Caldesmon-induced inhibition of ATPase activity of actomyosin and contraction of skinned fibres of chicken gizzard smooth muscle

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Caldesmon induces inhibition of MG^{2+}-ATPase activity of actomyosin and relaxation of skinned fibers of chicken gizzard smooth muscle without influencing the level of myosin light chain-1 phosphorylation. Both these effects are reversed by calmodulin at a high molar excess over caldesmon in the presence of Ca^{2+}.

Caldesmon *Calmodulin* *ATPase activity* *Skinned fiber* *Chicken gizzard*

1. INTRODUCTION

It is widely accepted that phosphorylation of myosin light chains-1 plays an important role in the activation of smooth muscle contraction [1]. In addition to the myosin-linked regulation, the existence of actin-linked regulation as a secondary mechanism has also been postulated [2]. The latter could operate through calmodulin and caldesmon, an actin-binding protein of M_r 150000 discovered by Sobue et al. [3].

Caldesmon was originally purified from chicken gizzard smooth muscle [3]. Immunocytochemical studies subsequently demonstrated its presence in many smooth muscle and non-muscle cells [4]. A protein with similar properties to caldesmon and a slightly lower M_r was recently isolated from aortic smooth muscle thin filaments [5].

It was found that, due to its binding to actin, caldesmon can inhibit superprecipitation of smooth muscle actomyosin as well as Mg^{2+}-ATPase activity of both skeletal and smooth muscle actomyosin [6–8]. Recently, it was suggested that this inhibition can be abolished by phosphorylation of caldesmon [8].

Here we describe the effect of caldesmon on Mg^{2+}-ATPase of actomyosin as well as on contraction of skinned smooth muscle fibers in the absence and the presence of Ca^{2+} and calmodulin.

2. MATERIALS AND METHODS

Caldesmon was prepared from fresh chicken gizzard muscle following the method of Bretscher [9]. Myosin light chain kinase (MLCK) was obtained from chicken gizzard muscle, according to Walsh et al. [10]. Calmodulin was isolated from bovine testis, as described by Gopalakrishna and Anderson [11]. Actomyosin was prepared from fresh chicken gizzard muscle essentially, according to Mrwa et al. [12] with the following modifications: 50 g minced muscle, homogenized with 20 vols of a solution containing 50 mM KCl, 15 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 0.5 mM DTT and 1% Triton X-100, was centrifuged for 10 min at 5000 × *g*. The pellet was washed with 20 vols of a solution containing 100 mM KCl, 10 mM Tris-HCl, pH 7.5 and 0.5 mM DTT and then extracted for 2 min with 2 vols of a solution containing

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5 mM ATP, 40 mM imidazole-HCl, pH 7.2, 4 mM EGTA and 0.5 mM DTT. After addition of $MgCl_2$ to a final concentration of 30 mM the extract was left for 2 h. Precipitated actomyosin was collected by a 10 min centrifugation at 15000 × g, dissolved and dialysed against a buffer containing 60 mM KCl, 0.5 mM EGTA, 10 mM Tris-HCl, pH 7.0, and 0.2 mM DTT. The purity of all these proteins was checked by 10% SDS-polyacrylamide gel electrophoresis performed in 7.5–20% polyacrylamide gradient slab gels, according to Laemmli [13]. The concentration of the proteins was determined either by the biuret method [14] or using $E^{276}_{1\%}$ of 3.0 for caldesmon [9] and 2.0 for calmodulin [15], respectively.

ATPase activity of actomyosin was determined according to Merkel et al. [16], in a medium containing 50 mM KCl, 2 mM $MgCl_2$, 10 mM imidazole-HCl, pH 7.0, 10 mM NaN_3 and 2 mM EGTA or 0.1 mM $CaCl_2$ with the protein concentration given in the legend to fig.1.

The extent of myosin light chain-1 phosphorylation in actomyosin and trichloroacetic acid-fixed skinned fibers was measured from scans of 2-dimensional gel electrophoresis according to Gagelmann et al. [17].

The fibers were prepared immediately after slaughtering (length 5 mm, width about 0.2 mm) and were skinned according to Sparrow et al. [18] following the modification of Wagner [19]. After glueing the fibers at one end to the carbon rod of a force transducer (type AME 801, Aksjeselskapet, Horten Electronics) and at the other to a glass rod attached to a micrometer drive, they were relaxed by immersion in a solution containing 50 mM KCl, 4 mM $MgCl_2$, 1 mM ATP, 25 mM imidazole-HCl, pH 7.0, 1 mM creatine phosphate, 0.4 mg/ml creatine kinase as well as 4 mM EGTA and $CaCl_2$ mixed in the proportion calculated to produce the desired free Ca^{2+} concentration [20].

