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REVIEW

Signal Transduction and Protein Phosphorylation in Smooth Muscle Contraction

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Abstract—Smooth muscles are important constituents of vertebrate organisms that provide for contractile activity of internal organs and blood vessels. Basic molecular mechanism of both smooth and striated muscle contractility is the force-producing ATP-dependent interaction of the major contractile proteins, actin and myosin II molecular motor, activated upon elevation of the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). However, whereas striated muscles display a proportionality of generated force to the $[Ca^{2+}]_i$ level, smooth muscles feature molecular mechanisms that modulate sensitivity of contractile machinery to $[Ca^{2+}]_i$. Phosphorylation of proteins that regulate functional activity of actomyosin plays an essential role in these modulatory mechanisms. This provides an ability for smooth muscle to contract and maintain tension within a broad range of $[Ca^{2+}]_i$ and with a low energy cost, unavailable to a striated muscle. Detailed exploration of these mechanisms is required to understand the molecular organization and functioning of vertebrate contractile systems and for development of novel advances for treating cardiovascular and many other disorders. This review summarizes the currently known and hypothetical mechanisms involved in regulation of smooth muscle Ca^{2+}-sensitivity with a special reference to phosphorylation of regulatory proteins of the contractile machinery as a means to modulate their activity.

Key words: smooth muscle, Ca^{2+} sensitivity, contraction, phosphorylation, signal transduction, actomyosin regulatory proteins

Smooth muscles are indispensable components of the circulatory system and visceral organs that support normal physiological activity of the whole body. Smooth muscle dysfunction is linked and, in some cases, is causal of such pathologies as hypertension, myocardium infarction, stroke, asthma, gastrointestinal and reproductive disorders, etc. Current schemes of pharmacological treatment and prevention of these conditions often employ therapeutics that target the circulatory or visceral smooth muscles. Thus, application of modern advances in the discovery of novel pharmacological compounds requires a detailed knowledge of molecular mechanisms of smooth muscle regulation, which still are not completely understood. The goal of this review is to survey and characterize the molecular components of these pathways.

The basis of muscle contraction in all known vertebrate muscles is an energy consuming sliding of actin filaments along myosin filaments. The molecular mechanism of force generation by cycling actomyosin cross-

Abbreviations: $[Ca^{2+}]_i$) intracellular free Ca^{2+} concentration; CaM) calmodulin; CaMK II) type II Ca^{2+}-calmodulin-dependent protein kinase; CPI-17) 17-kD C-kinase potentiated phosphatase inhibitor; ELC_{17}) 17-kD myosin essential light chain; ERK) extracellular signal regulated kinases; ILK) integrin-linked kinase; MLCK) myosin light chain kinase; KRP (telokin)) an independently expressed C-terminal domain of MLCK (myosin light chain kinase-related protein); MLCP) myosin light chain phosphatase; M20) 20-kD myosin-binding subunit of MLCP; MAP-kinase) mitogen-activated protein kinase; MEK) MAP-kinase activating protein kinase; MYPT) myosin binding regulatory subunit of MLCP (myosin phosphatase targeting subunit); PAK) p21-activated protein kinase; PKA) protein kinase A, cAMP-dependent protein kinase; PKC) protein kinase C, Ca^{2+}/phospholipid-dependent protein kinase; PKG) protein kinase G, cGMP-dependent protein kinase; PKN) protein kinase N, homolog of Rho-kinase; PP-1) type 1 protein phosphatase; Rho-kinase) small GTP-binding protein Rho-activated protein kinase; RLC) 20-kD myosin regulatory light chain; sHSP) small heat shock proteins; SM-1 and SM-2) smooth muscle myosin heavy chain isoforms; ZIP-kinase) zipper-interacting protein kinase.

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bridges is well described in the recent reviews [52, 87, 218] and therefore it will not be detailed here.

The rise in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by various external stimuli is a common signal to initiate the contraction in all muscle types [216]. However, whereas striated muscles display a proportionality between $[Ca^{2+}]_i$ and generated force, in smooth muscle multiple mechanisms function to modulate this relationship enabling the smooth muscles to develop and maintain the contraction at low $[Ca^{2+}]_i$ levels and energy utilization. Recent studies have indicated that these mechanisms involve protein phosphorylation as a critical component. This review will be particularly focused on description of these pathways and events.

