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## Smooth muscle signaling pathways in health and disease

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### Abstract

Smooth muscle contractile activity is a major regulator of function of the vascular system, respiratory system, gastrointestinal system and the genitourinary systems. Malfunction of contractility in these systems leads to a host of clinical disorders, and yet, we still have major gaps in our understanding of the molecular mechanisms by which contractility of the differentiated smooth muscle cell (dSMC) is regulated. This review will summarize recent advances in the molecular understanding of the regulation of smooth muscle myosin activity via phosphorylation/dephosphorylation of myosin, the regulation of the accessibility of actin to myosin via the actin binding proteins calponin and caldesmon, and the remodeling of the actin cytoskeleton. Understanding of the molecular “players” should identify target molecules that could point the way to novel drug discovery programs for the treatment of smooth muscle disorders such as cardiovascular disease, asthma, functional bowel disease and preterm labor.

### Keywords

myosin light chain phosphorylation; calponin; caldesmon; myosin phosphatase; CaMKII; actin cytoskeleton

### Introduction

All contractility is initiated by changes in the activity of, or interactions of, actin and myosin. In recent years, a multitude of signaling pathways have been suggested to regulate smooth muscle contractility; however, these pathways can be broken down into 3 major types of mechanisms (Fig. 1): **1.** mechanisms that regulate actin-activated myosin ATPase activity via changes in the phosphorylation state of the 20kDa myosin light chain (LC20); **2.** mechanisms that regulate the availability of actin to interact with myosin via the action of inhibitory actin binding proteins such as caldesmon (CaD) and possibly calponin (CaP); and, **3.** the less well studied possibility of mechanisms by which the cytoskeleton is remodeled to facilitate the transmission or maintenance of force developed by actomyosin interactions.

The first set of pathways are known to be involved in pathologies such as traumatic brain injury and post-hemorrhagic cerebral vasospasm [1,2] (discussed in detail in a review by Jose Rafols in this series), and in pulmonary hypertension [3]. The second set of pathways are strongly implicated in preterm labor [4]. The third set of pathways are implicated in asthma [5]. Thus, the diversity of signaling pathways that regulate contractility may well offer opportunities for discovery of potential disease-specific and organ-specific therapies.

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The *first* set of mechanisms is illustrated on the left-hand side of Fig. 1 (purple type), where depolarization of a smooth muscle cell, for example, by exposure to a physiological saline solution in which NaCl is replaced by KCl, opens voltage-dependent  $\text{Ca}^{2+}$  channels and increases  $\text{Ca}^{2+}_i$  levels. This leads to a  $\text{Ca}^{2+}_i$  / calmodulin (CaM) – dependent activation of myosin light chain kinase (MLCK), phosphorylation of myosin LC20, and an increase in myosin ATPase activity [6,7]. However, as soon as intracellular  $\text{Ca}^{2+}$  indicators were successfully applied to smooth muscle cells and cell permeabilization techniques were developed, it became clear that many agonists increase the relative amount of force produced at a constant  $\text{Ca}^{2+}_i$ , i.e. cause “ $\text{Ca}^{2+}$  sensitization” of force [8–17].  $\text{Ca}^{2+}$  sensitization of force can be caused by a  $\text{Ca}^{2+}$  sensitization of LC20 phosphorylation mechanisms and this is now known to involve pathways that inhibit myosin phosphatase [18,19] as well as pathways that increase the  $\text{Ca}^{2+}$  sensitivity of MLCK, such as the ERK1/2-mediated phosphorylation of MLCK [20–22], or, possibly by the direct phosphorylation of Ser19 on LC20 by kinases other than MLCK. These mechanisms are described in more detail below.

An observed “ $\text{Ca}^{2+}$  sensitization” of force can also occur in the absence of changes in LC20 phosphorylation [23]. In this case most evidence points to the *second* type of pathway— i.e. those that regulate the activity of inhibitory actin binding proteins that regulate the availability of actin to interact with myosin (Fig.1 right-hand side, blue type). These pathways include possible roles for ERK1/2, CaP and CaD [24]. These pathways are described below in more detail.

In recent years, the *third possibility*, (Fig. 1, top, gold type) i.e., that contractility may be modulated by remodeling of the cytoskeleton has been suggested, although the molecular mechanisms are far less well defined. Remodeling of the cytoskeleton is well known to occur in airway smooth muscle, and there is growing evidence that this also happens in some blood vessels, (see below), however, it is not yet clear exactly how cytoskeletal remodeling modulates contractility of either the airway or vascular cells. This topic will be discussed in detail below. Little is known regarding the molecular mechanism of the remodeling but several groups have suggested that, as occurs in nonmuscle cells, the process involves not only actin polymerization but also turnover of adhesion plaque proteins and activation of nonreceptor tyrosine kinases such as those in the Src family [25].

## Mechanisms that regulate LC20 phosphorylation

Smooth muscle myosin, unlike striated muscle myosin, requires phosphorylation at Ser 19 in order to show significant levels of actin-activated myosin ATPase activity.  $\text{Ca}^{2+}$ /CaM-dependent activation of MLCK is the primary and best known pathway by which changes in the phosphorylation level of smooth muscle myosin occur. However, it is worth mentioning that smooth muscle myosin, at least in vitro, is also known to be capable of being phosphorylated in a calcium-independent manner by additional kinases like Rho kinase [26], integrin-linked kinase (ILK) [27], and zipper-interacting protein kinase (ZIPK) [28,29] however, the relative in vivo significance of these pathways is not yet entirely determined [18,30,31]. MLCK itself has been recently reviewed elsewhere [32] and will not be focused on here. Recent studies have uncovered evidence for a multitude of additional complex pathways by which smooth muscle myosin phosphorylation levels can be regulated in vivo, including both regulation of dephosphorylation and the activation of a second  $\text{Ca}^{2+}$ /CaM-dependent kinase, CaMKII described below.

## Regulation of Myosin Phosphatase

LC20 dephosphorylation is catalyzed by a single myosin light chain phosphatase (MP). The smooth muscle MP complex consists of the catalytic subunit, PP1c delta, a regulatory subunit, myosin phosphatase target subunit 1 (MYPT1), also referred to as myosin binding subunit

(MBS), and a small, approximately 20kDa subunit of unknown function. Alternative splicing of two exons gives rise to four major MYPT1 isoforms, that differ in the presence of a central insert and/or a leucine zipper motif at the C-terminal end of the protein (LZ+ and LZ- isoforms) [33–36].

Initially, MP was assumed to be constitutively active and not subject to regulation. However, in recent years, an array of regulatory pathways leading to MP inhibition and activation has been described and is summarized diagrammatically in Fig. 2. The various MP regulators can be divided roughly into two groups: (1) protein scaffolds and (2) substrates of upstream kinases (or, phosphoproteins), with some regulators having a dual function.

