# Role of Caldesmon in the Ca2+ Regulation of Smooth Muscle Thin Filaments

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# Chapter #

# Role of Tropomyosin in the Regulation of Contraction in Smooth Muscle

**S. Marston* and M. EL-Mezgueldi**

## Abstract

Smooth muscle contraction is due to the interaction of myosin filaments with thin filaments. Thin filaments are composed of actin, tropomyosin, caldesmon and calmodulin in ratios 14:2:1:1. Tissue specific isoforms of α and β tropomyosin are expressed in smooth muscle. Compared with skeletal muscle tropomyosin, the cooperative activation of actomyosin is enhanced by smooth muscle tropomyosin: cooperative unit size is 10 and the equilibrium between on and off states is shifted towards the on state. The smooth muscle-specific actin-binding protein caldesmon, together with calmodulin regulates the activity of the thin filament in response to $Ca^{2+}$. Caldesmon and calmodulin control the tropomyosin-mediated transition between on and off activity states.

## Introduction

Thin filaments were first extracted from smooth muscle in 1976 and identified as containing actin and tropomyosin,[1] however subsequent experimentation showed that native thin filaments were $Ca^{2+}$-regulated due to the presence of additional proteins.[2,3] It is now well established that the $Ca^{2+}$-regulated smooth muscle thin filament is made up of actin, tropomyosin, caldesmon and calmodulin in molar ratios 14:2:1:1.[4-6] Smooth muscle thin filaments and the regulatory role of caldesmon has been reviewed several times;[7-12] in this chapter we will discuss the current state of knowledge of the mechanism of regulation of thin filaments in smooth muscle together with recent evidence that has established a critical role for tropomyosin.

## The Components of Smooth Muscle Thin Filaments

### *Actin*

In smooth muscles, actin filaments are major components of both the actin cytoskeleton and the contractile apparatus. These form separate cellular compartments which are linked through the dense bodies, homologous with Z lines, which provide anchorage points for the contractile actin filaments as well as for intermediate filaments of the cytoskeleton.[13,14] The contractile filaments of smooth muscle are made up from smooth muscle α (ACTA2 gene) and γ (ACTAG2 gene) actin whilst the cytoskeletal actin isoforms are cytoplasmic β (ACTB gene) and γ actin (ACTG1 gene). α-actin predominates in visceral and other phasic smooth muscles whilst γ-actin is the main isoform in tonic smooth muscles such as aorta. Actin is a highly conserved protein and all isoforms form filaments that are made up of a right handed helix of actin monomers with two strands that cross over every 36 nm; each crossover unit contains 13 actin monomers[15,16] (see

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Fig. 1). Although smooth muscles do not form regular arrays of thick and thin filaments like the sarcomeres of striated muscles, contractility is still due to the same sliding filament mechanism powered by myosin crossbridges interacting with actin filaments.[17] Smooth muscle myosin has a much slower rate of crossbridge cycling and a higher duty cycle compared with striated muscle and the thick filaments form side-polar rather than bipolar structures.[18-20] Nevertheless, at the molecular level the crossbridge cycle is essentially the same in smooth and striated muscles.

## *Tropomyosin*

Smooth muscle thin filaments, from both tonic and phasic muscle, contain approximately equal quantities of α-(TPM1) and β-(TPM2) smooth muscle tropomyosin;[21] 90% of tropomyosin is in the form of heterodimers in vivo.[22] The smooth muscle isoforms are produced from the same genes as striated and nonmuscle tropomyosin with tissue-specific alternative splicing. The smooth muscle α-tropomyosin is produced by splicing in exons 2a and 9d in place of exons 2b, 9a and 9b found in striated muscle tropomyosin. In β-tropomyosin exons 6a and 9d are expressed in place of 6b and 9a in skeletal muscle (see Vrhovski and Thiebaud chapter 2). The exons expressed in smooth

