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Aorta caldesmon inhibits actin activation of thiophosphorylated heavy meromyosin Mg^{2+}-ATPase activity by slowing the rate of product release

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Activation of aorta thiophosphorylated heavy meromyosin (HMM[SP]) Mg^{2+}-ATPase activity by aorta actin and the fraction of HMM[SP]-substrate intermediate complexes bound to actin were measured simultaneously. At 25°C the K_m for ATPase activation and the dissociation constant for the binding reaction were similar, irrespective of the presence or absence of tropomyosin. Aorta caldesmon (0.1 mol/mol actin) inhibited ATPase activation by 80–90% but did not alter the binding of HMM[SP]-product intermediates to actin. It is concluded that caldesmon inhibits by slowing the rate-limiting release of products from the actin-HMM[SP]·ADP·P_i complex.

Caldesmon; Actomyosin; ATPase; Regulation; (Smooth muscle)

1. INTRODUCTION

Muscle contraction is regulated by Ca^{2+} which acts on receptor molecules which are components of the contractile apparatus. In all muscles, the contractile apparatus is made from interdigitating filaments of actin and myosin. Force and movement are produced from cyclic interaction of these two proteins at the expense of ATP hydrolysis. Ca^{2+} acts to control the actin-myosin interaction and hence ATP hydrolysis.

The enzymic mechanism of actin-activated ATPase hydrolysis has been determined in many muscles [1,2] and the kinetic mechanism by which several of the control mechanisms, notably the troponin system of striated muscles [3] and the myosin phosphorylation system of smooth muscles [4], control the actomyosin ATPase has also been determined. There would appear to be a common mechanism: the actin-activated myosin Mg^{2+}-ATPase is switched on and off by a change in the rate of the release of products from an actin·myosin·ADP·P_i complex, which is the rate-limiting step of the reaction.

Smooth muscles contain two Ca^{2+}-dependent regulatory mechanisms: the myosin phosphorylation mechanism and a Ca^{2+}-dependent switch located in the actin-containing thin filaments [5]. Smooth muscle native thin filaments are Ca^{2+}-regulated when tested with unregulated myosin [6]. The regulatory factors present in smooth muscle thin filaments are a high molecular mass inhibitor, caldesmon, and a Ca^{2+}-binding protein which modulates caldesmon action in response to Ca^{2+} [7,8]. Our recent work has confirmed the regulatory role of caldesmon in aorta thin filaments since anti-caldesmon antibodies destroy the Ca^{2+} sensitivity of native thin filaments [9] and pure caldesmon has been shown to be able to inhibit isometric muscle contraction [10].

Here, we have investigated the mechanism by which caldesmon inhibits smooth muscle actomyosin Mg^{2+}-ATPase. Functionally smooth muscle caldesmon resembles striated muscle troponin I+T [11] and consequently we were particularly interested in determining whether the two systems

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have similar mechanisms. In recently published work it has been shown that caldesmon inhibition is associated with a large increase in the affinity of myosin·ADP·P_i for actin [12,13], an observation which is difficult to explain on the basis of the known mechanism of actomyosin ATP hydrolysis [1,2]. We have taken advantage of the properties of vascular smooth muscle caldesmon isolated by the technique of Smith et al. [7]. We find that this form of caldesmon inhibits ATPase without changing the affinity of myosin·ADP·P_i for actin. From this work we can conclude that caldesmon controls the rate-limiting step (product release) of the ATPase only and that the very tight binding previously observed is not related to the regulation of actomyosin Mg^{2+}-ATPase activity.

2. MATERIALS AND METHODS

All proteins were prepared from sheep aorta. Native thin filaments were isolated according to Marston and Smith [6]. Caldesmon and F-actin were obtained from the thin filaments.

Thin filaments at 10 mg/ml were treated with 0.8 M KCl and centrifuged. Pure actin sedimented and the supernatant contained caldesmon and other actin-binding proteins.

Caldesmon was isolated from the supernatant by two procedures: (i) the mixture was taken to pH 3 and sedimented at low speed. Caldesmon remained in the supernatant and was purified by 35–50% ammonium sulphate precipitation [7]. (ii) The caldesmon was concentrated and partly purified from the mixture by 35–50% ammonium sulphate precipitation. Final purification was on a 1 × 15 cm column of Q Sepharose as described in [14].

Aorta myosin was prepared as in [15] and thiophosphorylated according to [16]. Heavy meromyosin was prepared by chymotryptic digestion [4,15].