The most prominent MP regulator is the regulatory subunit, MYPT1, which behaves as both a scaffolding protein and a substrate of upstream kinases. In its function as a scaffold, MYPT1 can be classified as an activator of MP, since it targets the MP complex to its substrate, LC20 [37]. Moreover, binding of MYPT1 to PP1c delta also enhances its catalytic activity towards LC20 [38,39]. On the other hand, phosphorylation of MYPT1 on one or both of the two major inhibitory phosphorylation sites (corresponding to threonine-696 and threonine-853 in mammalian MYPT1), leads to inactivation of MP [40,41]. MYPT1 also contains an activating phosphorylation site – serine-695 – that inhibits subsequent inhibitory phosphorylation of threonine-696 [42].

Another phosphoprotein-type inhibitor of MP is CPI-17 (PKC-potentiated PP1 inhibitory protein of 17 kDa) [43]. This small protein acts as an MP pseudosubstrate when phosphorylated, and binds to the catalytic site of MP, thereby competing with LC20 for phosphorylation. A bimodal MP regulator of the scaffold-type is the myosin phosphatase-Rho interacting protein (M-RIP), which targets MYPT1, Rho and Rho kinase to actomyosin filaments. As far as the targeting of MYPT1 to the actomyosin filaments is concerned, this protein can be an activator of MP; however, since it also targets Rho kinase to the MP complex, it can also have an inhibitory effect on MP activity. These different possibilities are reflected by controversial reports about MRIP function [44–48].

The immediate MP regulators are, themselves, downstream elements of extracellular cascades. Inhibitory phosphorylation of MYPT1 is primarily mediated by the Rho pathway, via Rho kinase directly and/or via Rho kinase-mediated activation of ZIPK [41,49–51]. Whether kinases other than Rho kinase can activate ZIPK in smooth muscle is not known. The inhibitory effect of activated protein kinase C (PKC) on MP is primarily mediated by CPI-17 [52]. Apart from PKC, CPI-17 can also be phosphorylated by ZIPK and ILK [53,54]. As CPI-17 is more rapidly phosphorylated and dephosphorylated than MYPT1, these apparently redundant pathways might be necessary for fine-tuning of contraction; furthermore, each pathway provides contact points for offset signaling [55,56].

Counterbalancing the Rho and PKC pathways, which support contractility, the nitric oxide (NO) pathway leads to relaxation. NO elevates intracellular cGMP, which activates the type Ia cGMP-dependent protein kinase (PKG). PKG phosphorylates RhoA at Serine-188, an inhibitory phosphorylation site, thus disrupting RhoA signaling [57,58]. Furthermore, PKG phosphorylates MYPT1 at the activating phosphorylation site, serine-695, thus blocking subsequent inhibitory phosphorylation [42,59]. This effect on MYPT1 depends on the expression of the LZ+ isoform of MYPT1, however, it is controversial whether the interaction between MYPT1 and PKG is mediated by a leucine zipper-leucine zipper interaction [60–62]. Other downstream effectors of NO include protein phosphatase 2a (PP2a) [63], one of the phosphatases that dephosphorylate and thus inactivate CPI-17 [64,65].

These regulatory pathways form a network that tightly regulates MP and therefore, has a significant impact on LC20 phosphorylation and contractility. Disturbance of the balance

between LC20 phosphorylation and dephosphorylation is expected to, and is known to lead to pathologies. For example, vascular hyperconstriction of pulmonary arteries, caused by an overstimulated Rho pathway, leads to pulmonary artery hypertension (PAH) [3]. MYPT1 phosphorylation in particular has been shown to be sensitive to hypoxia, thus probably playing an essential role in hypoxic relaxation [66]. In hypertensive rat models, inhibition of the Rho pathway corrected the hypertension [67]. In the setting of neonatal circulatory transition and in persistent pulmonary hypertension of the newborn, the Rho pathway appeared to be less important, but instead a role for CPI-17 was shown [68].

## CaMKII

Ca<sup>2+</sup>/Calmodulin dependent kinase II (CaMKII) is a Serine/Threonine kinase that is ubiquitously expressed. It is a family of four closely related isoforms; alpha, beta, gamma and delta, the products of four separate genes. The alpha and beta isoforms are primarily restricted to neural tissues, but the gamma and delta isoforms are widely distributed. We have identified six variants of CaMKII gamma from an aorta cDNA library raising the possibility that each could be differentially targeted, have different substrate specificity and, thus, have different functions [69].

CaMKII consists of an N-terminal catalytic/regulatory domain and a C-terminal association domain. A linker domain connects these two conserved domains, which is variable in sequence (Fig. 3). The association domains of all isoforms of CaMKII interact to form large wheel-shaped holoenzymes [70]. Crystallographic data indicate that the holoenzyme is dodecameric [71].

As the name implies CaMKII is activated by Ca<sup>2+</sup> and calmodulin (CaM). In the inactive state, an autoinhibitory domain blocks the active site of the molecule, but the binding of Ca<sup>2+</sup>/CaM disrupts the autoinhibitory domain and the kinase becomes active. In vitro studies have shown that CaMKII can autophosphorylate at several sites [72,73]. However, autophosphorylation of Thr286 (numbering according to alpha isoform, Thr287 for beta, gamma and delta) is the best explored site and two important consequences have been proposed for autophosphorylation at this site [74]. First, autophosphorylation of Thr286 disables the autoinhibitory domain; as a result CaM kinase acquires “autonomous activity”, activity that is retained even after removal of Ca<sup>2+</sup>. Secondly, the affinity of CaMKII for CaM increases about 1000-fold, also called ‘CaM trapping’. The ability of the kinase to retain activity after being activated Ca<sup>2+</sup> at a previous point in time has been referred to as a “molecular memory” [75] that has been linked, for the alpha and beta isoforms to the processes of synaptic plasticity, learning, and memory per se, supported, most convincingly by the CaMKII alpha knockout mouse model [76]. In vascular smooth muscle a modified form of memory has been linked to a prolongation of vascular tone [77].

A distinct phase of autophosphorylation occurs at Thr305, Thr306 and Ser314 when Ca<sup>2+</sup>/CaM dissociates from the Thr286 phosphorylated protein [78]. Autophosphorylation of Thr305 and Thr306 are inhibitory as they prevent further binding of CaM. However, autophosphorylation at Ser314 has no effect on binding of CaM [73,79]. In vascular smooth muscle it appears that Thr305/306 phosphorylation is a significant regulator of vascular tone in that this site, which inhibits CaMKII activity, is, itself, inhibited by the action of alpha agonists [77] leading to a delayed reactivation of kinase activity, and increased vascular tone.

