% Coomassie Brilliant Blue R 250 (Sigma) and after destaining were scanned with a Molecular Dynamics computing densitometer.

Protein concentration. Concentrations of caldesmon and myosin were determined by measuring UV-absorbance with the following absorption coefficients and molecular mass values: $A_{278}^{1\%}$ 0.40 [32], 87 kDa and $A_{280}^{1\%}$ 0.50 [14], 474 kDa.

RESULTS

It has been shown that folded conformation of smooth muscle myosin is more resistant to papain proteolysis than extended conformation, although the pattern of the HC digestion is similar [11,33]. These differences in the digestion rates arise from the conformation-dependent changes in accessibility of the head-neck junction of the molecule to proteolytic attack. The effect of caldesmon on papain digestion of monomeric chicken gizzard myosin has been studied under conditions favouring either 10S or 6S conformation of its molecules. The time course of papain digestion was monitored by SDS-PAGE and the rate of the disappearance of myosin HC and RLC during digestion was determined by scanning the gels. Products of caldesmon cleavage did not preclude the monitoring of the HC and RLC digestion. Comparison of the extent of proteolysis of the HC of fully unphosphorylated and fully thiophosphorylated 10S myosin at low ionic strength solution (buffer A) containing 1 mM ATP and either 0.1 mM $CaCl_2$ or 1 mM EGTA in the presence and absence of caldesmon reveals marked difference. While irrespective of the presence or absence of Ca^{2+} the rate of HC digestion of folded unphosphorylated myosin is somewhat slower (Fig. 2), the cleavage of folded phosphorylated myosin is significantly faster in the presence of caldesmon (Fig. 3). In the latter case the sensitivity to proteolytic attack is higher in the presence of Ca^{2+} also due to the effect of this cation itself on the rate of proteolysis [34]. The decrease in the amount of HC is followed by accumulation of the 125 kDa rod portion of myosin which serves as a marker of myosin cleavage at the head/rod junction. Fig. 4 shows the alterations in production of 125 kDa fragment upon binding of caldesmon in the presence and absence of Ca^{2+}. The effect of caldesmon is the most pronounced for phosphorylated 10S myosin in the presence of Ca^{2+}.

Digestion of unphosphorylated RLC in 10S myosin is inhibited by caldesmon both in the presence and absence of Ca^{2+}, although the removal of Ca^{2+} increases the resistance of the