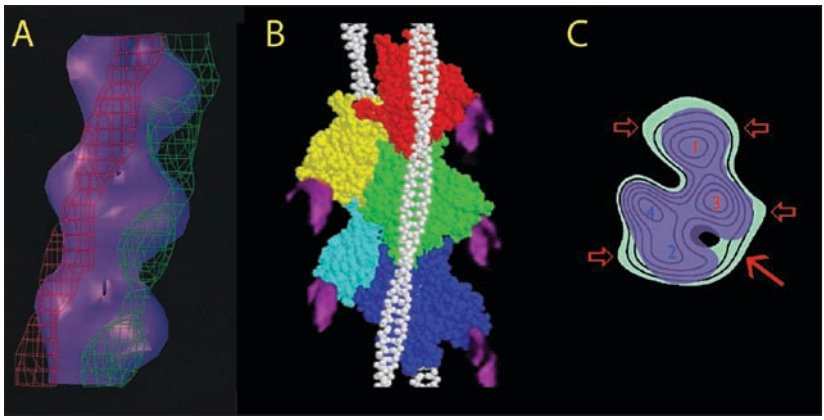


Figure 1. Structure of the smooth muscle thin filament. *A) Helical reconstruction of electron micrograph of smooth muscle thin filaments.* Reproduced from[15]. The location of tropomyosin and caldesmon densities (Red and green cages respectively) is shown superimposed on three actin monomers of one of the long-pitch actin filament helices (the other actin helix is removed for clarity). Tropomyosin is in the C position whilst caldesmon is clearly well separated from tropomyosin with no evidence of interaction. *B) Model of smooth muscle thin filament based on 3D helical reconstruction of actin-tropomyosin containing caldesmon domain 4.* The figure shows the structure of 5 actin molecules from F-actin according to the model of Lorenz et al[98] with tropomyosin in the location found for the inhibited smooth muscle thin filament.[99] These are drawn from atomic coordinates and rendered using RASMOL. The location of extra density due to caldesmon domain 4 seen in difference maps of helical reconstructions of negatively stained em images was fitted to the actin structure manually using Photoshop (purple objects).[100] Caldesmon domain 4 is located over the C-terminus of actin. *C) Transverse section through a helical reconstruction of negatively stained filaments made up of actin-caldesmon domains 3 + 4 superimposed on actin to show the location of caldesmon mass.* Because adjacent actin monomers on either side of the filament axis are staggered by half a subunit, sectioning through the center of subdomains 1 and 3 of one monomer will result in sectioning through subdomains 2 and 4 of the other monomer. The *open bold arrows* indicate regions of significant caldesmon density, most prominent on subdomain 1. The *red arrow* points to the interstrand density, derived from caldesmon domain 3 forming a bridge from actin subdomain 1 to subdomain 3 of the neighboring actin monomer in the other strand.[32]

muscle are thus the same as in nonmuscle tropomyosins, in fact smooth muscle β tropomyosin is identical to the cytoskeletal tropomyosin 1. Thus it is evident that the striated muscle exons are the exceptions and it has been proposed that they are adapted for interaction with troponin T.[23,24]

Exon 9d confers strong end to end interactions on smooth muscle tropomyosin, as a consequence cooperative activation of actin interaction with myosin is enhanced relative to skeletal muscle tropomyosin. The cooperative unit size is 10 compared with 7 for skeletal muscle and the equilibrium between on and off activity states of actin-tropomyosin is shifted towards the on state.[25,26] This is illustrated in Figure 2 which shows greater activation by myosin subfragment 1 compared with skeletal muscle tropomyosin and an increase in filament sliding speed in the in vitro motility assay due to smooth muscle tropomyosin. Tropomyosin is incorporated into smooth muscle thin filaments with a stoichiometry of 1 per 7 actin monomers and is located in a continuous strand along the actin helix. Like skeletal muscle tropomyosin, smooth muscle tropomyosin is able to take up a variety of positions on actin corresponding to the M, C and B states of the thin filament defined by Lehman (see chapter 8). Actin-smooth muscle tropomyosin is found in the B conformation and the addition of caldesmon or S-1 can move it to the M and C positions respectively.[16,27,28]

## *Caldesmon*

The third most abundant protein of smooth muscle thin filaments is caldesmon (Fig. 3). Caldesmon is an elongated molecule, with 793 amino-acids and a molecular mass of 93 kDa in human. The length of a single caldesmon molecule is 80 nm and it is made up of four structured domains separated by unstructured linkers that are sensitive to proteolysis. The first three domains approximate to a single alpha-helical structure which is stabilised by a repeating motif of acidic and basic side chains that form salt-bridges along the alpha helix. The fourth domain forms a more globular but also very flexible structure with few secondary structural elements. Domain 4

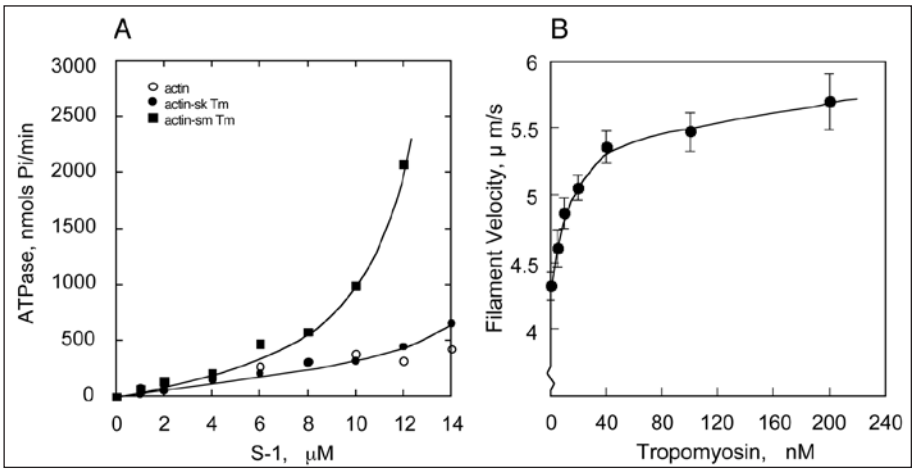


Figure 2. Activation of actomyosin interaction by smooth tropomyosin. *A*) *Dependence of ATP hydrolysis rate on S-1 concentration at a fixed actin concentration; the effect of different tropomyosin species*. Cooperative activation of ATPase by S-1 (increased slope) is much greater with smooth muscle tropomyosin than skeletal muscle tropomyosin. Conditions: 4 μM actin (Tm:actin 0.4 w/w), 5 mM MgATP, 50 mM KCl, 35 mM Imidazole-HCl, pH 7.4, 4 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 1 mM $CaCl_2$. B) *The effect of smooth muscle tropomyosin on actin filament velocity at 28°C measured by in vitro motility assay*. Actin-tropomyosin complexes were formed at a range of tropomyosin concentrations in 50 mM KCl, 25 mM Imidazole-HCl pH 7.4, 4 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT and the velocity of filaments analysed. Data points represent mean velocities with standard errors from 20 filaments.[59]

