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REVERSAL OF CALDESMON FUNCTION BY ANTI-CALDESMON ANTIBODIES CONFIRMS ITS ROLE IN THE CALCIUM REGULATION OF VASCULAR SMOOTH MUSCLE THIN FILAMENTS

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Direct evidence that caldesmon is the Ca^{2+}-regulated inhibitory component of native smooth muscle thin filaments is provided by studies using caldesmon-specific antibodies as antagonists. The antibodies reverse caldesmon inhibition of actomyosin ATPase and abolish Ca^{2+}-regulation of native aorta thin filament activation of myosin ATPase. This effect is a result of antibody binding to the caldesmon on the filament thereby inactivating it and not due to antibody-induced caldesmon dissociation from the filament. The antibodies, however, neutralise caldesmon only in systems using skeletal muscle myosin and not in those using smooth muscle myosin; this implies that smooth muscle myosin prevents appropriate antibody binding to caldesmon perhaps because smooth muscle myosin binds to caldesmon thus preventing access of antibody to antigenic sites.

The interaction of the contractile proteins of smooth muscles is controlled by Ca^{2+} concentration changes via Ca^{2+}-dependent regulatory mechanisms associated with both the myosin and the actin filaments[1]. Ca^{2+} control of smooth muscle actin-based thin filaments has been demonstrated with preparations of native thin filaments[2,3] and with synthetic thin filaments reconstituted from actin, tropomyosin, caldesmon and calmodulin[4,5]. We have proposed that the Ca^{2+}-sensitising components of the native smooth muscle thin filaments are caldesmon, which exerts an inhibitory effect, and a Ca^{2+} binding protein which relieves the inhibition by caldesmon upon binding Ca^{2+} [4,5]. The proposed role of caldesmon in smooth muscle thin filaments is supported by the observations that caldesmon is restricted to thin filaments[3] and is present at a stoichiometry of one per 28 actins. This value corresponds to the quantity of caldesmon bound to synthetic filaments which gives full inhibition of ATPase activity[2,5]. Nevertheless, such evidence is circumstantial and it

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has been suggested that the Ca^{2+}-dependent regulation of smooth muscle thin filaments does not involve caldesmon but is caused by other minor actin binding proteins[6].

In the present study we have tested the role of caldesmon directly by using an anti-caldesmon antibody which is capable of antagonising caldesmon's inhibitory action.

METHODS AND MATERIALS

Sheep aorta F-actin, tropomyosin, caldesmon and thin filaments were prepared as previously described[2,4,5]. Sheep aorta myosin was prepared and thiophosphorylated as described by Heaslip and Chacko[8] and rabbit skeletal muscle myosin was prepared by the method of Margossian and Lowey[9]. Antibodies specific to caldesmon used in this investigation were prepared and characterised in an earlier study[3]. IgG fractions were prepared from the anti-caldesmon serum and from a non-immune control serum[10].

The rate of MgATPase hydrolysis was measured in synthetic actomyosins consisting of myosin (either from smooth or skeletal muscle) and either smooth muscle thin filaments or purified smooth muscle F-actin and tropomyosin (0.4 tropomyosin/actin w/w). Actomyosin hybrids were prepared in 0.3M KCl and then dialysed in ATPase buffer (50mM KCl, 10mM PIPES pH7.0, 5mM $MgCl_2$, 10mM NaN_3); haemoglobin (10 mg/ml) was present in all reaction mixtures to minimise non-specific effects of IgG. After initiating the reaction with 2mM MgATP, the ATPase rate was measured as previously described[2]. Myosin MgATPase and background ATPases due to contaminants in the thin filament or other protein preparations were subtracted.

RESULTS

Effect of anti-caldesmon IgG on activation of skeletal muscle myosin MgATPase

The activation of skeletal muscle myosin MgATPase by actin-tropomyosin was inhibited approximately 65% following addition of caldesmon (Figure 1). Subsequent addition of increasing amounts of anti-caldesmon IgG to the inhibited system caused a corresponding increase of MgATPase approaching values obtained with caldesmon-free uninhibited actomyosin (Fig.1). Control studies showed that non-immune IgG had no effect in reversing caldesmon inhibition; moreover neither immune nor non-immune IgG affected the ATPase of the caldesmon-free actomyosin (Fig.1).

Native aorta thin filaments confer Ca^{2+}-dependence on skeletal muscle myosin ATPase (Fig.2). Addition of the anti-caldesmon antibody abolished the Ca^{2+}-dependence by elevating the MgATPase obtained in EGTA to that occurring in Ca^{2+} (Fig.2). Control studies showed that non-immune IgG had no effect on Ca^{2+}-dependence (Fig.2).

