Signal Transduction in Smooth Muscle
Invited Review: Cross-bridge regulation by thin filament-associated proteins

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Morgan, Kathleen G., and Samudra S. Gangopadhyay. Invited Review: Cross-bridge regulation by thin filament-associated proteins. J Appl Physiol 91: 953–962, 2001.—This minireview will cover current concepts on the identity and mechanistic function of smooth muscle actin binding proteins that may regulate actin-myosin interactions. The potential roles of tropomyosin, caldesmon, calponin, and SM22 will be discussed. The review, for purposes of brevity, will be nonexhaustive but will give an overview of available information on the in vitro biochemistry and potential in vivo function of these proteins. Preterm labor is discussed as a possible example of where thin filament regulation may be relevant. Considerable controversy surrounds the putative physiological significance of these proteins, and emphasis will be placed on the need for more experimental work to determine the degree to which tissue- and species-specific effects have clouded the interpretation of functional data.

caldesmon; calponin; tropomyosin; SM22; preterm labor

THIS REVIEW WILL FOCUS ON smooth muscle thin filament regulatory proteins. The thin filaments are defined as those filaments 6–8 nm in diameter and composed of filamentous actin. These filaments are to be distinguished from the intermediate filaments (10 nm), the myosin filaments (15–18 nm), and the microtubules (~24 nm) (102). Cross-bridge regulation can occur, in theory, by the action of molecules associated with either actin or myosin or by changes in the “load” imposed by any other cytoskeletal component. For this minireview, we will restrict our focus to actin binding proteins that might regulate cross-bridge cycling. Space limitations require that the review be nonexhaustive, and for that reason we will focus on the possible roles of certain proteins that are purported to bind actin and possibly regulate cross-bridge function: tropomyosin (Tm), caldesmon (CaD), calponin (CaP), and SM22. We will not deal with the issue of regulated actin filament assembly and disassembly, as this has been covered in other recent reviews [e.g., Gunst and Tang (38)].

IS THIN FILAMENT REGULATION IMPORTANT IN SMOOTH MUSCLE FUNCTION?

It is clear that smooth muscle cross bridges are regulated by phosphorylation of the regulatory light chains of myosin (LC20) (for reviews see Refs. 7, 8). However, recent studies have indicated that additional regulatory mechanisms are also present. Evidence for thin filament cross-bridge regulation includes 1) the demonstration of calcium sensitivity of myosin ATPase activity in preparations containing skeletal muscle myosin and smooth muscle thin filaments (74, 75); 2) reports that cross-bridge cycling rates can vary without detectable changes in LC20 phosphorylation (e.g., Refs. 36, 37, 96); and 3) reports of dissociations between LC20 phosphorylation and tension (20, and reviewed in Horowitz et al. (45)). Additionally, the suggestion that, under some conditions, unphosphorylated cross bridges are not completely turned off (39, 92, 112) provides a teleological need for a regulatory system in addition to thick filament regulation.
ACTIN

The actin concentration in smooth muscle is ~0.9–1.6 mM (40, 82) and accounts for 30–50% of the total noncollagenous proteins in smooth muscle (62). Cytoplasm, in general, mimics the conditions necessary for polymerization of actin (62), and actin is thought to exist primarily as filamentous F-actin in intact cells (102), but, given the presence of numerous proteins that could regulate polymerization in vivo, the percentage of monomeric G-actin in contractile smooth muscle cells is not definitively known.

In vertebrate tissues, six actin isoforms have been described and are products of separate genes. The α-, β-, and γ-isoform variants are separated based on iso-electric focusing, proceeding from the most acidic to the least acidic. Based on differences in amino acid sequence, α-skeletal, α-cardiac, and α-vascular; γ-enteric and γ-cytoplasmic; and β-cytoplasmic isoforms are distinguished (43, 62). In smooth muscle tissues, the β-isoform and the γ-cytoplasmic isoforms are often referred to as nonmuscle isoforms, whereas the α-vascular and γ-enteric isoforms are often referred to as smooth muscle actin isoforms. The smooth muscle isoforms have been associated with the contractile filaments, whereas the nonmuscle isoforms have been associated with the noncontractile cytoskeleton (27, 84) and subplasmalemmal cortex (87).