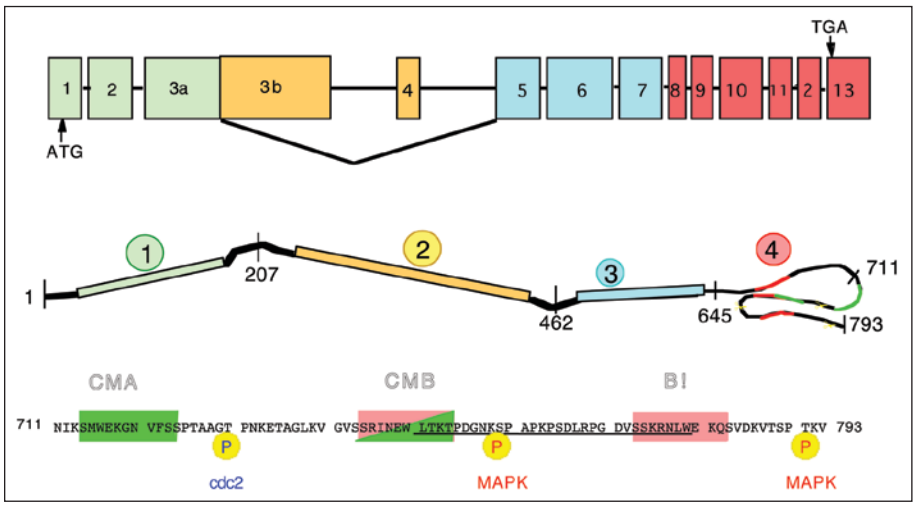


Figure 3. Caldesmon structure. Top) The exon structure of the human caldesmon gene is based on the genomic sequence.[101,102] The nonmuscle isoform message is produced by excluding exons 3b and 4. Centre) The domain structure of human smooth muscle caldesmon in relation to the exon structure. Amino acid numbers, defining the beginning of each domain, refer to the human caldesmon cDNA sequence. Actin binding segments in domain 4 are shown in red, $Ca^{2+}$-calmodulin binding segments are in green. Phosphorylation sites are in yellow. Bottom) The sequence of the C-terminal regulatory domain 4b, showing the calmodulin (green) and actin (pink) binding motifs and the phosphorylation sites. A color version of this image is available online at www.Eurekah.com.

contains the main actin and $Ca^{2+}$-calmodulin binding sites and is responsible for the regulatory property of caldesmon.

Caldesmon has been shown to bind to actin, tropomyosin, calmodulin and myosin in vitro.[10] The most important binding interaction is with actin; this binding is strongly influenced by tropomyosin, but this does not necessarily mean that there is a direct binding of tropomyosin to caldesmon in thin filaments. Evidence has been produced for tropomyosin binding to sites in all four domains of caldesmon; however the sites have differing affinities and characteristics (reviewed in 10). Thus the site in caldesmon domain 3 binds to skeletal muscle tropomyosin but not to smooth muscle tropomyosin[29] and the site in domain 2 has only been detected with recombinant fragments and is not detected in the same sequence obtained by proteolysis. Dependence of binding upon salt concentration also varies and a biosensor study has shown that the only site with significant affinity in physiological salt concentrations is in domain 4; this site corresponds to a sequence homology with troponin T.[30,31] X-ray diffraction of Bailey-type tropomyosin crystals containing added caldesmon or caldesmon domain 4 show discrete areas of extra mass along the tropomyosin molecules indicating the presence of a complex.[30]

Despite the evidence for binary actin-tropomyosin binding in vitro there is no direct evidence for caldesmon-tropomyosin contacts in the intact thin filament. In fact, helical reconstructions of smooth muscle thin filaments indicate that caldesmon forms a continuous strand along the actin filament, parallel to tropomyosin but separated by about 90°[15] (Fig. 1A). This is supported by helical reconstructions of actin complexed with domain 4 of caldesmon, which is located at the bottom of actin subdomain 1,[16,32] as far away as possible from the tropomyosin strands (Fig. 1B). NMR analysis of actin-caldesmon binding also places the main contact in subdomain 1 near the actin C-terminus.[33] Interestingly, the location of domain 4 of caldesmon on actin appears to be around the same place where the inhibitory C-terminus of troponin I has been proposed to bind.[34]

Although the structural evidence indicates no contacts between tropomyosin and caldesmon, there is a strong functional interaction. Caldesmon is an inhibitor of actomyosin ATPase and motility and both actin binding and caldesmon inhibition are greatly enhanced in the presence of tropomyosin.[35,36] The converse is also observed: caldesmon enhances the affinity of tropomyosin for actin.[37] Thus tropomyosin and caldesmon behave as allosterically coupled ligands of actin.