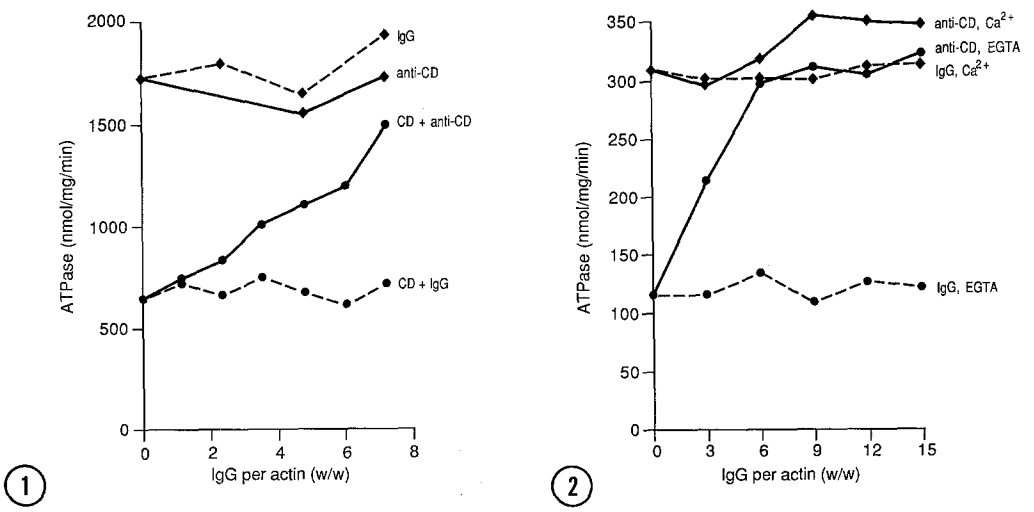

Figure 1 Antibody specifically antagonises caldesmon inhibition. MgATPase activity was measured at 37°C with skeletal muscle myosin (0.125 mg/ml), aorta actin (0.5 mg/ml) and aorta tropomyosin (0.2 mg/ml) with (●) or without (◆) aorta caldesmon (0.18 mg/ml) and in the presence of either anti-caldesmon IgG (—) or control non-immune IgG (---)

Figure 2 Antibody abolishes Ca^{2+}-regulation of thin filaments. MgATPase activity measured at 25°C with skeletal muscle myosin (0.5 mg/ml) and native aorta thin filaments (0.8 mg/ml) in the presence of either 0.1mM Ca^{2+} (◆) or 1mM EGTA [pCa>8] (●) and in the presence of either anti-caldesmon IgG (—) or non-immune IgG (---). The amount of IgG added is expressed as a w/w ratio to actin assuming molecular weights of 150 000 for IgG, 42 000 for actin and an actin content of 60% in the filament.

Antibody binding to thin filaments

Mixtures of aorta native thin filaments and anti-caldesmon IgG in proportions observed to antagonise caldesmon inhibition were sedimented at 100 000 xg for 30 minutes to pellet the thin filaments. Samples of the pellet and supernatant were analysed by SDS polyacrylamide gel electrophoresis. The caldesmon associated with the thin filament pellets was unaltered by the addition of anti-caldesmon. Thus, the anti-caldesmon IgG does not displace caldesmon from the filament and presumably antagonises caldesmon inhibition by binding to caldesmon on thin filaments.

The quantity of IgG bound to thin filaments was measured by sedimenting thin filaments with IgG covalently labelled with [^{3}H]-N-hydroxysuccinimidylproprionate. The radioactivity was measured in samples of pellet and supernatant to determine the quantity of labelled IgG which had cosedimented with the thin filaments. At IgG concentrations necessary to reverse caldesmon inhibition 3.0 nmols of anti-caldesmon IgG bound per mg of thin filaments compared with 0.9 nmols of control IgG per mg, equivalent to a specific anticaldesmon binding of 4 IgG molecules per caldesmon (based