Both pharmacological inhibitors and antisense knockdown of CaMKII in vascular tissue and cells have clearly shown that CaMKII activation is a significant regulator of vascular tone [20,80,81]. In vitro, CaMKII has been shown to phosphorylate many key proteins involved in smooth muscle regulation, including MLCK [82,83], LC20 [84], caldesmon and calponin [85–87], phospholipase alpha2 [88] and the alpha subunit of Ca<sup>2+</sup> channel [89]. The in vivo

significance of most of these proteins is unclear; however, *in vivo* studies have indicated that CaMKII increases the activity of the voltage dependent  $K^+$  channel [90], the voltage dependent  $Ca^{2+}$  channel [91] and decreases the activity of the  $Ca^{2+}$  activated  $Cl^-$  channel [92]. The transcription factor, cAMP-responsive element binding protein (CREB) also appears to be an *in vivo* substrate of CaMKII and the elevation of *c-fos* by CREB phosphorylation regulates gene expression in smooth muscle [93]. Phospholamban, a negative regulator of sarcoplasmic reticulum  $Ca^{2+}$ -ATPase is also a substrate of CaMKII that seems to have *in vivo* relevance; however, because of the apparent low abundance of phospholamban in smooth muscle, the magnitude of the effect may be less than that in cardiac muscle [94].

CaMKII-mediated regulation of MLCK appears also to have *in vivo* relevance but is not due to direct phosphorylation of MLCK but rather to the recruitment of a more complex pathway involving activation of ERK and the presumed ERK-mediated phosphorylation of and activation of MLCK at constant  $Ca^{2+}$  levels [20,81,95]. (Pathway 1, Fig. 1)

It is of interest that the knockdown of a specific variant of CaMKII gamma, CaMKII gamma G-2, inhibits depolarization-mediated contractions and the ERK-MLCK pathway described above. Whether this one variant is the only variant of the 6 known to be present in vascular tissue that regulates contractility, or whether other variants share this mechanism, is not yet known. Obviously, if G-2 is the sole variant effective in regulating vascular tone, it would be a potentially specific candidate molecule for drug discovery research. This variant is unusual in having a unique sequence of 99 amino acids that target the variant, when activated, to adhesion plaques. The prevention of CaMKII gamma G-2 targeting to adhesion plaques leads to significant inhibition of ERK activation as well as contractility [81].

A specific G-2 phosphatase, a small C-terminal domain (CTD) phosphatase-3 (SCP3) homologue, a PP2C-type phosphatase has also been reported [96]. This phosphatase is primarily expressed in vascular smooth muscle tissues and specifically binds to the association domain of the CaMKII gamma G-2. SCP3 dephosphorylation of CaMKII gamma G-2 is site specific, excluding the Thr287 site associated with  $Ca^{2+}$ /CaM-independent activation of the kinase. Thus, the selective dephosphorylation by SCP3 creates a constitutively active kinase which is regulated by phosphorylation-dependent targeting mechanisms [96].

Recent work on the delta isoform of CaMKII has shown a role for CaMKII delta in PDGF-stimulated vascular smooth muscle migration [97] and wound healing [98]. Vascular injury induced by balloon angioplasty has been shown to increase CaMKII delta isoform expression in smooth muscle cells and in fibroblasts [99]. Trafficking of iNOS was also shown to be dependent on the activation of CaMKII delta isoform [100]. Thus, the gamma and delta isoforms of CaMKII may have distinct, but equally important roles in vascular function.

## Mechanisms that regulate the access of myosin to actin

### Caldesmon

Caldesmon (CaD) is a highly conserved, actin [101] and myosin [102] binding protein that exists in two isoforms which are generated by alternative splicing. Whereas the heavy isoform (h-CaD) is restricted to smooth muscle cells, the light isoform (l-CaD) is expressed in non-muscle and dedifferentiated smooth muscle cells [103]. This thin-filament associated protein is capable of stabilizing actin filaments [104], blocking Arp2/3 mediated actin polymerization [105], inhibiting actomyosin ATPase activity [106] as well as actin-myosin interaction [107] and thereby regulates contractility of smooth muscle cells. The inhibitory effect of h-CaD can be reversed by either binding of (CaM)/ $Ca^{2+}$  to the C-terminal domain [108] or by phosphorylation of Serine residues 759 and 789 by either ERK1/2 [109] or cdc2 [110]. These events lead to a conformational change in the CaD structure which allows interaction between



actin and myosin [111]. While cdc2 phosphorylation plays an important role in cytokinesis [112], the phosphorylation of CaD by ERK1/2 appears to regulate smooth muscle contractility [113]. In this latter process ERK1/2 is activated by PKC (Fig. 1) which in turn can be stimulated by phorbol esters or GPCR receptor agonists. Interestingly, CaD is found in podosomes [114], structures that are known to represent sites of PKC activation and signaling and that are involved in actin cytoskeleton remodeling processes [115]. Thus CaD is an important mediator of smooth muscle contractility, regulated, among other mechanisms, by a PKC/ERK signaling pathway.

### **bCaP function in regulation of PKC alpha/epsilon and ERK1/2 signaling**

Calponin (CaP) is an actin-binding protein that was first isolated from gizzard smooth muscle cells and that can regulate myosin ATPase activity [116]. To date there are known three isoforms of this putative actin regulatory protein, called h1 CaP (smooth muscle-specific basic CaP, bCaP) [117], h2 CaP (neutral CaP) [118] and acidic CaP [119] which are encoded by three different genes. The most abundant isoform in dSMC is bCaP [120,121], although the other isoforms are also expressed in this cell type in a lower extent [119,122]. Moreover bCaP can be used as a differentiation marker of smooth muscle due to its downregulation in proliferating cells [87]. CaP proteins consist of a conserved so called CaP homology (CH) domain in their N-Terminus, a Troponin I (TnI) -like actin-binding domain and three C-terminal repeats [123,124]. The very C-terminus is the variable region in the three CaP-isoforms, whereas the N-terminal fragment is highly conserved (see Fig. 4). The CH-domain has been shown to bind acidic phospholipids [125] and ERK1/2 [126], although this structural motif is often implicated in actin binding in many other cytoskeleton proteins [127]. PKC alpha and epsilon can interact with bCaP through the C-terminal repeats [128]. Actin binding of CaP occurs through the TnI-like domain and in a weaker fashion through the C-terminal repeats [124] and is regulatable by phosphorylation of PKC at Serine 175 and Threonine 184 residues located within the C-terminal repeats [129,130].

The functional role of bCaP in regulation of smooth muscle contractility is controversial. One theory is that bCaP may directly regulate contractility by inhibiting actomyosin ATPase activity of myosin heads cross-linked to actin [116] and indeed experiments have shown that bCaP is able to negatively influence in vitro motility of actin filaments [131]. On the other hand, bCaP has been suggested to facilitate agonist-induced signal transduction perhaps by acting as a scaffold protein. This latter hypothesis is based on the findings in vascular smooth muscle cells that bCaP acts as an adaptor protein to directly interact with the signaling proteins PKC [128] and ERK1/2 [126], cotranslocates to the cell cortex upon stimulation together with ERK1/2 and PKC [132] and, in addition, seems to promote PKC activation [128]. Moreover it was shown that a knockdown of bCaP in differentiated vascular smooth muscle cells results in impaired ERK1/2 activity, h-CaD phosphorylation and contractility [133].