The amino acid sequences of the actin isoforms are remarkably conserved, with most sequence differences confined to the N-terminal end of the molecule. This part of the molecule is not involved directly in filament formation but is the site for interaction with various actin binding proteins, which may lead to isoform-specific targeting functions (43, 62).

THIN FILAMENT-ASSOCIATED PROTEINS

Tm

Tm is a coiled-coil α-helix. Tm in muscle tissue spans seven actin monomers. Consistent with this, the molar ratio of Tm to actin monomers is ~1.2:7 in smooth muscle (98). Individual molecules interact head to tail, thereby creating a continuous strand along the actin filament.

In striated muscle, it is clear that Tm confers properties of cooperativity along the contractile filaments by virtue of its spanning several actin monomers and that it also provides the calcium switch for turning myosin ATPase activity on and off by interacting with the troponin complex (63). The function of Tm in smooth muscle is less clear because troponin is not present. However, several lines of evidence suggest that Tm functions to provide cooperativity in smooth muscle as well. X-ray studies have suggested that the activation of smooth muscle leads to the movement of Tm in a manner similar to that in striated muscle (110). More recently, fluorescent resonance energy transfer studies have demonstrated that the binding of smooth muscle myosin heads to actin leads to the movement of smooth muscle Tm in a highly cooperative fashion (33). Cross-linking studies between actin and smooth muscle Tm are consistent with this movement (30). Furthermore, it has been shown that the movement of smooth muscle Tm by myosin binding is more easily facilitated by phosphorylated myosin than unphosphorylated myosin (34), providing for possible cross talk between thick and thin filament regulation. Functional studies have demonstrated that strong binding myosin cooperatively activates force in smooth muscle in a manner that would be consistent with the observed movements of Tm propagated down the actin filaments (103). Evidence also exists suggesting that the end-to-end interactions of smooth muscle Tm may be stronger than those in striated muscle (32), which may accentuate cooperative effects in smooth muscle and account for the steeper cooperative activation of gizzard muscle compared with rabbit skeletal muscle (64, 65). Finally, Tm is necessary for full inhibition of actomyosin ATPase activity by CaD (12, 99).

CaD

CaD is an actin, Tm, myosin, and calmodulin (CaM) binding protein. Its in vitro properties have been reviewed previously (45, 73). Briefly, carboxy-terminal domains are responsible for actin binding and inhibition of myosin ATPase activity in vitro (Fig. 1). Binding of CaM or phosphorylation of sites between the two C-terminal actin binding domains can reverse some of the inhibitory actions of CaD in vitro (see below). The N-terminal half of the molecule has been shown to bind myosin and, in vitro, tether myosin to actin in conjunction with C-terminal actin binding domains of CaD (11, 42, 48, 69, 114).

Although considerable work has been reported on the in vitro biochemistry and physical chemistry of CaD, relatively few in vivo cellular or physiological studies have been performed to test the relevance of CaD to the regulation of smooth muscle contraction. Two main approaches have been used to probe the function of endogenous CaD. First, a peptide antagonist approach has been used, based on the domain structure of CaD. A putative peptide antagonist of CaD, targeted in the higher affinity actin binding site and containing Gly\(^{651}\)-Ser\(^{667}\) of the gizzard sequence (GS17C; Fig. 1), was shown to induce a sustained elevation of basal contractile tone of single, permeabilized smooth muscle cells (50). These results were interpreted to indicate that the peptide competes with endogenous CaD for this actin binding site, resulting in a “nudging” of CaD locally and interfering with inhibitory actions of CaD on myosin ATPase activity.

