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# $Ca^{2+}$ can control vascular smooth-muscle thin filaments without caldesmon phosphorylation

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The $Ca^{2+}$-dependent regulation of the activation of myosin MgATPase by vascular-smooth-muscle thin filaments involves caldesmon. This effect may be due to the direct interaction of caldesmon with a $Ca^{2+}$-binding protein such as calmodulin or phosphorylation of caldesmon by a $Ca^{2+}$-dependent kinase. I have found that $Ca^{2+}$ switches on aorta thin filaments in less than 10 s, whereas the caldesmon in the thin filaments is phosphorylated only slowly (half-time > 10 min) and the maximum phosphorylation is very low (1 molecule per 7 molecules of caldesmon). I conclude that the phosphorylation of caldesmon hypothesis is untenable.

## INTRODUCTION

Contraction of smooth muscles is initiated by an increase in $Ca^{2+}$ in the vicinity of the contractile elements, actin and myosin. There are $Ca^{2+}$-dependent switches on both the myosin and the thin (actin-based) filaments (Marston *et al.*, 1980; Marston, 1982). We have isolated thin filaments from several smooth muscles and have demonstrated that their interaction with myosin is controlled by $Ca^{2+}$ (Marston & Smith, 1984; Marston & Lehman, 1985). The thin filaments are mainly made up of actin, tropomyosin and caldesmon in the molar proportions 28:4:1. Caldesmon is the major regulatory component: purified caldesmon is a potent inhibitor of actin–tropomyosin interaction with myosin (Sobue *et al.*, 1982; Smith & Marston, 1985; Dabrowska *et al.*, 1985). The mechanism by which $Ca^{2+}$ causes release of this inhibition is controversial. A number of workers have shown relief of inhibition when $Ca^{2+}$ complexed with calmodulin binds to caldesmon, and a mechanism analogous to that of troponin in skeletal muscle thin filaments has been proposed (Sobue *et al.*, 1982; Smith & Marston, 1985; Dabrowska *et al.*, 1985; Marston & Smith, 1985). One significant problem with this model is that current experiments have needed calmodulin concentrations greatly in excess of physiological in order to demonstrate relief of inhibition in the presence of $Ca^{2+}$.

Ngai & Walsh (1984, 1985) have observed that caldesmon can exist in phosphorylated and non-phosphorylated forms and that caldesmon is a substrate for a calmodulin-controlled kinase, distinct from myosin light-chain kinase, and a phosphatase, present in certain caldesmon preparations. It is also a substrate for protein kinase C (Umekawa & Hidaka, 1985). It has been proposed that phosphorylated caldesmon is not an inhibitor; thus $Ca^{2+}$-dependent release of caldesmon inhibition might be due to $Ca^{2+}$-stimulated phosphorylation of caldesmon, a reaction requiring a low calmodulin concentration (Ngai & Walsh, 1984).

I have examined this hypothesis by measuring changes in the extent of caldesmon phosphorylation when native vascular-smooth-muscle (v.s.m.) thin filaments are switched on by $Ca^{2+}$. The results indicate that caldesmon phosphorylation is very low and cannot account for its $Ca^{2+}$-dependent regulation.

## METHODS

Sheep aorta thin filaments and actin were prepared as described by Marston & Smith (1984), and thiophosphorylated sheep aorta myosin (myosinSP) was prepared as described by Heaslip & Chacko (1985). The extent of thiophosphorylation was 2.04 mol/450000 g of myosin. All measurements were made in an 'ATPase buffer', consisting of 50 mM-KCl, 5 mM-$MgCl_2$, 5 mM-$NaN_3$, 1 mM-dithiothreitol and 5 mM-Pipes buffer, pH 7.1, at 35 °C. ATP hydrolysis was measured by assaying $P_i$ released in 0.5 ml samples of the reaction mixture by the method of Taussky & Schorr (1953). Thin-filament phosphorylation was assayed as described by Walters & Marston (1981).

## RESULTS

At 37 °C, v.s.m. myosinSP had a MgATPase activity of 11 nmol/min per mg of myosinSP. This was activated to 58 nmol/min per mg by the addition of 2 mg of v.s.m. actin/ml, saturated with v.s.m. tropomyosin. This activation was essentially independent of the $Ca^{2+}$ concentration (Fig. 1). Thus the myosinSP was fully capable of being activated by actin filaments and lacked any intrinsic $Ca^{2+}$ regulation under these conditions, in agreement with Heaslip & Chacko (1985).

At 10 nM-$Ca^{2+}$, 2.8 mg of v.s.m. native thin filaments/ml did not activate v.s.m. myosinSP significantly, but, when the $Ca^{2+}$ concentration was raised to 30 $\mu$M, MgATPase activity immediately increased 6-fold. Examination of the time course indicated that the increase in activity had occurred within 10 s after [$Ca^{2+}$] was raised (Fig. 1). The extent of thiophosphorylation of myosin (2.04 mol of $^{35}S$/mol of myosin) was constant throughout the experiment. The same rapid change of ATPase rate in response to $Ca^{2+}$ was observed with skeletal-muscle myosin activating v.s.m. thin filaments.