Extracellular regulated kinase 1/2 (ERK 1/2) is a member of the Mitogen-activated protein kinase (MAPK) family, kinases that possess serine/threonine activity. Stimulation of most cell surface receptors by mitogens or GPCR agonists causes activation of MAPK signaling pathways where MAPKs such as ERK1/2 become phosphorylated at Thr and Tyr residues. Phosphorylated ERK1/2 proteins are able to form dimers, enter the nucleus, phosphorylate transcription factors and thereby promote cell proliferation of undifferentiated cells [134–136]. On the other hand, active MAPKs have also been found in differentiated, non-proliferating contractile smooth muscle cells [137,138], but their function in this cell types is still not fully understood. Furthermore it is interesting that an intact actin cytoskeleton seems to be necessary for ERK signaling [139] and that ERK1/2 itself shows actin-binding properties [126], suggesting that the actin cytoskeleton is involved in ERK1/2 signaling. More evidence for ERK1/2 playing a significant role in regulation of smooth muscle contractility comes from

studies in pregnant rats showing that ERK1/2 activity is connected to onset of labor [140]. In uterine smooth muscle, the kinase is activated in late pregnancy, possibly due to an increased cortical tension, after translocation to the cell surface. This event is followed by an increased h-CaD phosphorylation which contributes to the initiation of contractions [4,25].

Another interesting interaction partner of bCaP that seems to be involved in the ERK1/2 signaling pathway is the smooth muscle Archvillin (SmAV) protein. Recent findings show that SmAV binds to bCaP, ERK1/2 and Raf [141,142], translocates to the cell periphery in smooth muscle cells after agonist stimulation and colocalizes with ERK1/2 and bCaP. Furthermore, an antisense-mediated knockdown of SmAV decreases ERK1/2 activation and contractility of smooth muscle, similar to a bCaP knockdown [141].

The model shown in Fig. 5 brings these findings together. In this model bCaP acts as a scaffold, connecting ERK1/2 and PKC pathways to regulation of smooth muscle cell contractility [24] (Fig. 5): (1) In the first step a, yet unclear, stimulus leads to subsequent activation of PKC alpha/epsilon, which is facilitated by bCaP. bCaP has been shown in vitro to be able to stimulate PKC alpha/epsilon activation in the absence of lipids [128]. It can be speculated that either cell permeant phorbol esters bind to PKC alpha/epsilon located at the actin filaments to activate the molecule or, in the case of GPCR activation, that another PKC isoform such as PKC delta activates PKC alpha/epsilon by phosphorylation. Activated PKC alpha/epsilon would then be able to phosphorylate bCaP and attenuate its binding affinity to actin, so that (2) the PKC alpha/epsilon – ERK1/2 – bCaP complex translocates to the cell cortex, where it comes in contact with SmAV, acting as a scaffold for Raf and MEK. PKC alpha/epsilon at the surface membrane could then undergo a full activation through binding of diacylglycerol (DAG), produced by active phospholipase C (PLC) at the cell membrane. Active PKC alpha/epsilon can phosphorylate Raf [143], which in turn activates MEK, followed by activation of the ERK1/2 molecule. (3) Whereas PKC alpha/epsilon, bCaP and SmAV stay at the cell cortex, (4) phosphorylated ERK1/2 moves back to the actin cytoskeleton where it comes into proximity with the actin-bound h-CaD molecule and phosphorylates its substrate [144]. Phosphorylation of h-CaD results in a conformational change of the molecule, leading to partial dissociation of h-CaD from actin and, hence, to actomyosin interaction and contraction [113].

Less is known about bCaP and its involvement in vascular diseases, but hints come from mice with a mutated bCaP locus which express a C-terminal truncated form of the protein lacking the TnI-like domain as well as the three C-terminal repeats and the variable C-terminus. Surprisingly the most conspicuous phenotype of these mice was an increased bone formation, leading to the hypothesis that bCaP is involved in regulation of osteogenesis [145]. Further examination has revealed that these mice display a faster shortening velocity [146], a lower heart beat rate, an impaired  $\alpha$ -adrenergic vasoconstriction, an enhanced arterial baroreflex sensitivity [147], a lower active isometric force [148] as well as impaired arterial blood pressure regulation during exercise due to an enhanced muscular vasodilation [149]. Particularly the enhanced vasodilation in mice expressing the truncated bCaP form is consistent with our hypothesis that bCaP is required to activate ERK1/2 which in turn inactivates h-CaD, thereby facilitating contraction. However, it should be considered that the truncated bCaP protein expressed in the mouse model lacks only the actin-binding domains and the CH-domain is still expressed. So it should be examined if this truncated bCaP form is still able to interact with ERK1/2 and could therefore substitute for the wild type bCaP in some of its functions.

In other studies, it has been shown that blood vessels of mice with a mutated bCaP locus are more fragile [150] and therefore less resistant to metastasis of tumor cells [151,152]. Indeed downregulated bCaP expression is a poor diagnostic marker in many cancers [153–158], leading to the hypothesis that misregulated bCaP levels are connected to cancerous diseases. It has been speculated that downregulated bCaP levels in smooth muscle cells of blood vessel

tumors leads to a destabilization of actin filaments, thereby weakening adhesion of cells to neighbouring cells as well as to the extracellular matrix. This in turn could be a reason for the fragility and penetrability of metastatic tumor cells [150]. On the other hand it was also shown that restoration of bCaP expression in transformed cells can lead to a reduced proliferation rate, tumorigenicity and metastatic cell motility. It has also been speculated that a loss of bCaP leads to an unstable actin filament system, thereby facilitating cytoskeleton reorganisation which is necessary for cells to assume an invasive phenotype [159,160]. Finally, given the evidence (mentioned above) that CaP interacts with MAPKs and the known association of MAPKs with regulation of proliferation, this pathway could also contribute to this property of CaP. Thus, the exact mechanisms by which bCaP can act as a tumor suppressor remains to be elucidated.

## Mechanisms that regulate cytoskeletal remodeling

The actin cytoskeleton is defined as the collection of actin filaments and actin filament associated proteins, including adhesion plaque proteins. It has previously been assumed that the actin cytoskeleton in dSMC is largely static, performing a solely structural role, in comparison to the dynamic cytoskeleton of migrating, proliferating vascular smooth muscle cells [161]. Although this topic is still controversial, a body of evidence is growing to support the idea that the actin cytoskeleton is remodeled during contractile agonist activation and that this remodeling might modulate vascular contractility [162–164].

Actin can exist as either filamentous actin (F-actin) or globular actin (G-actin) in cells. G-actin spontaneously polymerizes to form F-actin above its critical concentration (~8 microgram/ml) [165]. The G-actin concentration in the cytoplasm of dVSM cells is above the critical concentration. However, the polymerization of actin is tightly controlled by a large number of actin binding proteins such as profilin, ADF/cofilin, capping proteins, and sequestering proteins etc. Signaling pathways that regulate these processes therefore regulate the actual ratio between G- and F-actin resulting in assembly or disassembly of the actin filament (Fig. 6) [165,166].