A second approach to investigating the physiological function of CaD has been to use a chemical loading procedure originally developed to load equinor into smooth muscle (80) to introduce antisense oligonucleotides against CaD into intact vascular strips (22). The effect of CaD antisense was a sustained elevation of basal contractile tone, consistent with a role of endogenous CaD to tonically suppress contractile tone. The consistency between the peptide studies with GS17C...
and the antisense studies, which are very different approaches, strongly suggests that, indeed, CaD plays a physiologically important role in suppressing smooth muscle tone.

Regarding the possible tethering ability of CaD, two peptides have been synthesized from the region of the N-terminal myosin binding domain of CaD (MY27C and IK29C; Fig. 1). The effect of these peptides was, specifically, to prevent agonist-activated contractions. After the contraction was established, the peptides had no effect on reversing the contraction. These results were interpreted to suggest a role for CaD in normally aligning actin and myosin (through a tethering effect), so that the C-terminal end of CaD will inhibit actomyosin interactions until agonist-mediated signaling pathways (see below) disinhibit this effect (58).

**Regulation of Actions of CaD in Cells**

In vitro studies have suggested two possible mechanisms by which inhibitory actions of CaD can be reversed: by the binding of Ca$^{2+}$/CaM and by phosphorylation.

**CaM.** Two CaM binding sites have been localized to the COOH-terminal domain of CaD (Fig. 1). Binding of Ca$^{2+}$/CaM to CaD decreases the binding of CaD to actin in vitro, as measured by a reduction of CaD-actin cross-linking (35). In situ, CaD is not observed to leave the actin filaments (52), but a local decrease in the affinity of CaD for actin may result in a positional change that results in reversal of its inhibitory actions. The CaM binding domains have been measured to have a binding constant of $2.3 \times 10^5$ M$^{-1}$ when prepared by conventional methods (122). It has been questioned whether sufficient free CaM is available to effect a significant change through the CaM binding sites of CaD. However, recently, three different lines of evidence have caused the CaM hypothesis to be reevaluated. 1) It is now recognized that, whereas the previous CaD binding constants for CaM were determined with respect to purified, heat-treated CaD, recombinant CaD that is not heat treated has a higher binding constant of $2.0 \times 10^7$ M$^{-1}$ (122). 2) The possibility has been raised that the amount of free CaM may be dynamically regulated. In experiments in which a CaM binding domain of CaD was used as a probe of free CaM concentration in living smooth muscle cells, activation of protein kinase C (PKC)-dependent pathways significantly increased the signal from the probe, indicating that, indeed, sufficient CaM is present, at least in agonist-activated cells, to bind CaD (46). 3) It has also been reported that a modified version of CaM exists in smooth muscle that has a significantly higher affinity for CaD than does unmodified CaM (85). Thus the possibility that Ca$^{2+}$/CaM may regulate the inhibitory actions of CaD on contractility deserves further investigation, most importantly at the in vivo level.

**Phosphorylation.** The question of whether phosphorylation of CaD regulates its activity in vivo has been particularly controversial and confusing. In vitro experiments have shown that phosphorylation of CaD by PKC or p21-activated kinase (PAK) can reverse the inhibitory activity of CaD on myosin ATPase activity and can also decrease the actin binding affinity of CaD (23, 47, 106, 111). In contrast, Childs et al. (15) reported only a small, but significant effect of extracellular regulated kinase 1 (ERK1) phosphorylation on actin cosedimentation of CaD. Furthermore, Krymsky et al. (56) have shown that ERK phosphorylation of gizzard CaD did not reverse its effects on myosin ATPase.

However, it has been shown that, when CaD is phosphorylated by ERK, CaD no longer inhibits movement in a motility assay (2, 25). Furthermore, Li et al. (68) have recently shown that ERK phosphorylation at both

![Fig. 1. Domain structure of caldesmon, modified from Lee et al. (58).](http://jap.physiology.org/)
S759 (homologous to gizzard S702) and S789 of a C-terminal fragment of human CaD (fragment 576–793) clearly prevents actin binding. Similar results were presented by Marston et al. (72). Thus, whereas in the intact molecule multiple actin binding domains appear to retain phosphorylated CaD on the actin filaments (52), there is the possibility that phosphorylation may cause a local conformational change in the 576–793 region, which may significantly alter the function of CaD.