1. EXCITATION–CONTRACTION COUPLING IN SMOOTH MUSCLE

Transduction of excitatory signals from plasma membrane and/or activated receptors to smooth muscle cell contractile machinery, i.e., excitation–contraction coupling [211] occurs via two major pathways. The first is referred to as the electromechanical coupling and is based on the increase in $[Ca^{2+}]_i$ and proportional activation of the contractile units. The second mechanism involves agonist-induced Ca^{2+} release from internal stores, activation of intracellular signaling, and modulation of Ca^{2+}-sensitivity of contractile units, thus representing the pharmacomechanical coupling [216].

Two mechanisms provide for the increase in $[Ca^{2+}]_i$ in smooth muscle cell (Fig. 1a). First, plasmalemmal Ca^{2+} channels mediate Ca^{2+} influx upon membrane depolarization or agonist ligation by cell surface receptors. Secondly, the activation of trimeric G-protein coupled receptors or tyrosine kinase receptors leads to activation of phospholipases Cβ and Cγ, respectively, and generation of $InsP_3$ (Fig. 1a). The binding of $InsP_3$ to specific receptors of sarcoplasmic reticulum membrane activates Ca^{2+} release from the reticulum (see [84, 211] and references therein). The increase in $[Ca^{2+}]_i$ can also stimulate sarcoplasmic ryanodine receptors and cause Ca^{2+}-induced unitary Ca^{2+} releases from reticulum known as Ca^{2+} sparks [97].

Two major Ca^{2+}-dependent regulatory systems further relay Ca^{2+} signal towards the contractile machinery (Fig. 1a). Calmodulin (CaM) is a key acceptor of Ca^{2+} in cytosol that binds four Ca^{2+} ions and further interacts with regulatory proteins, myosin light chain kinase (MLCK) and caldesmon [244]. Thus activated MLCK phosphorylates Ser^{19} of myosin regulatory light chain (RLC) and activates smooth muscle myosin [67, 68, 143, 244].

However, exposure of myosin-binding sites on an actin filament is required for the subsequent productive actomyosin interaction, i.e., the interaction resulting in filament sliding and force generation [168]. In resting muscle these sites are obstructed by tropomyosin-caldesmon complex located along actin filaments (Fig. 1a). Therefore, such a change of caldesmon conformation that liberates tropomyosin and allows unblocking of the myosin-binding sites on actin is a requirement for actomyosin activation. This is achieved upon binding of Ca^{2+}-calmodulin (Ca^{2+}/CaM) [1, 152] or closely related Ca^{2+}-binding protein [181a] to caldesmon. Thus, smooth muscle tension development depends on both myosin activation by direct phosphorylation and thin filament activation by the relief of caldesmon inhibition.

In most instances, the myosin RLC phosphorylation appears to be necessary and sufficient for the contraction development in smooth muscle [96], whereas caldesmon exerts rather a modulatory effect [84, 211]. The most likely explanation lies in the high affinity and cooperative binding of activated (phosphorylated) myosin heads to actin that accounts for the sigmoid shape of the force/$[Ca^{2+}]_i$ relationship [134] (Fig. 1b). Caldesmon retards, rather than cancels the cooperative binding of highly activated myosin heads simply slowing the force development and accelerating the relaxation [8]. However, under the circumstances of scantily activated myosin, for instance in force redevelopment of unstimulated tissue [37] or agonist-induced contraction with meager increases in $[Ca^{2+}]_i$ [108], caldesmon-mediated regulation of thin filaments may contribute significantly.

The decline of $[Ca^{2+}]_i$ results in CaM dissociation from MLCK and caldesmon, inactivation of MLCK, and restoration of caldesmon inhibition. Subsequent dephosphorylation of RLC by a specific, Ca^{2+}-independent myosin light chain phosphatase (MLCP) and transition of thin filaments into inactive state determine smooth muscle relaxation [66, 67, 190]. As in the case of the force development, the extent of myosin RLC dephosphorylation is the key determinant for relaxation to begin, whereas caldesmon affects relaxation parameters.