Actin exists primarily as F-actin (~ 80% of total actin) in unstimulated contractile intact vascular smooth muscle. The percentage of F-actin can go above 90% in alpha adrenergic receptor stimulated smooth muscle, indicating dynamic remodeling of the actin cytoskeleton [164]. This result is consistent with the findings reported by Gunst and colleagues for airway smooth muscle [167,168].

Actin is the most abundant protein in smooth muscle and it is highly conserved throughout evolution and across species [169]. Six actin isoforms, products of separate genes, are present in vertebrate tissues [170]. Among these isoforms, two smooth muscle type isoforms (alpha and gamma) and two cytoplasmic isoforms (beta and gamma) coexist in differentiated vascular smooth muscle [164,171]. The cytoplasmic beta and gamma actin isoforms are also sometimes referred to as nonmuscle isoforms in smooth muscle tissues [169]. Evidence exists, largely in cultured non-muscle cells that the different actin isoforms perform different cellular functions [169]. The amino acid sequences of these actin isoforms are remarkably conserved and most sequence differences are clustered at the NH<sub>2</sub>-terminal ends [172]. The NH<sub>2</sub>-terminal region of actin is known not to be involved in actin-actin monomer binding and hence does not directly regulate actin filament polymerization, however this region is known to be the binding site of many actin binding proteins including myosin [173] and many actin polymerization regulatory proteins. Recently, we showed that the NH<sub>2</sub>-terminal of actin isoforms also modulates the contractile function of vascular smooth muscle by using the NH<sub>2</sub>-terminal decoy peptides of actin isoforms [164]. This result supports the concept that different actin isoforms perform different functions, even though the detailed mechanisms involved are not clear yet.



The actin filaments that connect to either dense bodies or dense plaques (also known as focal adhesion complexes) in smooth muscle are likely to include both the contractile filaments, i.e. actomyosin-containing myofilaments, as well as actin filaments that are not associated with myosin, i.e. the nonmuscle actin cytoskeleton. However, the arrangement of the contractile filaments with respect to the overall cytoskeleton and the mechanism by which the nonmuscle cytoskeleton is coupled to the contractile apparatus are still a matter of debate [174]. A differential subcellular distribution of alpha-smooth muscle and beta-cytoplasmic actin in vascular smooth muscle [175] and a differential distribution for beta-cytoplasmic actin from actomyosin containing contractile bundles in chicken gizzard smooth muscle [174] have been reported. A separation of cytoskeletal actin from actomyosin-containing contractile actin has been reported by using immunoprecipitation [176]. In contrast, others have argued against any actin isoform specific domains in vascular smooth muscle [177–179]. It is quite likely that the connection between the two actin cytoskeletons is dynamic perhaps regulated by ‘a hierarchical slippage clutch’ that is, itself, regulated by signaling pathways [180].

Focal adhesion complexes are large structures, often a micron in size, containing, in different cell types, over a hundred proteins including integrin, actin, actin binding proteins, protein kinases, and signaling proteins (Fig. 6) [181]. At focal adhesions, the force generated by the contractile proteins is conveyed to the extracellular matrix (ECM) via integrin receptors. The integrin family consists of eighteen alpha and eight beta subunits which can form 24 unique integrin heterodimers [182]. The short intracellular domains of integrins are indirectly connected to the actin cytoskeleton by several proteins such as talin, vinculin, paxillin and by these connections, the extracellular events can be communicated into the cell and vice versa [183]. Further details on this topic can be found in other recent review articles [181,183–185].

There are several reports indicating that the connection between the actin cytoskeleton and components of focal adhesion complexes are dynamic and are remodeled during agonist-induced activation in differentiated airway smooth muscle. Integrin mechanotransduction and consequent signal transduction-dependent alterations in the adhesion plaque proteins, paxillin, talin, vinculin and FAK has been reported for both vascular and airway smooth muscle [163,186–195].

Actin cytoskeletal remodeling also has been reported to occur in vascular smooth muscle [162,164,189,190,196]. However, what has not been clear is exactly how, or if, cytoskeletal remodeling modulates contractility of smooth muscle. Different possible mechanisms have been suggested by different groups. Some groups have suggested that cytoskeletal remodeling may alter the transmission of cross-bridge generated force to the cell membrane [192]. Dynamic cytoskeletal processes within the cell may enhance the strength of the connections between membrane adhesion junctions and actin filament within the contractile apparatus and cytoskeletal network, thus providing a strong and rigid framework for the transmission of force generated by the interaction of myosin and actin filaments to the outside of the cell [167]. However, Seow and his colleagues have published that the length of total contractile filament complex is changed by addition or removal of myofilament units, suggesting the possibility of dynamic remodeling in actomyosin containing myofilament [197–202]. Other groups have suggested that cytoskeletal remodeling modulates force maintenance or optimizes the energetic cost of tension in the vascular smooth muscle cell [203].

Recently, we found that the actin cytoskeleton in vascular smooth muscle is differentially remodeled in a stimulus- and pathway-dependent manner. Interestingly, gamma-actin, the least abundant actin isoform in vascular smooth muscle, is the most dynamically remodeled by alpha adrenergic receptor stimulation [164]. This result suggests that the nonmuscle actin cytoskeleton is more dynamic than the actin cytoskeleton in contractile apparatus. The

reorganization of the cytoskeleton was shown to correlate with mechanisms for regulation of smooth muscle contraction.

To understand the functional specificity of individual actin isoforms and to identify the isoforms related diseases, several investigations utilized manipulation of the isoform specific genes [204–206]. Ablation of gamma-cytoplasmic actin in skeletal muscle causes progressive muscle necrosis and regeneration in mouse [207–209]. Vascular smooth muscle contractility and blood pressure homeostasis are impaired in the alpha smooth muscle actin null mouse [206].

It is conceivable that abnormal contractility in vascular smooth muscle may result from the perturbation of normal cytoskeletal remodeling processes. Recently, Guo et al. [210] published that 14% of inherited familial ascending thoracic aorta aneurysms leading to acute aortic dissections (TAAD) are caused by missense mutations in *ACTA2* (encodes alpha smooth muscle actin). By using structural and immunofluorescence analysis, this study show that interference with actin filament polymerization caused by this mutation leads to impaired smooth muscle contraction [210]. There is much experimental evidence supporting the concept that remodeling of the actin cytoskeleton is a crucial regulator of smooth muscle function. However, much remains to be determined regarding the mechanistic basis for the actin cytoskeletal remodeling in smooth muscle.

## Conclusions

Undoubtedly, the information above has communicated the concept that smooth muscle “excitation-contraction coupling” consists of far more than a simple calcium switch. Although the complexity of the multitude of, apparently, redundant signaling pathways which regulate smooth muscle contractility can be overwhelming, clearly the importance of proper functioning of smooth muscle systems requires the fine tuning made possible by this sort of complex system.