Several types of in vivo experiments have been performed to address the question of whether CaD function is regulated by phosphorylation: measurements of in vivo phosphorylation levels; permeabilized muscle experiments; the use of pharmacological antagonists in intact smooth muscle preparations; and imaging studies.

With respect to the measurement of changes in phosphorylation levels in vivo, Adam et al. (1, 3, 4) reported that, after the addition of phorbol esters to canine aortas or pig carotid arteries, CaD is phosphorylated at sites identical to those caused by ERK in vitro. Recent work from D’Angelo et al. (17), using a quantitative phosphoantibody approach, has produced somewhat different results. In hog carotid tissues, there was a high basal and no phorbol ester-induced increase in S789 phosphorylation and very little signal at the S759 site. In contrast, our laboratory has used an antibody to the same sites and has seen a time- and agonist-dependent three- to fivefold increase in the phosphoantibody signal from the ERK1/2 sites during agonist-induced contractions (52). In vitro, CaD, on the other hand, has been reported to colocalize with CaD during agonist-induced contractions (52).

Thus our bias is that ERK may well be the physiological CaD kinase, at least in some types of smooth muscle. It is also possible that the effects of CaM and of phosphorylation of CaD may be synergistic to reverse the inhibitory actions of CaD. On the other hand, the possibility remains that PAK (with PAK + CaD) may also serve an important role or that an as-yet-to-be-identified kinase may be involved.

CaP

CaP is a family of putative actin regulatory proteins that includes three isoforms that are separate gene products: 1) a smooth muscle-specific basic CaP, h1 (105), 2) a neutral CaP, h2, that has a very high sequence homology with h1 CaP but is less basic and is localized primarily in cardiac muscle (104), and 3) an acidic variant, which is not tissue specific, identified from rat brain and fibroblasts (6, 108).

Basic h1 CaP was originally identified as a protein specific for differentiated smooth muscle that interacts with F-actin and inhibits the actomyosin Mg-ATPase activity in vitro (for review see Gimona and Small (27)). It has also been reported that CaP binds to CaM (116), myosin (94), desmin (70), and phospholipids (9). It is a substrate for PKC and Ca^2+/CaM-dependent kinase II in vitro (117).

Regarding the structure of CaP (Fig. 2), the CaP homology (CH) domain, which has very high homology across isoforms, is followed by a variable region that, in the basic isoform, contains an actin binding region (78) with homology to troponin I (TnI), referred to as the TnI-like sequence. This is followed by three C-terminal repeats. The C-terminal repeats are also reported to be involved in actin binding (79).

The function of CH domains in proteins is somewhat unclear, as the domain is present in actin binding proteins as well as in signaling proteins (26). An ABDb-type actin binding domain (ABD) has been described, which consists of two CH domains: a CH1 domain followed by a CH2 domain. There are sequence similarities between CH1 and CH2 domains, but they are not identical. In single CH domain-containing proteins, such as CaP, SM22, IQGAP, and Vav, the CH domain sequence is quite different from either the CH1 or CH2 of ABDs. The single CH domain-containing proteins...
are not thought to bind actin (29). In contrast, the main actin binding site of CaP was mapped to be within the TnI-like domain (78). Furthermore, we have identified the CH domains of both CaP and α-actinin to be ERK binding domains (67) (Fig. 2). The modular organization of CH domains and their presence in both cytoskeletal and signaling molecules, as well as the coexistence of CH domains with other actin binding domains, raises the possibility that the function of the CH domain may be to localize signaling molecules to the actin cytoskeleton.

The role of CaP in muscle contractility is controversial. Two mechanisms have been proposed for its function. On one hand, it has been suggested that CaP may directly inhibit actin-activated Mg-ATPase activity of myosin (117). This hypothesis is based on the in vitro properties of CaP (see above) and on effects obtained with CaP and CaP peptides in permeabilized smooth muscle preparations [reviewed in Horowitz et al. (45)]. However, some investigators have seen relatively little effect of CaP on contractile force in permeabilized preparations [see references in Horowitz et al. (44)], and the subcellular distribution of CaP may be at odds with this concept (71, 87).