The scheme described above represents the classic mechanism of electromechanical coupling and Ca^{2+}-dependent regulation of smooth muscle contraction that is mainly employed following depolarization of plasma membrane, for example by KCl (Fig. 1b). Yet it is recognized that the force of the agonist-induced contraction is not directly proportional to $[Ca^{2+}]_i$ [78, 105]. Various stimuli may similarly elevate $[Ca^{2+}]_i$ but evoke diverse contractile responses and, conversely, comparable contractions may be induced by agonists that increase $[Ca^{2+}]_i$ to different levels. These observations demonstrate an important role of the pharmacomechanical coupling mechanisms that sensitize the contractile machinery to Ca^{2+} and affect the force/$[Ca^{2+}]_i$ relationship [84, 211, 212]. By altering Ca^{2+} sensitivity of the contractile elements, smooth muscle as if "simulates" changes in $[Ca^{2+}]_i$ while it is actually constant. In such a way the majority of physiological stimuli and phorbol esters sensi-

tize the contraction to Ca^{2+}, leading to greater production of force at given $[Ca^{2+}]_i$ level than that caused by depolarization (Fig. 1b). Desensitization to Ca^{2+} can also take place when muscle is treated with agents that elevate intracellular level of cyclic nucleotides (forskolin, isoproterenol, sodium nitroprusside) [104] or stimulate phosphorylation of certain proteins.

It has been found under physiological conditions that contraction of smooth muscle is accompanied by phosphorylation of several contractile and regulatory proteins suggesting its potential role in the pharmacomechanical coupling and modulation of Ca^{2+} sensitivity. Apart from the requisite phosphorylation of myosin RLC by MLCK, the phosphorylation of MLCK itself and that of MLCP, caldesmon and several other actin or myosin associated proteins that can affect actomyosin interaction, has been observed. Below we will review mechanisms of the pharmacomechanical coupling identified to date, intracellular signaling cascades and protein kinases involved in direct phosphorylation of the regulatory proteins, and possible functional consequences with respect to modulation of Ca^{2+} sensitivity of smooth muscle contraction.

2. PHARMACOMECHANICAL COUPLING AND TONIC CONTRACTION IN SMOOTH MUSCLE

Evidently from physiological data, the tension response of smooth muscle to an agonist can be divided into two components (Fig. 2a). The first transient phase is relatively rapid and results chiefly from the electromechanical coupling sketched in the previous section. Thus, after the $[Ca^{2+}]_i$ increases above a threshold level, sufficient for activation of MLCK and inactivation of caldesmon, a typical Ca^{2+}-dependent contraction occurs.

Thereafter, the mechanisms for removal of Ca^{2+} from cytosol are activated causing reduction in $[Ca^{2+}]_i$ and in the level of RLC phosphorylation [36, 162]. However, relaxation does not necessarily follow and the muscle develops tonic contraction (Fig. 2a) [220]. In such a state it often long maintains force while the ATP hydrolysis rate and energy consuming costs are very low [73, 185]. Two hypotheses were proposed to explain this phenomenon either by Ca^{2+} sensitization of the contractile units [84, 211, 212], or in terms of smooth-muscle-specific properties of actomyosin (see section 2.3).

Two major pathways are now thought to mediate Ca^{2+} sensitization during agonist-induced smooth muscle contraction (Fig. 2b). The first involves activation of protein kinase C (PKC) by diacylglycerol that is produced either by phospholipase C from PtdIns(4,5)P_2 along with $InsP_3$, or by phospholipase D from phosphatidylcholine. The other mechanism involves activation of monomeric G-proteins of the Rho family, signal transduction by protein kinases to phosphorylation and inhibition of MLCP (see section 4.1.2). To date there is no complete agreement regarding the specificity of these signaling pathways to particular surface receptors; however, some data suggest that trimeric $G_{q/11}$ or $G_{12/13}$ protein coupled receptors transduce signals to PKC and Rho, respectively (see [213]). Thus, although involvement of each mechanism may be tissue specific and/or depend on the nature of agonist, their role is likely quite individual. At the same time, there is a crosstalk between both membrane G-proteins and their downstream effectors; however, this issue is less clear [213] and will not be discussed here in detail.

2.1. Role of protein kinase C. The hypothesis that protein kinase C is involved in tonic contraction was initially based on the fact that phorbol esters, functional mimetics of diacylglycerol, activate PKC and induce tonic contraction in smooth muscle [22, 31, 98, 99, 188]. Although some reports indicate that phorbol esters increase $[Ca^{2+}]_i$ [56, 192], in many cases the phorbol stimulated contraction was not accompanied by rises in $[Ca^{2+}]_i$ and levels of RLC phosphorylation [94, 98, 99, 226]. Thus the use of phorbol esters in experiments allows to separate artificially and to study specifically the tonic component of contraction [95] (Fig. 2).