Abbreviations used: v.s.m., vascular smooth muscle; myosinSP, thiophosphorylated v.s.m. myosin.

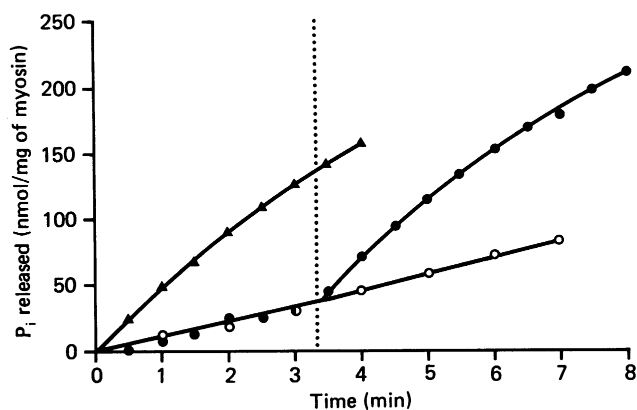


**Fig. 1. Time course of $Ca^{2+}$ stimulation of v.s.m. thin filaments and actin–tropomyosin activation of myosinSP MgATPase**

MyosinSP (0.8 mg/ml) (○), myosinSP (0.8 mg/ml)+2.8 mg of thin filaments/ml (●) or myosinSP (0.8 mg/ml)+2 mg of actin saturated with v.s.m. tropomyosin/ml (▲) were incubated at 35 °C with ATPase buffer plus 0.2 mM-EGTA (10 nM-$Ca^{2+}$). The reaction was initiated by adding 0.1 vol. of 20 mM-MgATP solution, pH 7. At 3 min 20 s (dotted line), [$Ca^{2+}$] was increased to 30 $\mu$M by the addition of 0.23 mM-$CaCl_2$. MgATPase activity of the thin filaments alone (5 nmol/min) has been subtracted.

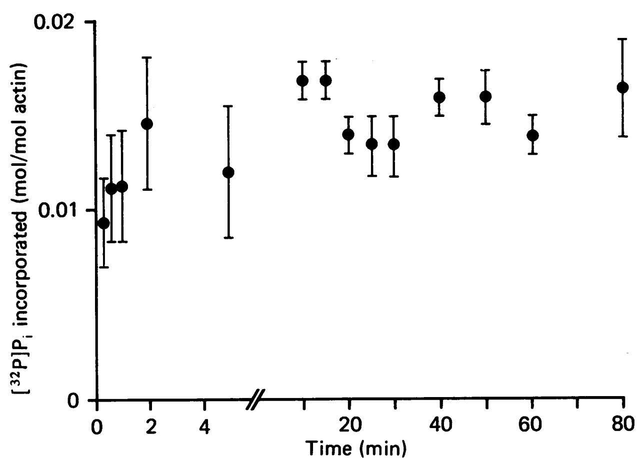


**Fig. 2. Time course of phosphorylation of thin filaments**

Thin filaments (2.8 mg/ml) in ATPase buffer, were incubated at 35 °C with 10 $\mu$M-$Ca^{2+}$, 10 $\mu$g of calmodulin/ml and 0.5 mM-[$\gamma$-$^{32}$P]ATP. Results are plotted as means ± S.E.M. for six experiments using different protein preparations. Value for $P_i$ incorporated per mol of actin are based on actin $M_r$ of 42000 and 63% actin content in the thin filament (Marston & Smith, 1984).