On the other hand, it has also been proposed that CaP may facilitate agonist-induced signal transduction. This hypothesis is based on the observations that, in ferret vascular smooth muscle, in the early time points after agonist activation, ERK and PKC coimmunoprecipitate and cotranslocate with CaP to the vicinity of the plasmalemma (77) and that ERK and PKC directly bind to CaP in vitro (66, 67). Furthermore, the binding of CaP (Fig. 2) is to the regulatory domain of PKC and appears to facilitate the activation of PKC (66). Taken together, these results suggest a role for CaP as a signaling molecule that facilitates activation of PKC and links targeting of ERK and PKC to the surface membrane in the manner of an adaptor protein (67, 77). Our laboratory has also previously shown that, in this cell type, after docking and being activated at the cell surface, ERK redistributes to the contractile filaments (52), phosphorylates CaD, and triggers a contraction (19). This model is illustrated in cartoon fashion in Fig. 3.

The technical difficulties surrounding the application of molecular techniques to contractile smooth muscle have made it difficult to determine whether either or both of these proposed mechanisms are true. A CaP knockout mouse has been generated (120). The most obvious phenotypic change observed was, surprisingly, an increase in bone formation. The observation of high expression of h1-CaP in developing skeleton and in undifferentiated osteoblasts led to the suggestion of a role for CaP as a negative regulator of bone formation. In an extensive study of bladder and vas deferens in the CaP knockout mouse, a faster unloaded shortening velocity was notice in these phasic smooth muscle tissues (76). These results are consistent with the hypothesis that CaP is a direct regulator of actomyosin interactions at the myofilament level. However, these smooth muscles also displayed decreased actin expression by 25–50%, which might be expected to decrease the mechanical load because of internal connections within the cytoskeleton of the cell and thus could also explain the high unloaded shortening velocity. Other changes in protein levels of desmin and CaD in these mice also suggest that the animal may have compensated for the physiological challenge presented by the loss of CaP (76).

On the other hand, our laboratory has recently reported preliminary data using an antisense approach (87) that resulted in an inhibition of agonist-induced contraction of a tonic vascular smooth muscle of the ferret. Thus it may be, as has been suggested before, that CaP plays different roles in the regulation of contractility in phasic and tonic smooth muscle (113).

**SM22**

SM22 is an abundant, smooth muscle-specific, 22-kDa protein, the function of which is unknown. It has been found in both visceral and vascular smooth muscle, and its expression is very high in aorta, lung, uterus, and intestine. It is detectable in some primary cultures of smooth muscle (101) but is rapidly lost with further time in culture with the switch from the contractile to the synthetic phenotype. Three isoelectric isoforms have been described as reported by Lees-

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**Fig. 2. Domain structure of calponin.**

PKC, protein kinase C; TnI, troponin I.
Miller et al. (60, 61) in chicken gizzard, namely $\alpha$, $\beta$, and $\gamma$-isoforms. The $\alpha$-isoform has been identified from different species including bovine (95), mouse (67), rat (51), and human (10) and variably designated as transgelin (57), WS3–10 (107), or mouse p20 (5). SM22 has some sequence similarities with CaP. It has one amino terminal CH domain and one CaP-like carboxy terminal repeat but lacks the TnI-like consensus actin binding motif that is present in basic CaP (26).

SM22 appears late in the embryonic development of chicken relative to other components of the smooth muscle cytoskeleton (21). Because of this specific expression, it serves as an early marker of smooth muscle tissue. It is detected in the aorta after day 4 and before the appearance of CaP (21).