Caldesmon interaction with actin is thus central to caldesmon's function in regulating thin filament activity. We have consistently observed biphasic binding curves consisting of a high affinity ($>10^7$ $M^{-1}$), low stoichiometry component with a stoichiometry of around 1 per 14 actins and a low affinity component ($10^6$ $M^{-1}$) which saturates at a total amount bound of 1 caldesmon per actin.[35,38-40] It is the tight binding component that is associated with the inhibition of actin-tropomyosin and this component of binding is completely dependent on tropomyosin. This biphasic binding has also been found for troponin I+C binding to actin-tropomyosin. Zhou et al[41] report 0.14 mols troponin I+C per mol actin binding at $6 \times 10^6$ $M^{-1}$ and 0.86 troponin I+C/actin binding at $3 \times 10^5$ $M^{-1}$. It is therefore probable that this biphasic pattern of binding is characteristic of cooperative regulatory actin binding proteins. It should, however, be noted that at physiological ratios of actin to caldesmon or troponin only the tight binding sites can be occupied so the low affinity binding is not physiologically relevant.

It is well established that domain 4, the C-terminal 170 amino acids, contains all the actin binding sites of caldesmon.[42] Extensive structure-function analysis using recombinant peptides derived from various parts of domain 4 have shown that there are three actin-binding segments, each about 9 amino acids long and that they act together since only peptides containing two or more segments have caldesmon-like regulatory properties.[43-45] Figure 3B shows a model of how the peptide chain of domain 4 might be folded when it is bound to actin-tropomyosin. We have proposed that the three regions which contribute to inhibition are positioned to form a single actin binding zone.[43] The placement of the three actin binding sequences close together is supported by nuclear magnetic resonance measurements which showed that amino acids from all three putative inhibitory segments are within 1.5 nm of the unique cysteine 636 that is at the junction between domains 3 and 4.[46] The actin binding sites essential for inhibition are located in the C-terminal half of domain 4 (termed domain 4b) and are designated CMB and B' in Figure 3C. Both sites plus the linking peptide form a minimal actin-binding inhibitory domain.[9,33,39,43,47,48]

### *$Ca^{2+}$-Binding Protein*

Smooth muscle thin filaments contain a $Ca^{2+}$-binding protein that confers $Ca^{2+}$ dependent regulation on caldesmon inhibition. The $Ca^{2+}$- binding protein was identified as calmodulin and it binds to caldesmon both in the presence and absence of activating $Ca^{2+}$.[49,50] $Ca^{2+}$-calmodulin binds to caldesmon through two short sequences in domain 4b, termed CMA and CMB in Figure 3C.[51-53] It is noteworthy that the binding sequence CMB is shared by $Ca^{2+}$-calmodulin and actin, therefore it is likely that reversal of caldesmon inhibition is achieved by displacing actin from site CMB .

## Kinetic Pathway of Myosin-Thin Filament Interaction and Its Regulation

Smooth muscle contraction results from actin activation of myosin ATPase[54] (Fig. 4A). In the absence of ATP, smooth muscle myosin head binds tightly to actin. ATP binding at the myosin active site weakens the affinity of myosin for actin and dissociates the actomyosin complex. ATP hydrolysis at the active site leads to the formation of a stable myosin.ADP.Pi complex. Actin binding to this complex triggers phosphate and ADP release. The basic ATPase mechanism is similar to that of skeletal muscle myosin, but the product-release steps that determine the overall rate of the ATPase are more than 20 fold slower.[18]

In the presence of caldesmon and tropomyosin, the actomyosin ATPase is strongly inhibited. The inhibition is very limited in the absence of tropomyosin showing that caldesmon inhibition is dependent on tropomyosin.[55] Equilibrium and transient kinetics investigations have shown