The role of PKC in tonic contraction has been explored in isolated ferret smooth muscle cells that contracted in response to phenylephrine without detectable changes in $[Ca^{2+}]_i$ and RLC phosphorylation level [83, 158], but were inhibited by PKC pseudosubstrate peptide inhibitor [26]. Constitutively active PKC fragment caused slow sustained contraction sensitive to this PKC inhibitor [26]. Similar effects with selective inhibitors of PKC were obtained in other smooth muscles [46, 149, 169].

Suggested involvement of PKC is consistent with the fact that various agonists stimulate a sustained elevation of diacylglycerol, the major physiological activator of PKC. The kinetics of diacylglycerol accumulation, intracellular PKC translocation, and activation in response to smooth muscle cell stimulation also substantiate a role of PKC in the second phase of agonist-induced contraction [64, 83, 113].

On the contrary, some reports shed doubt on the role of PKC in smooth muscle contraction. These are based on experiments showing no effect of PKC downregulation by chronic phorbol ester treatment on subsequent contraction [60, 242]. However, about a dozen of PKC isoforms have been described and their expression in different smooth muscles as well as in different species is likely to be quite variable [163, 173]. Therefore, tissue specificity in downregulation of distinct PKC isoforms coupled with individuality of their engagement in regulation of tonic contraction may underlie the lack of the downregulation effect.

PKC directly phosphorylates myosin RLC *in vitro*, but the modified sites (Thr^9 and Ser^{12}) are distinct from Ser^{19}, whose phosphorylation is required to activate

myosin [13, 89]. Moreover, phosphorylation by PKC inhibited, *in vitro*, both the subsequent phosphorylation of Ser^{19} by MLCK and the actin-activated ATPase of myosin phosphorylated at Ser^{19} [89]. The phosphorylation of RLC at PKC sites has been demonstrated in intact smooth muscle, although only in response to phorbol ester, but not natural agonists [103, 208]. Yet, the phosphorylation of RLC by PKC hardly affects the ability of phorbol esters to elicit powerful tonic contraction.

In vitro experiments have demonstrated that major regulators of smooth muscle contraction, i.e., MLCK, MLCP, and caldesmon can be the direct targets of PKC (see section 4). However, later studies *in vivo* have disregarded this possibility and suggested instead the involvement of additional signal transduction pathways downstream of PKC to the contractile machinery. Presently most reliable evidence is obtained for the role of PKC in phosphorylation and activation of CPI-17, an endogenous protein inhibitor of MLCP (see section 4.1.2).

Beyond direct phosphorylation of the regulatory proteins, PKC activates intracellular MAP-kinase pathway (Fig. 2b) [3, 24, 55] and thus obliquely mediates phosphorylation of other regulatory proteins and Ca^{2+} sensitization. Because the subsequent to PKC activation of MAP-kinases by MAP-kinase-kinases involves simultaneous phosphorylation at both tyrosine and threonine residues, the inhibition of the contraction by tyrosine phosphorylation inhibitors has been attributed to the effect on MAP-kinase activation [100]. Possible involvement of MAP-kinases in modulation of smooth muscle Ca^{2+} sensitivity further suggests a role for low molecular weight G-proteins as the upstream components of this signaling pathway.

2.2. Role of monomeric G-proteins. There is a growing body of evidence that the Rho proteins are involved in Ca^{2+} sensitization of the agonist-induced contraction. When added to mildly permeabilized smooth muscles, GTPγS increases the sensitivity of subsequent contraction to Ca^{2+}, and this is inhibited by bacterial toxins that chemically modify and inactivate G-proteins of the Rho family [45, 141, 187]. Discovery of a kinase activated by Rho (Rho-kinase) and demonstration of its ability to phosphorylate and inhibit MLCP [118, 172] allowed to formulate a hypothesis for a role of this cascade in elevation of the level of RLC phosphorylation while $[Ca^{2+}]_i$ is low and, therefore, in the smooth muscle tonic contraction [213]. This hypothesis has been further supported by the use of Y-27632, a selective cell permeable inhibitor of Rho-kinase that inhibits the Ca^{2+}-sensitized contraction [44, 225, 234].