The importance of pathways that regulate plasticity of the cytoskeleton and those that regulate the availability of actin to interact with myosin is only now becoming clear. Similarly, the relative importance of the mix of pathways that regulate myosin phosphorylation levels in dSM tissues is just now playing out. The good news is that this complex network of pathways offers a multitude of possible leads for novel drug targets, with the hundreds of proteins present in adhesion plaques representing, potentially, quite accessible targets. Added to this the well known tissue- and organ-specific nature of smooth muscle signaling molecules and pathways, it is feasible that such targets could lead to quite specific, as well as effective, new therapeutics.

## Abbreviations

Ca<sup>2+</sup><sub>i</sub>, intracellular ionized calcium  
 CaMKII, Ca<sup>2+</sup>, Calmodulin dependent kinase II  
 CaD, caldesmon  
 CaM, calmodulin  
 CaP, calponin  
 CPI-17, potentiated PP1 inhibitory protein of 17 kDa  
 CREB, c-AMP-responsive element binding protein  
 dSMC, differentiated smooth muscle cell  
 ECM, Extracellular matrix  
 ILK, integrin-linked kinase  
 LC20, 20kDa myosin light chain  
 MBS, myosin binding subunit

MLCK, myosin light chain kinase  
 MP, myosin light chain phosphatase  
 M-RIP, myosin phosphatase-Rho interacting protein  
 MYPT, myosin phosphatase target subunit 1  
 PKC, protein kinase C  
 PKG, cGMP-dependent protein kinase  
 PP2a, protein phosphatase 2a  
 ZIPK, zipper-interacting protein kinase

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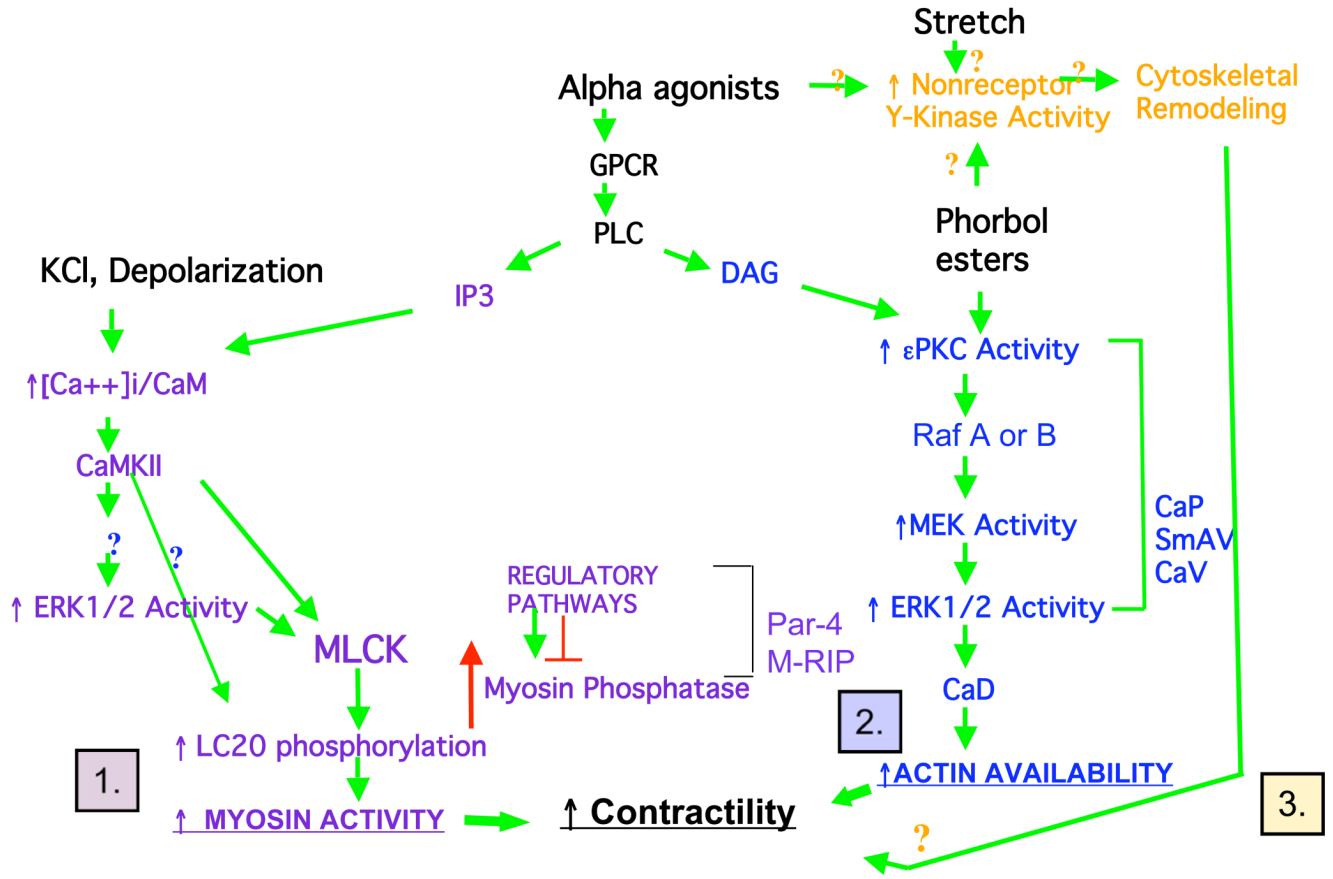
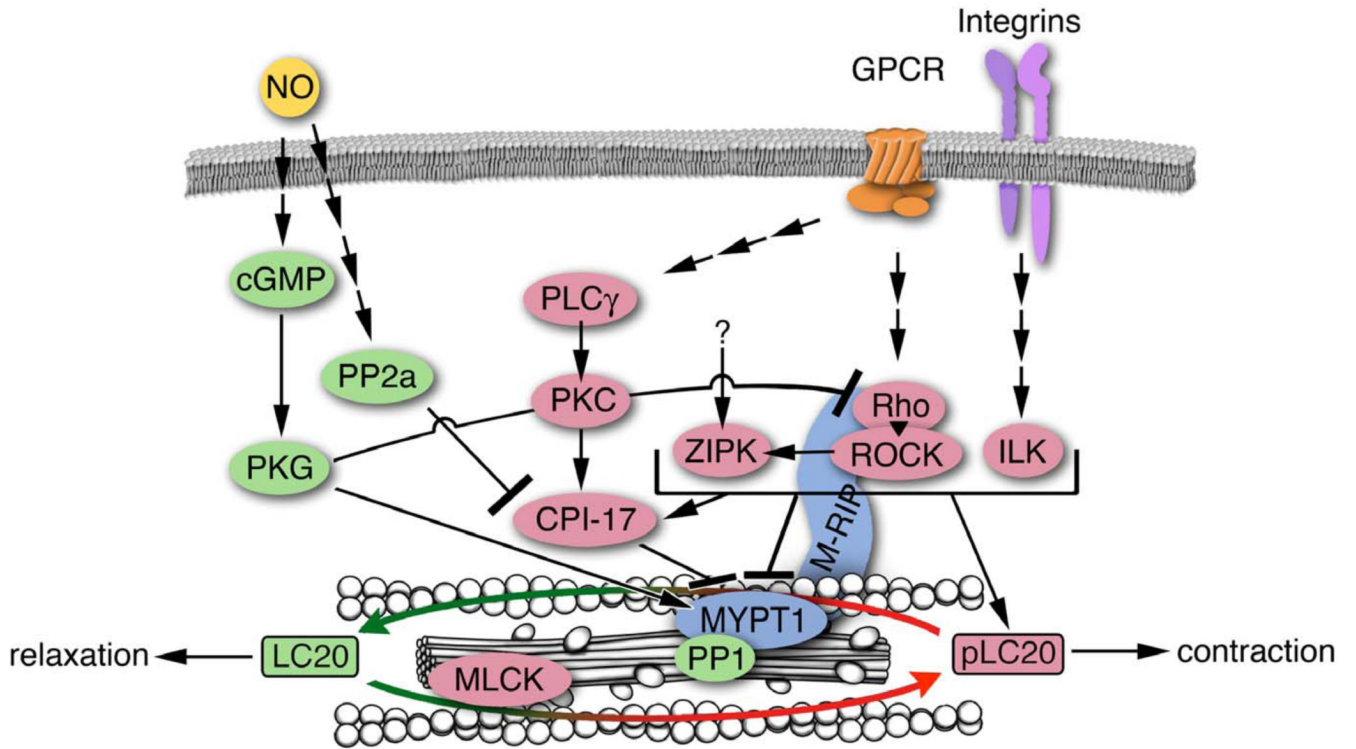
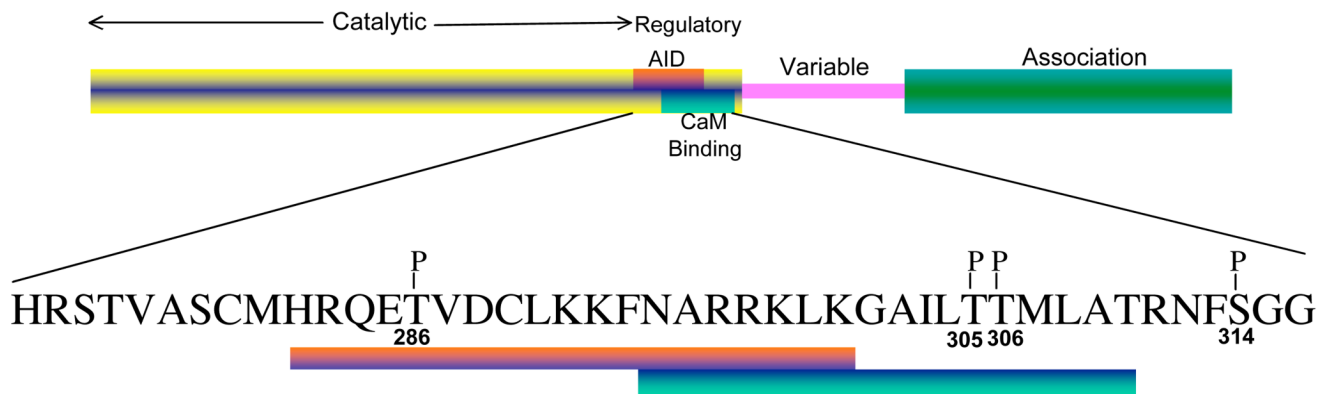