3. RESULTS

Actomyosin from chicken gizzard muscle, containing besides actin, myosin and tropomyosin, traces of MLCK and caldesmon (as shown in the inset to fig.1), was preincubated for 10 min in a solution containing 0.1 μM MCLK, 1 μM calmodulin, 2 mM ATP and 0.1 mM $CaCl_2$. During this time myosin light chains-2 were almost

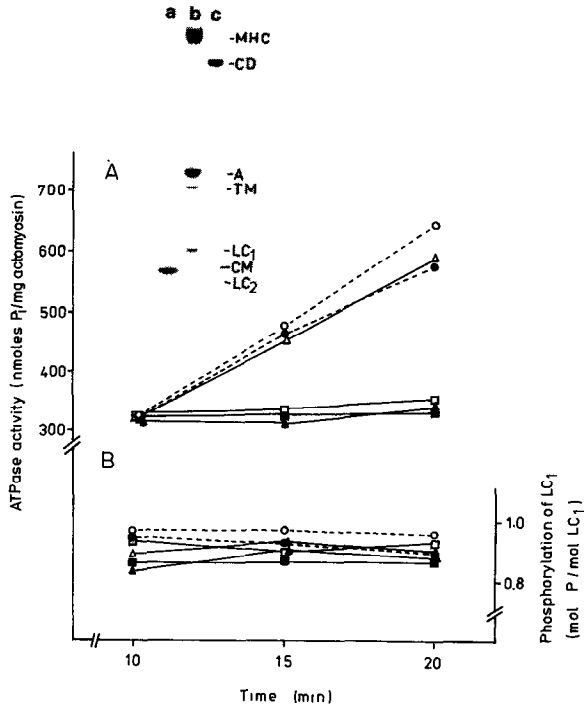

Fig.1. Ca^{2+}-calmodulin regulation of the effect of caldesmon on ATPase activity of chicken gizzard actomyosin. (A) ATPase was assayed as described in section 2 in a medium containing 50 mM KCl, 2 mM $MgCl_2$, 10 mM imidazole-HCl, pH 7.0, 10 mM NaN_3 and either 2 mM EGTA (solid symbols) or 0.1 mM $CaCl_2$ (open symbols) at 1 mg/ml actomyosin, 13.5 μg/ml MLCK and 17 μg/ml calmodulin. Caldesmon was added after 10 min preincubation at a molar ratio to calmodulin of 5:1 (■, □) and 1:5 (▲, △). (– – –) Control ATPase (without caldesmon). (B) For monitoring the level of myosin light chain-1 (LC-1) phosphorylation during measurements of ATPase activity aliquots of reaction mixtures were withdrawn at the indicated times and electrophoresed on 2-dimensional gels. The relative proportions of phosphorylated to non-phosphorylated myosin light chains-1 were determined from the scans of the gels [17] expressed as mol P/mol LC-1 or LC-1 phosphorylated in % of total LC-1 (mean ± SE, n = 6–7): The gel inset shows the purity of the proteins used in the experiments: (c) caldesmon, (b) actomyosin, (a) calmodulin. MHC, myosin heavy chain; CD, caldesmon; A, actin; TM, tropomyosin; CM, calmodulin. LC-1, phosphorylatable light chain; 20 kDa; LC-2, 17 kDa light chain.

completely phosphorylated (fig.1B). Fig.1A shows the time course of Mg^{2+}-ATPase activity of actomyosin containing phosphorylated myosin measured in the presence of caldesmon and 2 different ratios of calmodulin to caldesmon. In the absence of Ca^{2+}, caldesmon causes nearly complete inhibition of actomyosin ATPase. This inhibition could be reversed in the presence of Ca^{2+} when a 5-fold molar excess of calmodulin over caldesmon was added. When caldesmon was present in 5-fold excess over calmodulin or even at equimolar ratios the inhibition of ATPase, despite the presence of Ca^{2+}, was maintained. In either the presence or absence of Ca^{2+} the level of myosin light chain-1 phosphorylation during ATPase assays was stable showing that the actomyosin preparation was devoid of any contamination by myosin light chains-1 phosphatase as well as that caldesmon did not influence the state of myosin phosphorylation.