The question remains still uncertain as to how the active membrane associated Rho-kinase is coupled to actomyosin bound MLCP. It can be suggested that direct interaction of these proteins does not happen in smooth muscle and some intermediate should exist. Recently ZIP-like kinase has been shown as a possible candidate [145]. In contrast, in undifferentiated or non-muscle cells Rho-kinase may phosphorylate MLCP directly [109], probably because of some membrane localization of MLCP due to its binding to phospholipids [93]. However, the coupling of membrane bound MLCP to cytoskeleton also remains unexplored.

The physiological significance of the Rho-kinase mediated pathway in agonist-induced contraction remains indefinite since it assumes that generated force would be directly proportional to the extent of RLC phosphorylation. All the same, agonists stimulate transient increases in RLC phosphorylation (Fig. 2a), and also an opinion exists that in the intact muscle notable changes in MLCP activity would not lead to significant changes in the force–RLC phosphorylation relationship [220]. An additional argument against the sole role of Rho-kinase is that activation of Rho occurs upon an engagement of the $G\alpha_{12/13}$ heterotrimeric G-proteins, while most agonists activate the $G\alpha_{q/11}$ coupled receptors [213]. Attempts to identify direct coupling of Rho to the $G\alpha_{q/11}$ have not been successful [212]. Yet, tonic contraction stimulated by growth factors resulted in activation of phospholipase Cγ and/or MAP-kinase pathway [100], with no involvement of heterotrimeric G-protein signaling to the Rho-kinase pathway [86]. Finally, agonist-induced contraction was reduced by inhibitors of tyrosine kinases [35, 86, 219] the role of which in signalization through the Rho proteins has not been established.

However, taking into account the multitude of Rho family members (Cdc42, Rac1, Rac2, RhoA, RhoB, RhoC) and their possible functional crosstalk, as well as a specificity of contractile responses to a particular agonist, their direct [79] or indirect involvement in modulation of Ca^{2+} sensitivity might be expected. Specific targeting of their downstream components may be of a potential use in the development of antihypertensive therapeutics [234].

2.3. Latch phenomenon. As stated above, the maintenance of high isometric force at significantly decreased $[Ca^{2+}]_i$ and level of RLC phosphorylation is characteristic for the second phase of agonist-induced smooth muscle contraction (Fig. 2a). Discovered more than twenty years ago, this phenomenon was initially termed latch [36] to reflect similarity to mechanical properties of mollusk catch muscle developing long tonic contraction at very low energy costs [233]. The latch mechanism suggested by Murphy and colleagues [63, 167, 220] does not necessitate modulation of Ca^{2+} sensitivity, but postulates an existence of smooth muscle specific dephosphorylated non-cycling cross-bridges. It is suggested that myosin becomes dephosphorylated while strongly bound to actin, i.e., within the cross-bridges, and this results in a drastic decrease in the myosin head dissociation constant (k_{off}) and creation of the latched bridges [63]. This mechanism is based on the assumption that myosin RLC phosphorylation is required for the formation of strongly bound

cross-bridges, which has been indeed demonstrated *in vitro* [200]. The idea that the latch state is characteristic only for smooth muscle because of unique intrinsic properties of myosin gained a support from the Somlyo's group [212] who found that smooth muscle myosin has high affinity to Mg-ADP whose accumulation during active contraction results in increased lifetime of the cross-bridges [116, 117]. Furthermore, distinct affinities of myosin from phasic and tonic smooth muscles (see section 3) to Mg-ADP contribute to differences in their contraction and relaxation rates [115]. However, whether the dephosphorylated cross-bridges do really exist and if their affinity to Mg-ADP is higher than that of phosphorylated, as required by the latch hypothesis, remains unclear.

The proteins that simultaneously interact and cross-link actin and myosin filaments could make an additional contribution into tension maintenance under low levels of $[Ca^{2+}]_i$, RLC phosphorylation, and energy expenditure. The presence of actin- and myosin-binding sites in opposite ends of the molecules of MLCK and caldesmon makes such an interaction possible *in vitro* [220, 240] and, in the case of caldesmon, *in vivo* [58]. However, the cross-linking activity of these proteins is unlikely to grow during active contraction suggesting that it would rather be manifested by slowing down the tension and/or relaxation development as well as by increasing the fiber stiffness. Finally, the binding of regulatory proteins can additionally affect the mechanical properties of actin and myosin filaments [19].