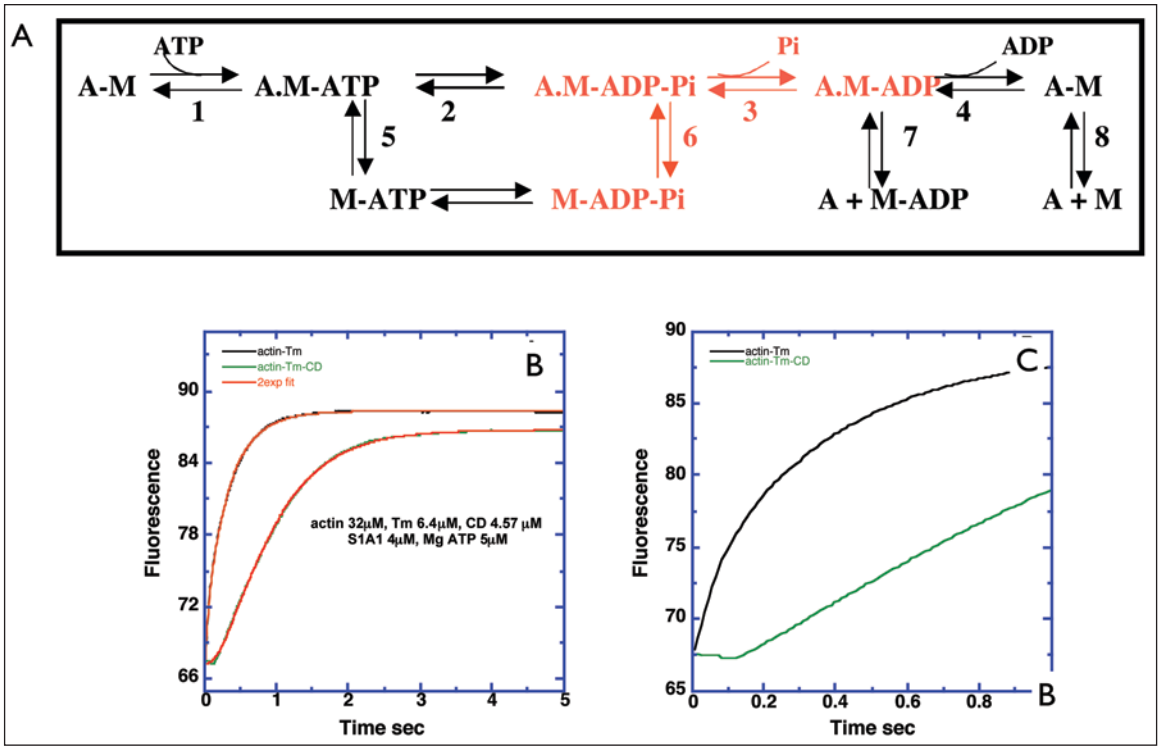


Figure 4. Kinetics of smooth muscle thin filament activation. A) Acto-myosin ATPase cycle. Highlighted in red are the steps involved in phosphate release. B) Shows the time course of Pi release measured by the change in the fluorescence of MDCC-PBP upon mixing 4 μM skeletal muscle S1 with 5μM ATP in a delay line for 1sec and then mixing this solution with an equal volume of 32 μM actin and 7 μM smooth muscle Tm in the absence or presence of 4.6 μM caldesmon. A fit to a double exponential function is superimposed on the experimental traces. C) Shows an expansion of the initial part of the transients. In the presence of caldesmon the transient clearly display a lag of around 150 ms followed by a slow exponential function.

that the caldesmon-tropomyosin complex bound to actin does not affect ATP induced acto-S1 dissociation, S1.ADP.Pi binding to actin or ADP release.[56,57] Caldesmon-tropomyosin, however, drastically inhibits the rate of phosphate release from actin.S1.ADP.Pi. In the presence of caldesmon-tropomyosin, the transient of phosphate release also showed a substantial lag (Fig. 4).[56] The presence of a lag is an indication that a prior slow step on the pathway is taking place and can be simply interpreted by a cooperative equilibrium. In the cooperative-allosteric mechanism (Fig. 6) this step would be the transition between the on and off states. At the start of the reaction the fraction of actin in the on state is very low due to caldesmon binding to the off state but as strong binding complexes are formed they will switch the thin filament to the on state leading to an acceleration of the reaction. In confirmation of this hypothesis, when actin-tropomyosin-caldesmon filaments were incubated with S-1 (1 per 4 actin) to switch on all the thin filaments before starting the reaction, the lag was no longer present.[56]

## Regulatory Mechanism of Smooth Muscle Thin Filaments

The $Ca^{2+}$-sensitive regulation of smooth muscle thin filaments and the requirement of actin, tropomyosin, caldesmon and a $Ca^{2+}$-binding protein in this regulation is well established[2,3,12] but the mechanism of $Ca^{2+}$- regulation has been a controversial topic. Like skeletal muscle thin filaments, the thin filaments of smooth muscles are negatively regulated: the function of caldesmon binding to actin-tropomyosin is to inhibit the activity of a constitutively active filament and the function of $Ca^{2+}$ binding to the CaBP is to reverse the inhibition. Caldesmon inhibition is cooperative with up to 14 actins being inhibited by the binding of one caldesmon molecule to actin-tropomyosin; moreover, $Ca^{2+}$ and calmodulin (CaM) interacting with caldesmon potentiate thin filaments to up to 150% of the activity of actin tropomyosin rather than simply neutralising the inhibitory effect of caldesmon[35,49,50] (Fig. 5AB).

In a series of papers we have given evidence that the only model of regulation that can account for all the observed regulatory characteristics is a cooperative allosteric mechanism[9,12,49,55,57-59] (Fig. 6). In this model actin-tropomyosin exists in two activity states, on and off that are linked by a concerted cooperative equilibrium. Caldesmon acts as an allosteric inhibitor by preferentially binding to actin-tropomyosin in the off state whilst at activating $Ca^{2+}$ concentration the $Ca^{2+}$-CaM-Caldesmon complex acts as an activator of actin-tropomyosin activity by preferentially binding to the on state.[60,61] This mechanism is very similar to that established for troponin-tropomyosin as recently described by Lehrer and Geeves:[62] caldesmon is equivalent to troponin I or troponin in the absence of $Ca^{2+}$ and $Ca^{2+}$-CaM-Caldesmon is equivalent to troponin in the presence of activating $Ca^{2+}$. Alternative models have been proposed that include a role for mutually exclusive competitive binding of caldesmon or myosin heads to actin-tropomyosin in determining thin filament interaction with myosin. The original model of Sobue[63] proposed a purely competitive "flip-flop" mechanism which was ruled out by measurements showing that caldesmon and S-1.ADP.Pi could bind simultaneously to actin-tropomyosin.[58] However several studies appeared to show a relationship between caldesmon inhibition and S1.ADP.Pi displacement.[64-69]