Next, we studied the effect of caldesmon on contraction of skinned fibers of chicken gizzard smooth muscle. These fibers contracted maximally at p*Ca* 5.2 even in the absence of exogenous calmodulin while the extent of myosin light chain-2 phosphorylation increased only from 8.7 ± 1.4% (basal level) to 16.5 ± 1.9% (table 1, row A), as shown by Wagner [19]. In all (6) experiments, this contraction was not inhibited by 0.2 μM exogenous caldesmon. When the Ca^{2+} concentration

Table 1

Caldesmon-induced inhibition of chicken gizzard skinned fibers

	p*Ca*	CaM (μM)	CaD (μM)	Force (4)	P-LC-20 (%)
(A)	5.2	0	0	100 (6)	16.5 ± 1.9 (6)
(B)	5.8	0	0	77 ± 4.9 (6)	9.6 ± 1.6 (6)
(C)	5.8	0	0.2	6.3 ± 2.7 (6)	8.1 ± 1.0 (6)
(D)	5.8	5	0.2	95–100 (3)	–

Force is expressed as per cent of tension attained at maximally activating calcium concentrations (p*Ca* 5.2). P-LC-20, phosphorylated regulatory light chains (20 kDa) in per cent of total LC-20. The last row indicates the extent of rapid force recovery by calmodulin of fibers relaxed by caldesmon. Values are given as the mean ± SE; numbers in parentheses show number of preparations used. Conditions as in fig.2

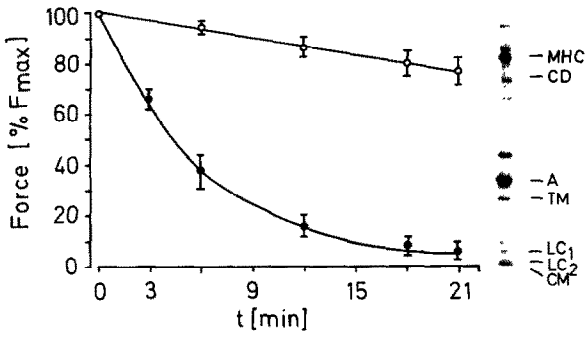

Fig.2. Effect of caldesmon on the relaxation of chicken gizzard skinned fibers. After maximal precontraction at p*Ca* 5.2 in the absence of exogenous calmodulin, the concentration of Ca^{2+} was lowered to p*Ca* 5.8 in the presence (●) or absence (○) of 0.2 μM caldesmon. Maximal tension development amounted to 0.7 kg/cm^2 at p*Ca* 5.2. The gel inset shows the SDS-gel electrophoretic pattern of proteins in skinned fibers.

was lowered to p*Ca* 5.8, however, fibers relaxed within 20 min to 6.3 ± 2.7% of the initial tension in the presence of 0.2 μM exogenous caldesmon, but only to 77 ± 4.9% in control experiments where caldesmon was not added (fig.2; table 1, rows B and C). The latter result is somewhat surprising since, as revealed by SDS-polyacrylamide gel electrophoresis, skinned muscle fibers, unlike actomyosin preparations, contain appreciable amounts of caldesmon (see inset to fig.2). Phosphorylation of the regulatory light chains of myosin measured after 20 min relaxation in the presence of caldesmon amounted to 8.1 ± 1.0% vs 9.6 ± 1.6% in control experiments (also at p*Ca* 5.8) in which caldesmon was not added. This suggests that caldesmon-induced relaxation was not due to the inhibition of the Ca^{2+}- and calmodulin-dependent MLCK activity. Nonetheless, a calmodulin antagonistic action of caldesmon was implicated by the finding that relaxation of skinned fibers could be reversed by 5 μM calmodulin (table 1, row D).

4. DISCUSSION

The present results clearly indicate that caldesmon is capable of causing both inhibition of actomyosin ATPase activity and relaxation of precontracted fibers of chicken gizzard smooth

muscle. The stable level of myosin light chain phosphorylation during ATPase assays and measurement of force development of skinned fibers, independently of the presence of caldesmon, indicates that its effect is not related to dephosphorylation of myosin.

According to the flip-flop mechanism of actin-myosin regulation proposed by Sobue et al. [6], caldesmon binds to actin and prevents its interaction with myosin at low Ca^{2+} concentration (p*Ca* 8.0), whereas in the presence of a high concentration of Ca^{2+} (p*Ca* 5.2) it binds to calmodulin and releases actin for interaction with myosin. However, on studying the effect of caldesmon on actomyosin ATPase at various concentrations of calmodulin we found that calmodulin added in equimolar ratio to caldesmon in the presence of Ca^{2+} is not able to neutralize its inhibitory effect. The addition of a high molar excess of exogenous calmodulin is necessary to reveal its antagonistic action to caldesmon, i.e. to reverse inhibition of actomyosin ATPase activity and relaxation of skinned fibers. In view of the high content of caldesmon in smooth muscle and its calcium/calmodulin-dependent interaction with actin, future work is required to investigate the mechanism by which caldesmon influences contractility and phosphorylation in skinned fibres independently of myosin phosphorylation.

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