Apparently neither of the suggested mechanisms of tonic contraction is solely realized *in vivo*. Both Ca^{2+} sensitization and the involvement of dephosphorylated cross-bridges or protein cross-links are unable to explain all aspects of smooth muscle physiology. Most likely, the tonic response is achieved by their combination and may be fairly tissue specific. Some species specific deviations in the regulation of tonic contraction may also take place.

3. PHASIC AND TONIC SMOOTH MUSCLES

Historically and physiologically smooth muscles are classified into two types of fast contracting phasic and slow contracting tonic muscles [216]. Phasic muscles generate action potentials, shorten rapidly, and produce spontaneous contractions. Their response to membrane depolarization is relatively rapid but transient. The typical phasic muscles are the mammalian gastrointestinal *ileum* and *taenia coli*, *uterus* in the urogenital system, *portal vein* in the circulatory system, and the avian *gizzard* muscle.

As a rule, tonic smooth muscles respond to agonist stimulation by gradual depolarization and have low shortening rates. They do not generate an action potential and spontaneous contractions, but can effectively maintain the contracted state for a long time, i.e., to produce tonic contraction [216]. Tonic smooth muscles include most circulatory (aorta, arteries, and arterioles) and airway muscles such as tracheal, as well as the gastrointestinal and urogenital sphincters [84].

Many differences between phasic and tonic smooth muscles can be accounted for by distinct biochemical properties of their contractile proteins. Besides the mentioned above different myosin affinities to Mg-ADP, the contractile apparatus of tonic muscles is more sensitive to $[Ca^{2+}]_i$ and MLCP inhibitors than that of phasic muscles [78, 120]. The relative defiance of phasic muscles to phosphatase inhibitors is because of increased endogenous activity of MLCP [59], which would restrain tone maintenance and aid relaxation. Higher activities of MLCK and MLCP in phasic relative to tonic muscles were actually found [78] assenting the transient mode of contraction due to increased turnover of the phosphate associated with myosin RLC.

Apart from the regulatory signaling pathways, an isoform diversity of the contractile proteins may be causal of different mechanical reactions of phasic and tonic muscles. The cross-bridge turnover rate governs the rate of contraction development and this, in turn, depends on the ATPase rate of the cross-bridges. As representative of the myosin II family, smooth muscle myosin is composed of two heavy chains and two pairs of light chains—20 kD RLC and 17 kD essential light chains (ELC_{17}). Several isoforms of the heavy chains [111, 171, 248], RLC [160, 170], and ELC_{17} [74, 107, 137] have been described to date.

Two heavy chain isoforms designated as SM-1 (~204 kD) and SM-2 (~200 kD) differ by presence or absence, respectively, of an additional C-terminal sequence [111]. However, the two isoforms appear functionally identical in an *in vitro* motility assay and with respect to filament formation [110, 111]. Thus the significance of the heavy chain isoform diversity remains unclear.

Heavy chain isoforms differing in the N-terminal sequence are also known. In contrast to myosin from tonic muscle of blood vessels, gizzard myosin has an isoform containing an additional seven amino acid insertion close to the ATP-binding site and thus it exerts an increased ATPase activity and actin filament movement in an *in vitro* motility assay. These differences may contribute to the faster and briefer responses displayed by gastrointestinal relative to circulatory smooth muscles [110].

It is suggested that ELC_{17} isoforms provide for differences in the smooth muscle shortening rates [215]. Thus, fast phasic gastrointestinal muscles contain only ELC_{17a} isoform, while tonic tracheal, aortic, carotid, and pulmonary arteries contain relatively high amounts of ELC_{17b} isoform [74, 150]. Hasegawa and Morita [69] found that a replacement of ELC_{17b} for ELC_{17a} in the pig aorta myosin leads to increased actomyosin ATPase activ-

ity. However, although the same difference has been identified between avian phasic and tonic muscle myosins, the same ELC_{17} exchange in chicken aortic myosin that does not contain the seven amino acid N-terminal insert, did not result in altered actin filament movement in an *in vitro* motility assay [112]. Therefore, physiological manifestation of ELC_{17} isoform divergence may be coupled to the heavy chain isoform polymorphism. For instance, a higher rate of the uterus muscle contraction of rats fed estrogen was associated not only with the increased ELC_{17a} content [161], but also with an concomitant elevation of SM-1 isoform constituent [76, 217].