Recently we have reported direct and unambiguous evidence for the cooperative-allosteric mechanism. We made measurements of the effects of caldesmon and $Ca^{2+}$-CaM-Caldesmon on the actin-tropomyosin on-off transition using pyrene-conjugated tropomyosin. The excimer fluorescence of pyrene-tropomyosin is sensitive to the activity state of actin-tropomyosin[70] (Fig. 5 C and D)

The changes in actin-tropomyosin state as monitored by excimer fluorescence correspond to the changes in thin filament activity as determined from measurements of activation of S-1 ATPase activity (Fig. 5). Fluorescence and ATPase are reduced by low concentrations of caldesmon relative to actin-tropomyosin similar to the physiological ratio found in native thin filaments (1:16)[71] and increased by similar concentrations of $Ca^{2+}$-calmodulin. $Ca^{2+}$-calmodulin increased ATPase and fluorescence to 18-30% above the levels for actin tropomyosin alone in accord with our earlier measurements.[35,49,50]

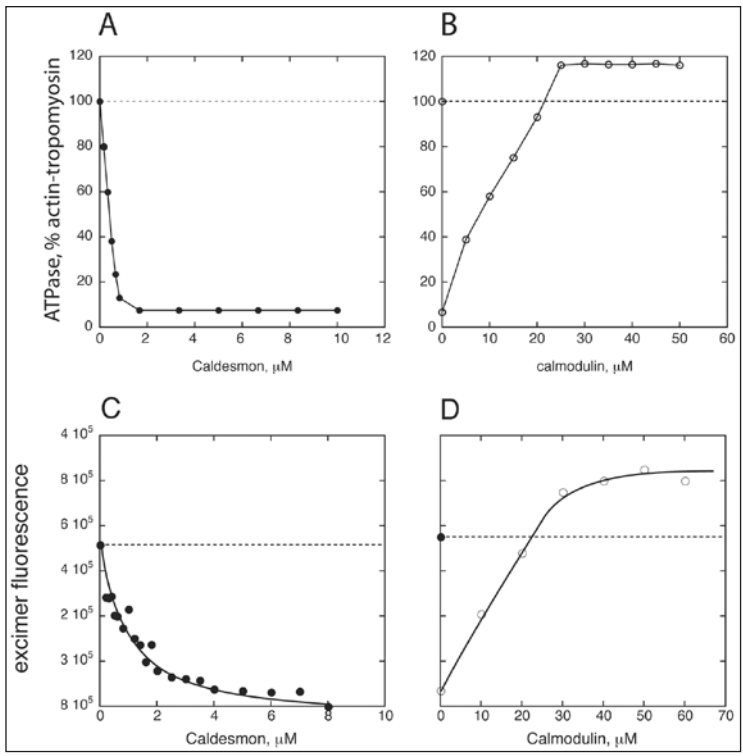


Figure 5. Caldesmon inhibition and CaM activation of smooth muscle thin filaments. *A, B Control of actin-tropomyosin ATPase activation of S-1 ATPase by caldesmon and $Ca^{2+}$-calmodulin.* Actin-tropomyosin activation of skeletal muscle myosin S-1 MgATPase activity was measured at 37°C. Conditions: 2 μM skeletal muscle S1, 12 μM skeletal muscle actin, 4 μM smooth muscle tropomyosin, in 120 mM KCl, 2.4 mM $MgCl_2$, 5 mM PIPES.$K_2$, pH 7.1, 1 mM $NaN_3$, 1 mM DTT, reaction initiated with 5 mM MgATP. Results expressed as percent of uninhibited ATPase activity. Mean uninhibited ATPase was 0.35 $sec^{-1}$. A) Inhibition of ATPase activity by 0-10 μM caldesmon, full inhibition is obtained at less than 0.2 caldesmon per actin. B) $Ca^{2+}$-calmodulin re-activation of ATPase activity in the presence of 0.8 μM caldesmon. ATPase is enhanced to 118% of uninhibited activity. *C, D) Caldesmon and calmodulin control the thin filament on-off equilibrium.* The on-off equilibrium was detected by changes in pyrene-tropomyosin excimer fluorescence. Change is actin-tropomyosin state correlates with change in actin-tropomyosin-activated ATPase. C) Caldesmon switches actin-tropomyosin to off state (low excimer fluorescence). 12 μM actin, 2 μM pyrene-labelled tropomyosin, 0-8 μM caldesmon. D) $Ca^{2+}$-calmodulin switches thin filaments to the on state (high excimer fluorescence). 12 μM actin, 2 μM pyrene-labelled tropomyosin, 8 μM caldesmon, 0-60 μM calmodulin.