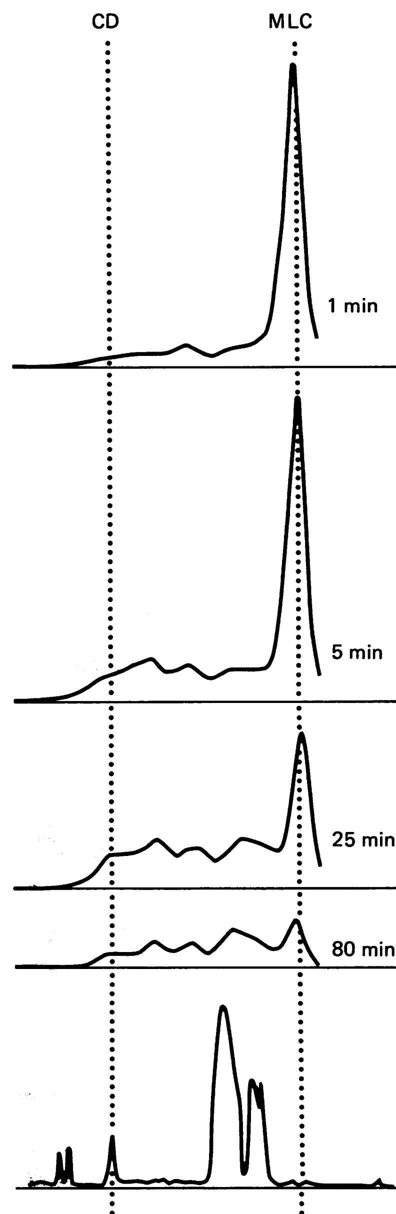


**Fig. 3. Location of $^{32}$P incorporated into thin filaments**

Samples were incubated as in Fig. 2 but with a high specific radioactivity of 0.5 mM-[$\gamma$-$^{32}$P]ATP. After termination of the reaction with 5% trichloroacetic acid, samples were separated by electrophoresis on a 0.1%-SDS/4–30%-polyacrylamide gradient slab gel. $^{32}$P radioactivity was detected by autoradiography using Agfa Osray X-ray film and the developed autoradiograms were scanned by an LKB Ultrascan densitometer. A densitometer scan of Coomassie-Blue-stained thin filaments is shown at the bottom. The direction of migration of proteins was left to right. Abbreviations: CD, caldesmon; MLC, myosin 20000-$M_r$ light chain.

The time course of covalent incorporation of [$^{32}$P]$P_i$ from [$\gamma$-$^{32}$P]ATP into native thin filaments was measured under the same conditions as the ATPase measurements with 10 $\mu$M-$Ca^{2+}$ and added calmodulin. Measurements were made with six protein preparations. $P_i$ incorporation per actin was calculated by assuming an actin $M_r$ of 42000 and 63% actin content in thin filaments (Marston & Smith, 1984). Very low incorporation was observed; the maximum was always less than 1 molecule/50 molecules of actin. Phosphorylation was initially rapid (half-time less than 1 min), and the amount of rapid phosphorylation was quite variable. After the rapid phase phosphorylation remained constant or increased or decreased slowly (maximum change ± 50% in 60 min). In one experiment thin filaments were preincubated for 15 h at 4 °C with alkaline phosphatase (1.5 $\mu$g/ml) and

acid phosphatase (2 $\mu$g/ml), a treatment which we have shown dephosphorylates smooth-muscle myosin. This treatment had no effect on the extent or rate of $P_i$ incorporation. Because of the variability between preparations, I have pooled results (Fig. 2).

The phosphorylated thin filaments were separated by SDS/polyacrylamide-gel electrophoresis and the $^{32}P$-labelled components were identified by autoradiography (Fig. 3). In the rapid initial phase virtually all the $[^{32}P]P_i$ was incorporated into myosin light chain, which is a minor contaminant of these preparations. No caldesmon phosphorylation was detected. Over the first 30 min of the reaction, $P_i$ incorporation into other proteins, including caldesmon, was observed, but the extent of myosin light chain phosphorylation declined. Caldesmon phosphorylation was never more than 25% of the total, which was estimated to be equivalent to 1 molecule of $P_i$/200 molecules of actin [1 per 7 molecules of caldesmon, based on the actin/caldesmon ratio of 28:1 (Marston & Smith, 1984; Marston & Lehman, 1985)]. The very low total $P_i$ incorporation and the fact that this involved a number of different proteins, some of which were contaminants, probably accounts for the variability in the measured time course of incorporation (Fig. 2).

## DISCUSSION

Activation by native v.s.m. thin filaments of v.s.m. myosinSP or skeletal myosin MgATPase activity is swtiched on rapidly by an increase in $[Ca^{2+}]$ (Fig. 1). This increase could not be due to activation of the myosinSP, since this was fully active at the start and the extent of thiophosphorylation did not change during the experiment (Fig. 1). The myosin contaminant of thin-filament preparations was rapidly phosphorylated when $[Ca^{2+}]$ was increased. On the basis of the amount of $P_i$ incorporated into myosin light chain (Figs. 2 and 3), contaminant myosin was 0.4–0.5 $\mu$M (active sites), compared with 4.5 $\mu$M added myosin in Fig. 1. Thus the contamination could not account for the 6-fold activation of thin-filament myosin ATPase by $Ca^{2+}$ (Fig. 1). I conclude that $Ca^{2+}$ stimulation acted primarily on the thin filament and its caldesmon-mediated regulatory system.

The activation of MgATPase activity was not associated with any phosphorylation of caldesmon (Figs. 2 and 3); thus $Ca^{2+}$ acted directly on the thin filament. $Ca^{2+}$ probably acted by binding to a $Ca^{2+}$-binding protein such as calmodulin, since $Ca^{2+}$–calmodulin has been shown to be capable of reversing caldesmon inhibition in reconstituted thin filaments (Sobue *et al.*, 1982; Smith & Marston, 1985; Marston & Smith, 1985; Dabrowska *et al.*, 1985).

Although the present work rules out a role for caldesmon phosphorylation in the acute $Ca^{2+}$ regulation of v.s.m. thin filaments, it does not exclude the possibility of long-term modulation of caldesmon by phosphorylation. *In vitro*, caldesmon can incorporate $P_i$ (Ngai & Walsh, 1984, 1985; Umekawa & Hidaka, 1985; Fig. 2), albeit rather slowly and under conditions where other contractile proteins such as actin and tropomyosin are also phosphorylated (Silver & DiSalvo, 1979; Walsh *et al.*, 1981). However, extents of caldesmon phosphorylation *in vivo* are unknown, and it has not yet been directly demonstrated that phosphorylation of caldesmon can alter its regulatory properties.

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