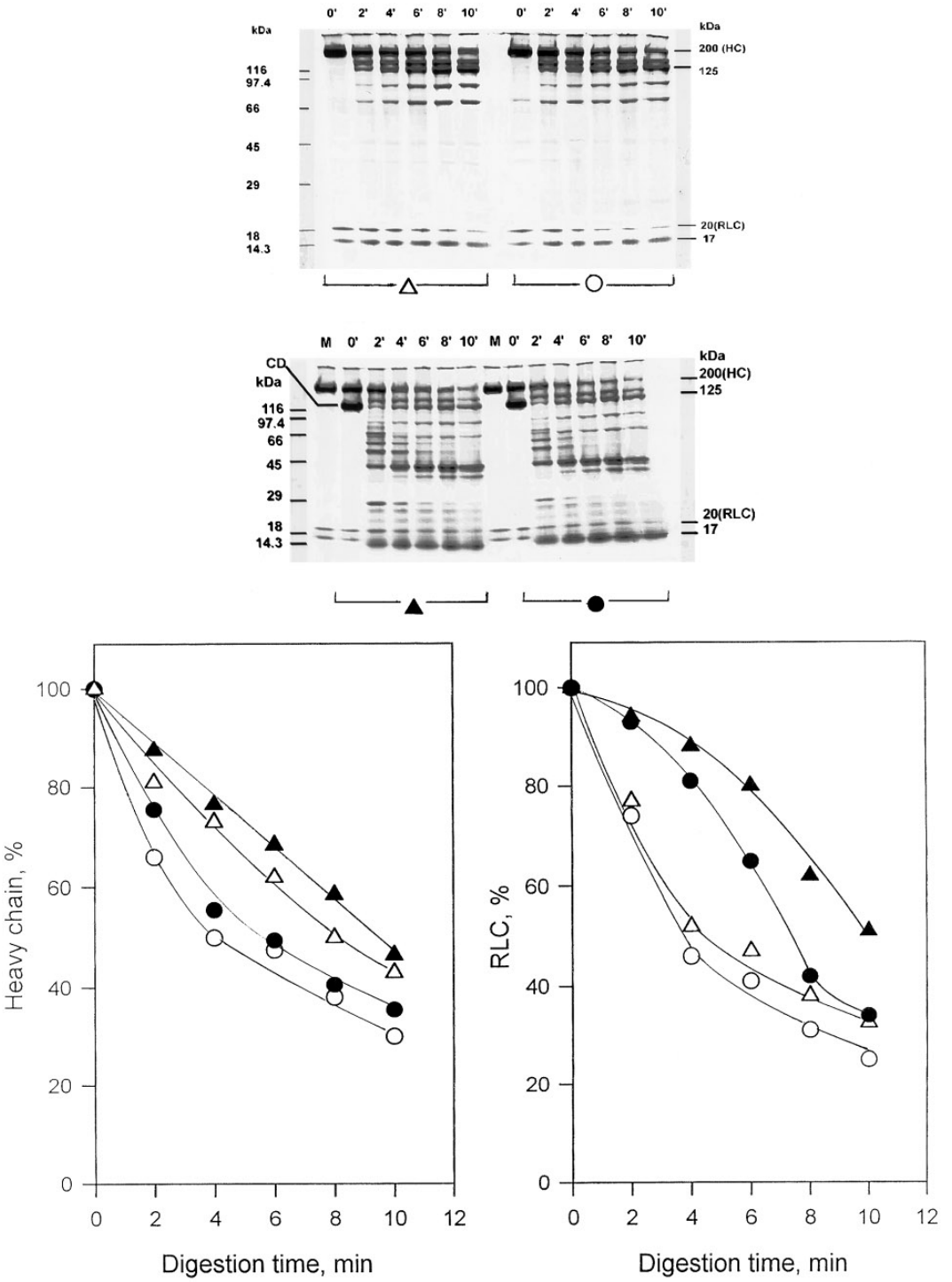

FIG. 2. The effect of caldesmon on papain digestion of HC and RLC of folded unphosphorylated gizzard myosin. Proteolysis was performed in buffer A with 1 mM ATP and either 0.1 mM Ca^{2+} (circles) or 1 mM EGTA (triangles) in the absence (open symbols) or presence (closed symbols) of caldesmon (at the molar ratio to myosin 1.5:1). The papain:myosin weight ratio was 1:1000. (Top) SDS-PAGE patterns of different times of proteolysis. (Bottom) The percentage of undigested myosin HC and RLC determined from the gels (see Material and Methods). M, myosin; CD, caldesmon.