Figure 1. Pathways that regulate contractility (demonstrated and putative)



**Figure 2. Regulatory pathways leading to myosin light chain phosphatase inhibition and activation**  
 Activating signaling molecules (leading to relaxation) are shown in green, inhibitory molecules (supporting contractility) are shown in pink. Molecules shown in blue are bimodal and can be both, an inhibitor or an activator.





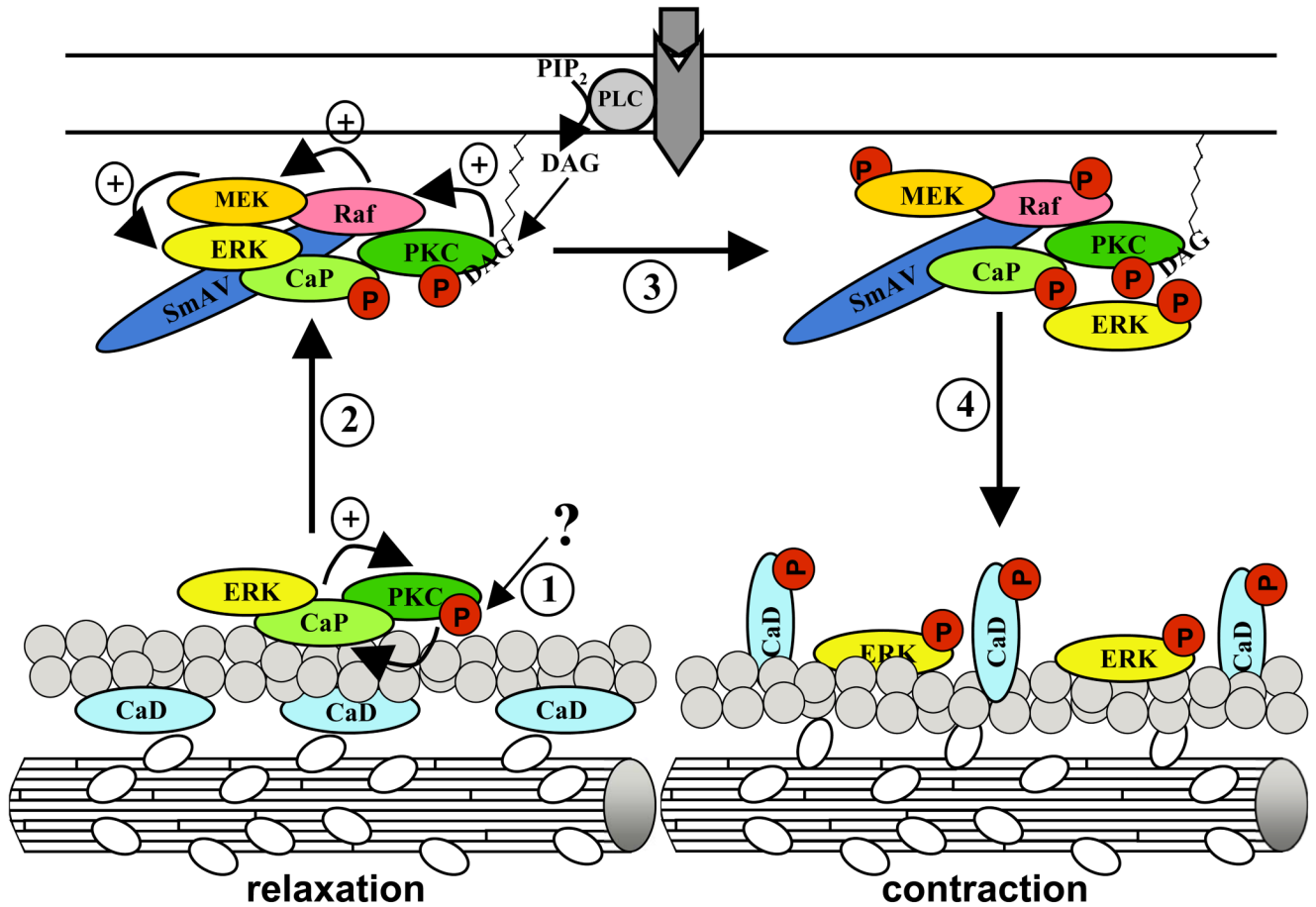
**Figure 3. Domain structure of CaMKII**