Measurements of MgATP hydrolysis and binding were made in 5 mM K_2Pipes, 2.5 mM $MgCl_2$, 1 mM DTT, 0.3 mg/ml serum albumin, 2 μM thiophosphorylated HMM (HMM[SP]) and 0–150 μM actin; pH 7.1 at 25°C. 200-μl samples were made up for each assay point and were split into 2 equal portions. One half was used to measure Mg^{2+}-ATPase. MgATP was added to 3 mM and after 10 min, 0.5 ml of 5% trichloroacetic acid was added to terminate the reaction. The P_i released was assayed as in [19]. Control samples containing actin and caldesmon but no HMM[SP] were run at the same time and these values were subtracted to obtain the ATPase activity. The other half of the sample was used for binding measurements. MgATP was added to a final concentration of 3 mM and immediately centrifuged for 20 min at 150000 × g in a Beckman airfuge or for 35 min at 150000 × g in 10-ml tubes in a preparative ultracentrifuge. The actin and any HMM bound to the actin was pelleted. 40-μl aliquots of the supernatant after sedimentation were taken and the quantity of HMM present was assayed by measuring EDTA ATPase activity according to [4]. A negligible quantity of HMM[SP] sedimented in the absence of actin; it was therefore assumed that the fraction of HMM sedimented in the presence of actin was equal to the fraction bound to actin.

3. RESULTS

Aorta actin activated the Mg^{2+}-ATPase activity of thiophosphorylated aorta HMM. Under our low ionic strength conditions at 25°C activation always followed simple saturation kinetics (fig.1) with a V_{max} in the region of 40 min^{-1} and a K_m of 47 ± 27 μM actin monomer (n = 5). These values are similar to our previous data with gizzard proteins [1].

Caldesmon prepared by the method of Smith and Marston [7] was a potent inhibitor of actin activation, despite the absence of tropomyosin, which was found to be an essential cofactor at higher ionic strengths. This observation is in agreement with [12,13]. At a constant 20 μM actin we observed maximum inhibition levels of at least 80 and 50% of maximum inhibition was obtained at 1:40 CD/actin (comparable with [7]). When actin activation of HMM[SP] Mg^{2+}-ATPase was measured in the presence of a maximally inhibiting ratio of caldesmon to actin, inhibition was observed throughout the actin concentration range (fig.1).

The binding of HMM[SP]·ADP·P_i to actin was measured simultaneously with ATPase activation. We found that binding followed a simple binding curve which extrapolated to 96 ± 12% (n = 10) of HMM[SP] bound to actin at infinite actin concentration. The estimated dissociation constant for the binding reaction (K_d) was similar to the K_m for the ATPase activation (fig.1, table 1). This same pattern of results was seen when we substituted skeletal muscle for smooth actin and in the presence and absence of aorta tropomyosin; however, with skeletal HMM K_d was substantially higher than K_m, as has been previously observed [18,19].

When the activation was inhibited by caldesmon, the binding was not altered. This result was always observed with smooth and skeletal actins and smooth and skeletal HMM (fig.1, table 1).

Since this result differs from that obtained by Lash et al. [12], we considered whether the mode

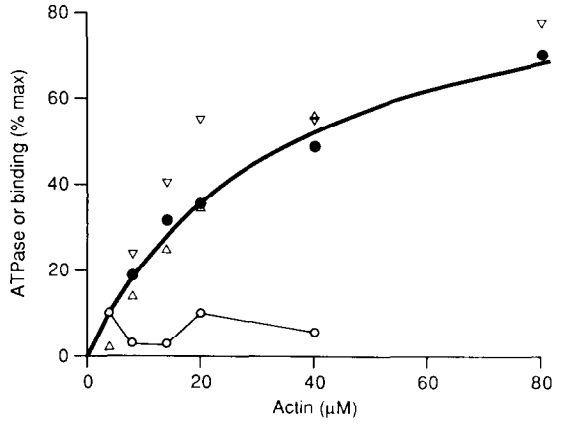

Fig.1. Effect of caldesmon on actin activation of HMM[SP] Mg^{2+}-ATPase activity and actin-HMM[SP] binding. Conditions: HMM buffer at 25°C, 4 μM aorta HMM[SP], 0–80 μM actin, 3 mM MgATP. (●) Activation of ATPase by actin; (○) activation of ATPase by actin + caldesmon (0.08 caldesmon/actin mol/mol); (▽) binding of HMM[SP] to actin; (△) binding of HMM[SP] to actin + caldesmon. Actin activation data were fitted to the Michaelis-Menten equation (solid line) K_m = 35.5 ± 7 μM, V_{max} = 16 ± 4 min^{-1}. Actin activation data plotted as % of V_{max}; binding results are plotted as % sedimented (equal to % bound).