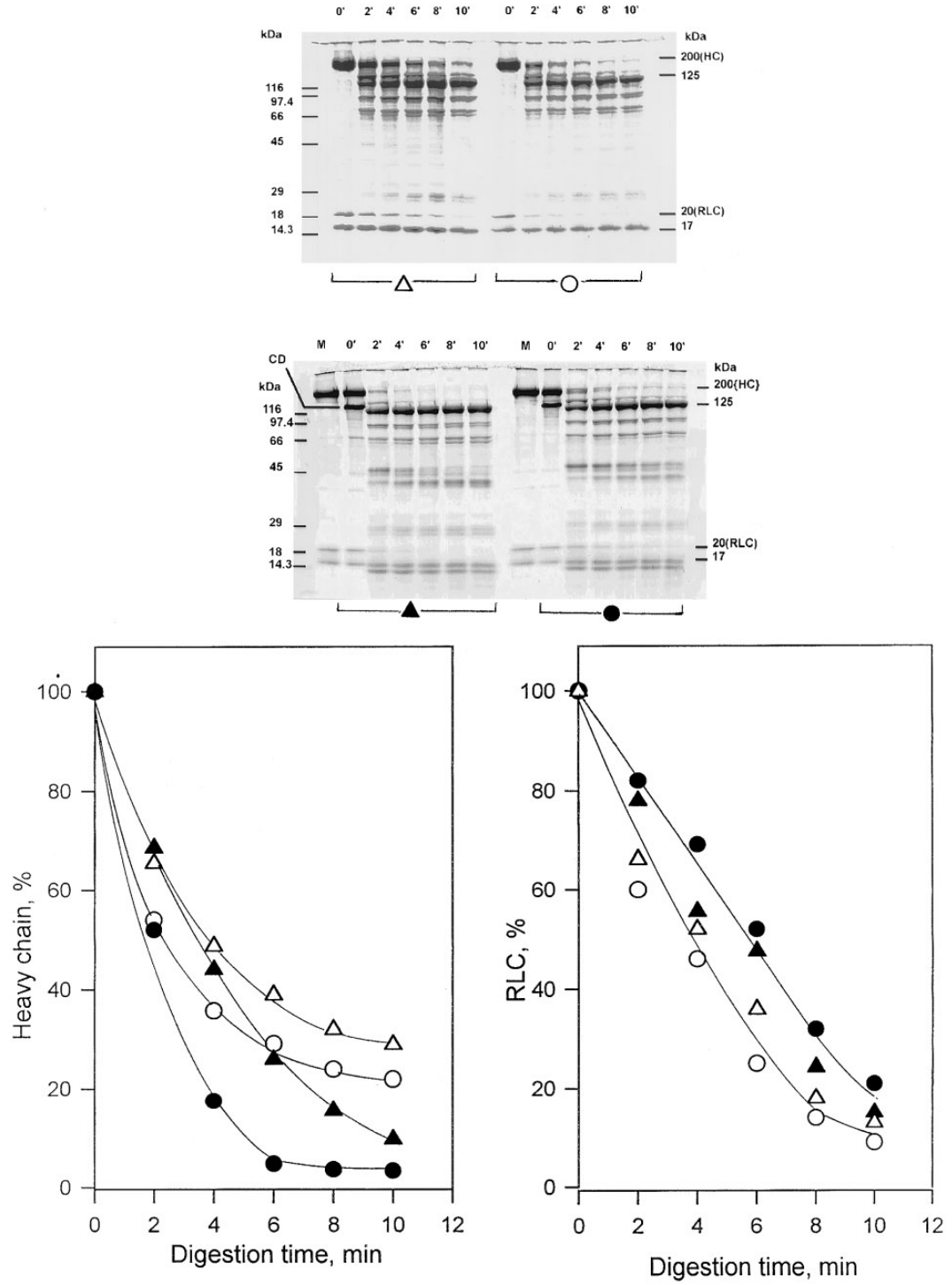

FIG. 3. The effect of caldesmon on papain digestion of HC and RLC of folded thiophosphorylated chicken gizzard myosin. Other conditions and symbols are the same as described in the legend to Fig. 2.

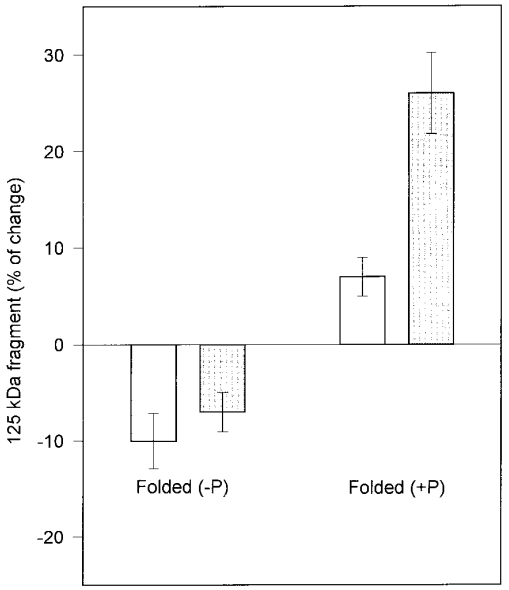

FIG. 4. The effect of caldesmon on production of 125 kDa rod fragment during papain digestion of folded unphosphorylated (−P) and thiophosphorylated (+P) chicken gizzard myosin. Digestion time 10 min. Empty and grey bars denote the effect in the absence and presence of Ca^{2+}, respectively. Digestion conditions as described in the legend to Fig.2. The amount of 125 kDa fragment formed in the absence of caldesmon was taken as a reference point.

RLC to papain attack (Fig. 2). Phosphorylated RLC in 10S form of myosin is more susceptible to proteolysis and its cleavage is much less inhibited by caldesmon (Fig. 3).

The described effects of caldesmon on papain proteolysis of HC at the head/rod junction and of RLC in folded phosphorylated myosin disappear upon increasing ionic strength (up to 150 mM), i.e. under conditions which favour unfolding of myosin molecule . Also, the increase in Mg^{2+} concentration (up to 6 mM) abolishes the effect of caldesmon (data not shown). The lack of the effect of caldesmon on papain digestion of myosin in the extended conformation can be explained by the results of experiments on the elution of phosphorylated and unphosphorylated folded myosin from caldesmon-affinity column. Elution profiles presented in Fig 5 indicate that myosin is removed from the column under ionic conditions favouring the 10S→6S transition, i.e. at about 150 mM NaCl for phosphorylated and 250 mM NaCl for unphosphorylated form.