Whether or not SM22 is a functional actin binding protein in smooth muscle cells is controversial. Aorta SM22 homologs are reported to bind to actin monomers in vitro (55, 93) to cause the gelation of actin in vitro and to bind to stress fibers when added exogenously to permeabilized rat fibroblasts (93). However, others have been unable to detect any binding of SM22 to actin. Gimona and Mital (26) reported that SM22 failed to bind actin in cosedimentation assay and did not colocalize with actin in transfected fibroblasts. On the other hand, Fu et al. (24) reported that SM22 cosediments with actin (at low ionic strength) and colocalizes with actin in cultured smooth muscle cells.

Interestingly, it has been reported that PKC efficiently phosphorylates SM22 in vitro and that this phosphorylation inhibits actin cosedimentation in vitro (24). However, phosphorylation of SM22 in vivo has not been detected in smooth muscle cells (28), and whether it is physiologically relevant is unknown.

Recently, an SM22-deficient mouse was developed, and no obvious functional abnormalities in either visceral or vascular smooth muscle were detectable (121). However, electron micrographs showed a pronounced change in the actin filament distribution, suggesting a possible role for SM22 in the organization of the cytoskeleton. It is also possible that the animal compensated for any defect with the recruitment of other proteins of redundant function.

**KINASES**

It is worth mentioning that kinases, such as myosin light chain kinase, PKC-$\epsilon$, and ERK have also been reported to bind actin (67, 88, 100). This raises the interesting possibility that, at the interface of actin and myosin, within the contractile filaments, considerable complex signaling events may be regulated and facilitated by close geometric proximity.

**POTENTIAL APPLICATIONS TO HUMANS**

A major question is the relative importance of thin filament regulation of smooth muscle contractility. The answer is likely to be influenced by the well-known tissue and species specificity of smooth muscle. Furthermore, a signaling pathway that plays a relatively minor role under physiological conditions may be a determining factor contributing to the etiology of a pathophysiological situation.

One example of a clinical situation that may point to a possibly important role for CaD is preterm labor. Premature delivery is one of the major causes of perinatal morbidity and mortality, affecting roughly 1 in
10 births in the U.S. (81). Surprisingly, there has been no major new therapeutic approach to this problem over the last 30 yr, despite the tremendous economic cost of treating premature newborns and the devastating birth defects that are often associated with premature birth. It is generally acknowledged that the lack of effective therapeutic agents reflects a poor understanding of the cell biology underlying the mechanism of normal and preterm labor.

Interestingly, there is evidence pointing to a possibly important role for CaD in the suppression of contractility during pregnancy and the promotion of labor at the end of the pregnancy. Word et al. (118) have demonstrated that CaD levels are increased during pregnancy in human myometrium. Because the growing fetus provides a stretch stimulus that would be expected to provoke a myogenic contraction, an increase in CaD levels could contribute to the contractile quiescence typical of, and desirable for, preterm myometrial smooth muscle. Furthermore, preliminary data have been published that demonstrate a statistically significant decrease in CaD levels during labor in human myometrium (90, 91). Regulation of signaling pathways that modify CaD activity has not been tried as a therapeutic approach to the treatment of preterm labor, but perhaps this merits attention.

FUTURE RESEARCH DIRECTIONS

From the above, it should be clear that the field of thin filament regulation of smooth muscle contractility is filled with controversy. Considerable cellular and in vivo work will be necessary to prove or disprove the suggestions raised by in vitro protein chemistry. Present apparent contradictions in the literature could be simply a consequence of species and tissue differences, but additional work is needed to demonstrate this possibility. Furthermore, it is clear that the investigation of mechanisms of contractility of smooth muscle is made difficult by the fact that contractility is lost, even in primary culture, when there is a profound phenotypic change from the contractile to the synthetic phenotype (13). This makes the application of molecular approaches difficult but not impossible. Transgenics and antisense approaches offer possible solutions to these challenges. Often transgenic animals compensate to hide physiological responses, and antisense rarely produces more than a 50–70% decrease in protein levels. Clever, new approaches incorporating retroviral and aden-associated virus approaches (86) undoubtedly will be developed in the coming years that will make it easier to finally determine the molecular mechanism and relative importance of thin filament regulation.

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INVITED REVIEW