Table 1

Comparison of the binding of HMM[SP]·ADP·P_i to actin with the Mg^{2+}-ATPase activity in the presence and absence of caldesmon

	K_{mA}/K_{dA}	% inhibition by caldesmon	K_{dA}/K_{dA+CD}	n
Aorta actin + aorta HMM[SP]	1.6	88	0.7	3
Skeletal actin + aorta HMM[SP]	1.6	93	1.06	1
Aorta actin + skeletal HMM	0.2	92	1.16	1
Aorta actin + aorta HMM[SP] + aorta tropomyosin	1.0	78	1.04	2
Aorta actin + aorta HMM[SP] column-purified caldesmon	2.1	97	1.2	2

K_m and K_d determined with actin (A) or actin + caldesmon (A + CD) by fitting the data (6–12 points) to an equation for simple binding. SE is about 20%. Where several experiments have been done the mean is given. % inhibition is the mean of data points (2–4 pairs per experiment) measured in the range 20–50 μM actin

of caldesmon preparation might affect the result. We prepared caldesmon by a procedure which omitted our usual pH 3 treatment and included an additional ion-exchange purification step. This caldesmon was usually a less potent inhibitor than our usual caldesmon but, again, it did not alter the binding of HMM[SP] to actin (table 1).

4. DISCUSSION

We have investigated the kinetics of vascular smooth muscle HMM[SP] Mg^{2+}-ATPase activation by actin by steady-state methods. The general finding is that activation follows simple Michaelis-Menten kinetics and that the K_m is approximately equal to the dissociation constant of the HMM[SP] from the actin (K_d).

The enzymic pathway of actomyosin is well characterised [1,2,18]. MgATP binds to actin-myosin rapidly and is hydrolysed whilst bound at the myosin active site; the substates myosin*·ATP and myosin·ADP·P_i are in rapid equilibrium with the corresponding attached complexes actin·myosin*·ATP and actin·myosin·ADP·P_i. The rate-limiting step of the reaction is a conformational change in actin·myosin·ADP·P_i leading to sequential and rapid release of ADP and P_i. In this study we found that vascular smooth muscle proteins $K_m = K_d$ for the equilibrium between attached and detached states. This implies that all the steps up to the formation of actin·myosin·ADP·P_i are rapid relative to the rate-limiting step and the intermediate complexes are at equilibrium. A similar analysis has been given for the gizzard system [1,4]. In the case of skeletal HMM, $K_d \gg K_m$ (table 1) because hydrolysis is slow relative to the rate-limiting step [18,19].

Caldesmon controls actin activation. The material we normally work with inhibits actin activation without altering K_d (fig.1, table 1). On the basis of the above pathway, therefore, caldesmon must act only by reducing the rate of the slowest step. This is a finding of considerable general interest since it is known that other regulatory mechanisms, skeletal muscle troponin, and myosin smooth muscle phosphorylation also control ATPase hydrolysis predominantly at this step [3,4].

It has been reported that gizzard caldesmon induces a 40-fold decrease in K_d in actin + smooth

HMM systems [12,13]. A large decrease in K_d cannot be reconciled with the enzymic mechanism of actin activation of HMM[SP] described above and in [1,2,4]. Our experiments with caldesmon prepared from aorta by the Smith-Marston method clearly show that the low K_d is not a necessity for caldesmon inhibition of actin activation. It has been tentatively suggested that the tight binding of HMM[SP] to caldesmon-containing actin filaments involves a site and a mechanism which is separate from the acto-HMM ATPase mechanism [8,13,20]. Our results support this hypothesis.

The tight binding of HMM to actin-caldesmon seems to be a rather labile property of caldesmon; whilst it is frequently reported in gizzard systems it is rarely seen in aorta. We usually do not observe tight binding (table 1) in reconstituted systems but tight binding is regularly observed in aorta native thin filaments [8]. Furthermore, tight binding is critically dependent on myosin type, being much more prominent with smooth muscle HMM than smooth S1 or skeletal HMM [13].

In contrast caldesmon inhibition is a very stable property of caldesmon [7,8], consequently it may be dissociated from the tight binding phenomenon. This has enabled us to establish that caldesmon resembles troponin and myosin phosphorylation in its regulatory mechanism: all three systems control actin activation of myosin Mg^{2+}-ATPase by modulating the rate of product release [2–4].

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