DISCUSSION

Our results indicate that caldesmon exposes the head/rod junction of folded phosphorylated smooth muscle monomeric myosin to papain attack but somehow screens it in folded unphosphorylated myosin. Thus, despite the similarity in the gross of hydrodynamic properties and electron microscopic performance of 10S forms of unphosphorylated and thiophosphorylated myosin at low ionic strength in the presence of ATP [7,29], there are subtle differences in the conformation of these two species which are distinguished by caldesmon. Also the position of RLC in unphosphorylated and phosphorylated folded myosin seems to be different as indicated by different degree of RLC's protection by caldesmon against proteolytic attack. The lack of the effect of caldesmon on the rate of papain hydrolysis of both HC and RLC in the extended form of myosin may be explained not only in terms of different myosin conformation but also by weakening of caldesmon binding to myosin at higher ionic strength (Fig. 5) [35].

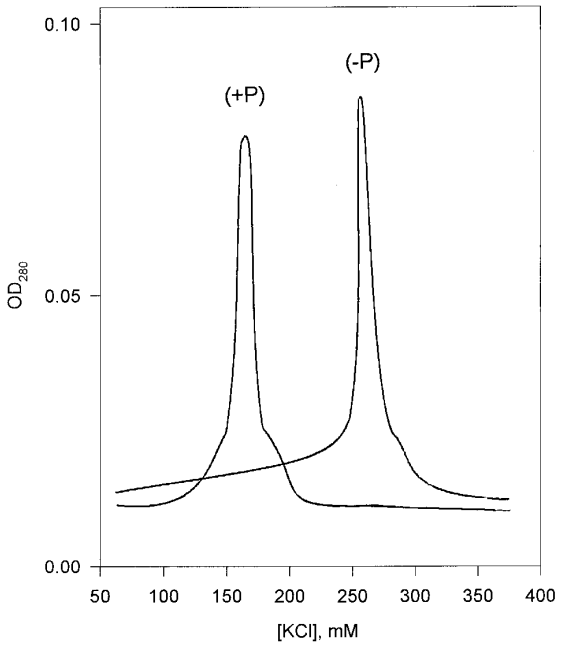

FIG. 5. Elution profiles of unphosphorylated (−P) and phosphorylated (+P) myosin from a caldesmon-Sepharose 4B affinity column. The column was equilibrated with buffer A containing 0.5 mM ATP and 1 mM EGTA. Thiophosphorylated 10S or unphosphorylated 10S myosin, prepared as described under Material and Methods, was applied on the column, allowed to react for 20 min, and then eluted with KCl gradient (50−500 mM) in the same buffer.

It is worthwhile to note that exposure of the head/rod juction of smooth muscle phosphorylated myosin in 10S conformation to papain attack is observed also in the presence of Ca^{2+} (Fig. 3) that is in agreement with observed earlier acceleration of papain cleavage of 10S phosphorylated myosin by Ca^{2+} [34]. It is known that EF-hand in domain I of RLC is capable to bind Ca^{2+} but, since this binding site is not specific for Ca^{2+}, it is supposed *in vivo* to be occupied by Mg^{2+} [36,37]. However, isotherms of Ca^{2+} binding to gizzard myosin revealed that at 1 mM Mg^{2+} it still binds Ca^{2+} (half saturation occurs at 50 mM Ca^{2+}) and this binding is completely eliminated at only 6 mM Mg^{2+} [34]. The effects of Ca^{2+} and caldesmon, probably divergent in nature, reflect alterations in conformation of phosphorylated RLC or/and its vicinity in 10S conformation of myosin.

It has been recently shown by Lu and Chalovich [38] that in the presence of 1 mM ATP the two heads and the neck region of chicken gizzard myosin form a single, high affinity binding site for caldesmon. At present it is difficult to determine the reason of caldesmon effect on the regulatory domain of phosphorylated myosin in 10S conformation. It can be due, for example, to the competition of caldesmon with the myosin tail for the binding site at the head-rod junction or its effect on the head-head interaction that was recently proved to be essential for the ''off-state'' of smooth muscle [39,40]. Each of these effects could result in rising myosin heads and, in consequence, in partial unfolding of myosin.

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