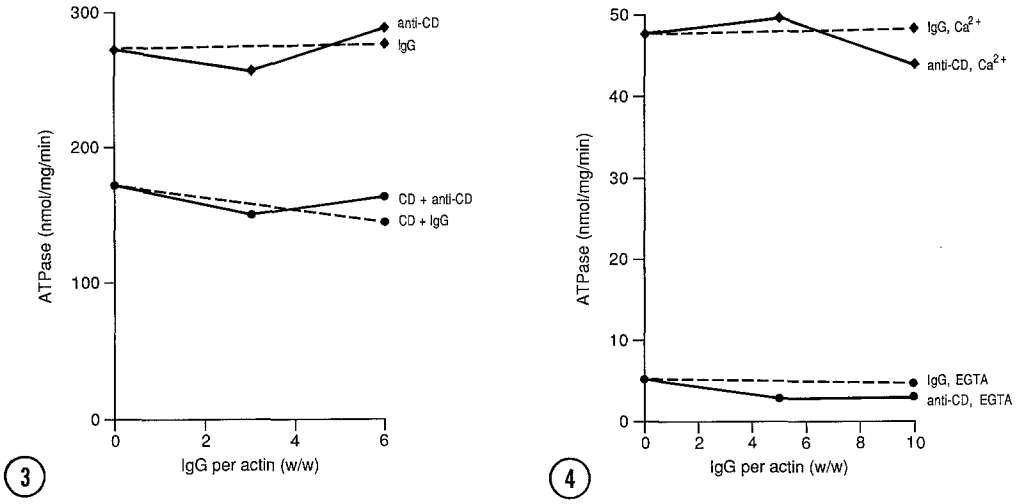

Figure 3 Antibody fails to antagonise caldesmon inhibition. MgATPase activity was measured at 37°C using smooth muscle myosin (0.4 mg/ml), aorta actin (1.25 mg/ml) and aorta tropomyosin (0.6 mg/ml) with (●) or without (◆) caldesmon (0.09 mg/ml) and in the presence of either anti-caldesmon IgG (—) or non-immune IgG (---).

Figure 4 Antibody has no effect on thin filament Ca^{2+}-regulation. MgATPases were measured at 25°C using smooth muscle myosin (0.4 mg/ml) and native aorta thin filaments (1.25 mg/ml) in the presence of either 0.1mM Ca^{2+} (◆) or 1mM EGTA [pCa>8] (●) and in the presence of either anti-caldesmon IgG (—) or non-immune IgG (---). The amount of IgG is expressed as in Fig.2.

on molecular weights of 150 000 for IgG, 120 000 for caldesmon and a caldesmon content of 6% w/w in the thin filament[2]). We estimated that about 5% of the total IgG bound to the caldesmon in native thin filaments.

Effect of anti-caldesmon IgG on activation of smooth muscle myosin MgATPase

The results of parallel ATPase studies using smooth-muscle instead of skeletal-muscle myosin are strikingly different from those reported above. Although caldesmon inhibited actin-tropomyosin activation of smooth muscle myosin ATPase, the inhibition was not reversed by anti-caldesmon antibody (Fig.3). Moreover, in most cases the Ca^{2+}- dependency of smooth muscle thin filament activation of smooth muscle myosin MgATPase was also unaffected by anti-caldesmon (Fig.4). In some instances, however, antibody treatment interfered with Ca^{2+}-activation and the ATPase of the thin filament-smooth muscle myosin system remained inhibited in both the absence and presence of Ca^{2+} (results not shown). In no case did antibody induce increased ATPase activity in experiments using smooth muscle myosin.

DISCUSSION

Caldesmon inhibition of actin-tropomyosin activated myosin MgATPase is specifically antagonised by the anti-caldesmon IgG when skeletal muscle myosin is used (Fig.1). This effect does not arise from antibody-induced dissociation of caldesmon from the actin-tropomyosin but instead by the antibody binding to the caldesmon on the filament and presumably inactivating it. This binding is very specific, involving as few as 4 IgG molecules.

Native smooth muscle thin filaments are Ca^{2+}-regulated[2] and activate the ATPase of Ca^{2+}-insensitive myosin only in the presence of Ca^{2+}. We have proposed that the control mechanism responsible for the Ca^{2+}-dependency involves caldesmon, exerting an inhibitory effect, and a Ca^{2+}-sensitising protein which can relieve inhibition[4,7,11]. Our demonstration that antibody specific to caldesmon abolishes the Ca^{2+}-dependency of the MgATPase exhibited by mixtures of smooth muscle thin filaments and skeletal muscle myosin corroborates our hypothesis directly (Fig.2). Antibody binding to and inactivating caldesmon reverses Ca^{2+}-dependency by increasing ATPase from the inhibited level to that of the Ca^{2+}-stimulated level.

The antibody fails to antagonise caldesmon inhibition in either "synthetic" actin-tropomyosin-caldesmon or native thin filament systems when myosin from smooth muscle, instead of skeletal muscle, is used (Figs.3 and 4). This suggests that the antibody is prevented from binding to caldesmon by a distinct property of smooth muscle myosin. Recent work on the binding of smooth muscle heavymeromyosin(SP)ADP.P_i to caldesmon-containing filaments indicate that the heavymeromyosin forms especially tight bonds with the filaments, involving a myosin domain separate from the ATPase enzymatic site[11,12]. This region of smooth muscle myosin is thought to interact with caldesmon and therefore might block antigenic sites on caldesmon, presumably accounting for the antibody having no effect in relieving inhibition when smooth muscle myosin is used. This interaction is not likely to be directly related to ATP hydrolysis[11] and may be associated with tension maintenance (latch) displayed by many smooth muscles[13]. Competition between smooth muscle myosin and antibody would not necessarily influence the interaction of the Ca^{2+}-sensitising protein and caldesmon, possibly explaining the variability observed in Ca^{2+}-activation when antibody is present. We anticipate that the ability of this antibody preparation to recognise an inhibitory domain, a putative myosin binding domain and perhaps also a domain involved in Ca^{2+}-dependent release of inhibition will enable us to make detailed structure function analysis of caldesmon in synthetic and native preparations.

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