The direct evidence for caldesmon regulation by a troponin-like mechanism is supported by measurements of how S1 and caldesmon binding to smooth muscle thin filaments depends on the on-off equilibrium. Weak binding (S1.ADP.Pi) binding to actin-tropomyosin is not affected by the binding of inhibitory concentrations of caldesmon, indicating that on and off states have equal affinities for S1.ADP.Pi[58,72] whilst the binding of strong binding complexes (S1.ADP, S1.AMP.PNP) is inhibited by caldesmon and becomes cooperative:[57] this effect parallels troponin-inhibited actin-tropomyosin[73] and is due to strong binding complexes having a much higher affinity for the on state than the off state leading to cooperative switching on of thin filaments[62,74] (Fig. 6).

The basis of cooperative inhibition is that the inhibitory ligand has a higher affinity for the off state than the on state. In vitro, Caldesmon binding to actin-tropomyosin in the on state is weakened at least 20-fold and becomes cooperative, whilst caldesmon binding to actin-tropomyosin in the off state is similar to binding to actin-tropomyosin alone, indicating that caldesmon preferentially binds to the off state as expected.[61] The activating effect of $Ca^{2+}$.CaM.CaD produces the opposite effect on affinity for actin-tropomyosin: $Ca^{2+}$.CaM.CaD binding to actin-tropomyosin in the on state is similar to binding to actin-tropomyosin alone, but $Ca^{2+}$.CaM.CaD binding to actin-tropomyosin in the off state is weakened about 20x and is now cooperative, indicating that the activated complex binds preferentially to the on state as predicted by the cooperative-allosteric model. Finally, as already described, the time course of the Pi release step (Fig. 4) of actin-tropomyosin ATPase inhibited by caldesmon can only be accounted for by the cooperative allosteric mechanism.

The role of tropomyosin in caldesmon inhibition remains controversial and seems to depend critically on ionic conditions. Whilst every laboratory reports a consistently low caldesmon:actin stoichiometry for inhibition in the presence of tropomyosin (see Fig. 5), inhibition in the absence of tropomyosin seems to be rather variable. At one extreme no caldesmon inhibition in the absence of tropomyosin was reported,[35] others reported full inhibition required 1 caldesmon per actin and several papers show less than 1 caldesmon per actin is required.[9,36,58,65,67,75-77] At the other extreme we have found that at very low ionic strength fully cooperative caldesmon inhibition can be obtained in the complete absence of tropomyosin.[78]

If thin filaments can be cooperatively regulated without tropomyosin in the absence of added KCl, what is the function of tropomyosin in native thin filaments? Tropomyosin is not a typical protein ligand. It is located at a radius of 3.8 nm from the actin filament axis and appears to make only a few contacts with actin.[79] Individual tropomyosin molecules have a very low affinity for actin ($10^4$ $M^{-1}$) and binding affinity only becomes high when tropomyosin molecules are joined end to end.[80] Charge plays a large role in binding since the affinity of tropomyosin for actin is optimal

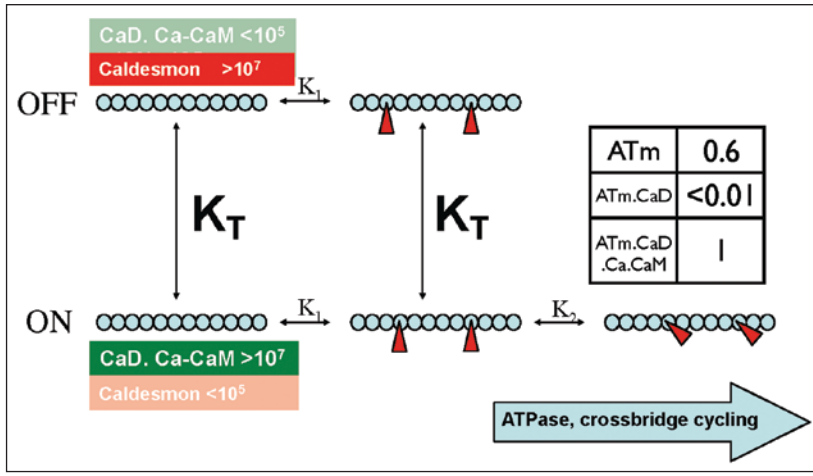


Figure 6. Cooperative allosteric mechanism for $Ca^{2+}$-regulation of smooth muscle thin filaments. Adapted from Lehrer and Geeves.[62] The thin filament is a cooperative two-state (on-off) system. Myosin ADP.Pi can bind equally to either state ($K_1$) but the transition to the strong binding state that is associated with crossbridge movement and force generation, $K_2$, can only take place from the on state. The population of the on state is controlled by caldesmon and calmodulin. Caldesmon binds strongly to the off state and so shifts the equilibrium $K_T$ from 0.6 to <0.01. CaD.$Ca^{2+}$-CaM binds strongly to the on state and so shifts $K_T$ from 0.6 to 1.