The phospho-sites are indicated with 'P' on top of the amino acid residues. The numbering is according to the alpha isoform.



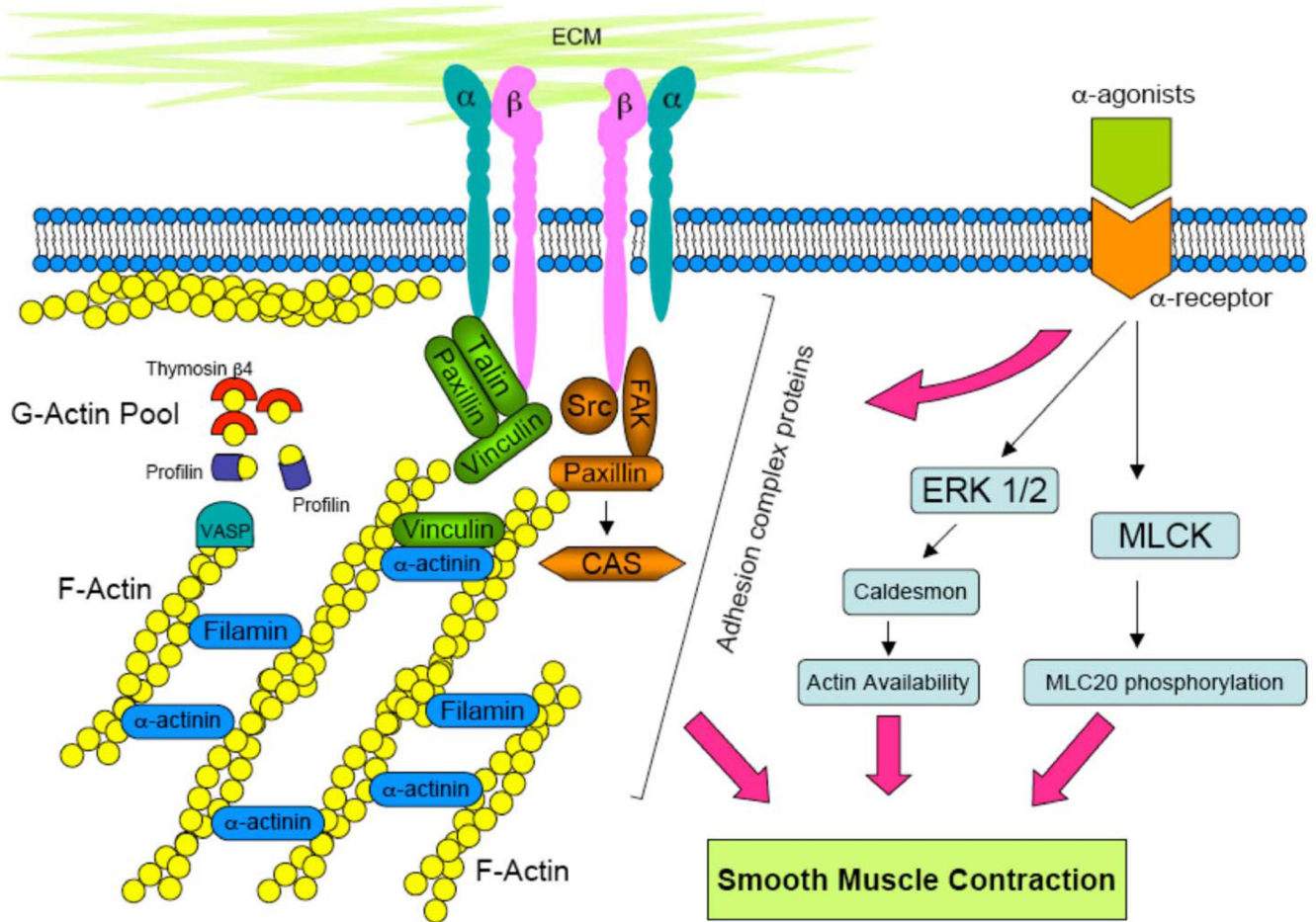
**Figure 4. Domain structure of Calponin**

Calponin proteins consist of a conserved N-terminus including the CH-domain (blue), the TnI-like domain (yellow) as well as the three C-terminal repeats (green). However, the very C-terminus is a highly variable region (red) that differs in size and amino acid sequence within the three Calponin isoforms.



**Figure 5. Model of bCaP function in regulation of smooth muscle cell contractility**

(1) Starting with a yet unidentified stimulus, PKC alpha/epsilon gets subsequently activated, an event that is further supported by bCaP binding. PKC alpha/epsilon may now phosphorylate bCaP, leading to an impaired actin binding property of bCaP. (2) Hence the ERK1/2 - PKC alpha/epsilon - bCaP complex translocates to the cell cortex where it binds to SmAV, a protein acting as a scaffold for Raf and MEK. Moreover the PKC alpha/epsilon molecule gets fully activated by membrane bound DAG that is produced by activated PLC coupled to GPCR. The activated PKC alpha/epsilon molecule phosphorylates Raf, which in turn phosphorylates MEK that now activates ERK1/2. Whereas the SmAV - bCaP - PKC alpha/epsilon complex stays at the membrane, (4) activated ERK1/2 moves back to the actin filaments where it comes in contact with its substrate h-CaD. Phosphorylation of the h-CaD molecule leads to its conformational change, resulting in enabled actin - myosin interaction and hence to contraction. For detailed information see text/article.



**Figure 6. Cytoskeletal remodeling at focal adhesions and regulation of smooth muscle contraction**  
 Integrins connect the extracellular matrix to actin filaments within the cell. Actin filaments are linked to cytoplasmic domain of integrin by linker proteins (green). Mechanical and/or contractile stimuli induce the cytoskeletal remodeling by recruiting signaling proteins (orange) to focal adhesions.