around physiological ionic strength and very low at both low and high ionic strengths.[81,82] Holmes et al[83] have proposed a model in which the negatively charged tropomyosin strand 'floats' over a bed of positively charged residues on the surface of actin with its position being determined by the pattern of surface charges on actin (itself defined by the allosteric ligands that bind to actin). Thus the evidence from the study of the actin-tropomyosin-caldesmon interaction suggests that the fundamental cooperative interactions takes place directly from actin to actin, predominantly via charge—charge interactions and that the function of tropomyosin is to shield the actin surface from the solvent so that these interactions could take place in physiological conditions. In this hypothesis the different positions of tropomyosin associated with different activity states are a consequence of allosteric transitions in actin rather than their cause.[78]

## Caldesmon Phosphorylation and the Molecular Mechanism of Thin Filament Regulation

Phosphorylation of caldesmon may be an alternative mechanism that would relieve caldesmon inhibition at low $Ca^{2+}$. Three sites have been identified in the regulatory domain 4b at threonine 730, serine 759 and serine 789 in the human sequence (Fig. 3).[84,85] Interestingly, the phosphorylation sites are in the middle of linking peptides, 10-14 amino-acids downstream from the tryptophan at the core of the regulatory segments CMA, CMB and B' respectively. In mammalian smooth muscle serines 759 and 789 are substrates of MAP kinase and threonine 730 does not get phosphorylated. Phosphorylation at either or both of these sites leads to loss of inhibition and a reduction in actin-tropomyosin binding affinity.[86] The structural effects of phosphorylation were studied in detail in a minimally inhibitory peptide (LW30, underlined in Fig. 3) using NMR spectroscopy.[87] The peptide linking the sites CMB and B' forms a structured turn which is hypothesised to position the two sites for docking onto actin in the switched off conformation.[33] The effect of phosphorylation (or mutation of the serine to aspartic acid) at serine 759, which is close to the turn, is to destroy its structure and hence eliminate two-sited binding.[32,87]

The concept of obligatory two-site binding for caldesmon inhibition unites structural and functional studies on caldesmon (Fig. 7).[89,90] We propose that the CMB-peptide-B' segment of domain 4 forms a structure which binds to actin—tropomyosin only in the off state, thus caldesmon inhibits actin-tropomyosin activity. Inhibition may be relieved either by $Ca^{2+}$-calmodulin binding to sites CMA and CMB, thus displacing site CMB from actin, or by phosphorylation of serines 759 and/or 789 that disrupts the structure necessary for two-site binding to actin-tropomyosin. Caldesmon is not dissociated from actin by $Ca^{2+}$-calmodulin or phosphorylation because there is an additional actin binding site, C, in the N-terminal half of domain 4 (Fig. 3B) and $Ca^{2+}$-free calmodulin can remain bound to caldesmon through site CMA. To account for the activating effect of $Ca^{2+}$-calmodulin, we presume that caldesmon. $Ca^{2+}$-CaM complex binds through site C to actin

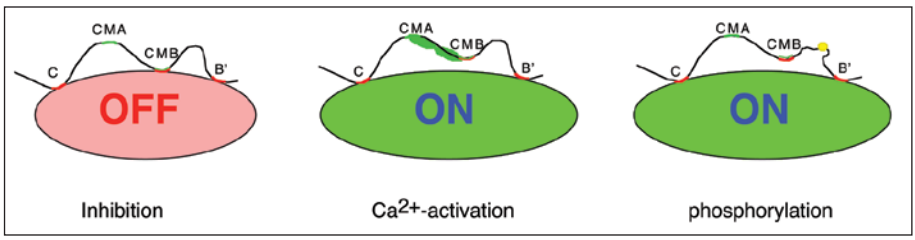


Figure 7. 2 site binding model for caldesmon regulation of actin-tropomyosin. Inhibition of actin-tropomyosin is due to two-site docking of caldesmon sites CMB and B′ specifically onto actin in the off state. Inhibition may be released by $Ca^{2+}$-CaM (green) binding to CMA and CMB thus displacing caldesmon from CMB, which abolishes two-site binding, or by phosphorylation that destabilises the linker peptide, also preventing two-site docking. Site C tethers actin to caldesmon in activating conditions and site CMA tethers calmodulin to caldesmon in relaxing conditions.

preferentially in the on state. This model is supported by studies of a caldesmon peptide, named H2 (683-767), containing sites C, CMA and CMB but lacking site B' necessary for inhibition. H2 is a tropomyosin-dependent activator of ATPase and has been shown to switch the filament to a state resembling the switched on state obtained with rigor crossbridges.[43,91]

## Conclusions

Tropomyosin plays an essential part in the regulation of smooth muscle thin filament activity by the troponin-like concerted cooperative mechanism but the physiological role of this regulation is yet to be fully clarified. Compared with troponin, caldesmon has a lower affinity for actin-tropomyosin in all activity states and the calculated cooperative unit size is reduced by the binding of caldesmon, whereas it is increased by binding troponin;[92] these properties mean that caldesmon is not able to switch activity in the same way as troponin does. Smooth muscle is a dual regulated muscle and the primary mechanism of $Ca^{2+}$-regulation is via the activation of myosin by phosphorylation of the regulatory light chain by $Ca^{2+}$-CaM dependent myosin light chain kinase. $Ca^{2+}$-dependent caldesmon inhibition seems to play a secondary role in modulating $Ca^{2+}$-sensitivity[93] by accelerating relaxation[94,95] and in maintaining basal relaxation.[96,97]

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