Another important factor that determines the functional differences between phasic and tonic smooth muscles is the contrastingly distinct expression levels of endogenous proteins that inhibit either phosphorylation (KRP/telokin), or dephosphorylation (CPI-17) of myosin. The physiological importance of these proteins will be highlighted below (section 4.1).

Divergence in excitation–contraction coupling mechanisms may also define the unlikeness of phasic and tonic muscles. The smooth muscle cell plasma membrane contains a variety of ion channels, exchangers, and ATPases whose distribution and properties considerably depend on the tissue type [156, 177]. Different biochemical properties and sensitivity to membrane potential may result in distinct regulation of Ca^{2+} homeostasis and electromechanical coupling, while different sensitivity to second messengers and posttranslational modifications would affect pharmacomechanical coupling and dependence of the contractile response on $[Ca^{2+}]_i$ [97, 177, 211, 216].

Most likely deviations in mechanisms of intracellular signaling, either in their organization and in involvement of certain signaling cascades, or in intracellular arrangement of their components significantly contribute to the natural variety of smooth muscle contractile responses. Specific mechanical characteristics of a certain muscle are likely to result from a combination of many factors, including specifics in the content and distribution of membrane proteins, intracellular signaling pathways, and isoforms of the contractile proteins. Different responses of phasic and tonic smooth muscle to a same stimulus can be used to search for and identify key events in intracellular signaling that are involved in tonic contraction [129, 239]. In perspective these studies may result in development of highly specific compounds for treatment a number of circulatory, pulmonary, and birth labor diseases.

4. MOLECULAR MECHANISMS FOR REGULATION OF SMOOTH MUSCLE Ca^{2+} SENSITIVITY

The force produced by all muscle types is a sum of the forces generated by elementary actomyosin interactions known as cross-bridges. Specifically, smooth muscles employ Ca^{2+}-sensitizing mechanisms of pharmacomechanical coupling to considerably modify the final mechanical output. These can be classified into two groups. The first comprises the mechanisms that alter dependence of RLC phosphorylation level on $[Ca^{2+}]_i$, while the second includes mechanisms that modify a relationship of the force to the RLC phosphorylation level [198] (Fig. 3). Closer inspection reveals that mechanisms of the first group are based on the myosin-associated regulatory systems and often are physically coupled to myosin filaments [212, 213]. The second group mechanisms mostly target the actin-associated regulatory proteins that can enhance or uncouple the interaction of actin with myosin at constant level of RLC phosphorylation [20, 84]. In many instances protein phosphorylation appears to be critically involved in modulation of Ca^{2+}-sensitivity of the regulatory proteins and muscle contraction.

4.1. Ca^{2+} dependence of RLC phosphorylation. A shift of the RLC phosphorylation–$[Ca^{2+}]_i$ dependence occurs upon alteration of the MLCK/MLCP activity ratio as well as upon phosphorylation of Ser^{19} of myosin RLC by other protein kinases. Before analyzing significance of phosphorylation of MLCK and MLCP themselves, the structure and an *in vivo* function of these proteins should be summarized. As several excellent reviews on MLCK [50, 143, 222, 244] and MLCP [66-68] have recently appeared, we will only sketchily regard these as a prelude to each following section.

4.1.1. Phosphorylation and modulation of MLCK activity. The catalytic core of MLCK is homologous to that of the other Ser/Thr protein kinases and contains ATP- and RLC-binding sites (Fig. 4). The regulatory segment is located further C-terminal and contains a binding site for Ca^{2+}/CaM that is required for MLCK activation, and a pseudosubstrate sequence that is autoinhibitory in the absence of Ca^{2+}/CaM [123, 143]. The C-terminus of MLCK contains a myosin-binding site [205] and aslo is independently expressed in smooth muscles as the kinase-related protein (KRP), also known as telokin [25, 48]. Several immunoglobulin (Ig) and fibronectin (FN) like repeats are located in the N-terminal part of MLCK [49] and separate the catalytic core from the N-terminal tandem repeats and actin-binding sequence [50] (Fig. 4). It is accepted that in the absence of bound CaM, the regulatory segment is folded back so that the pseudosubstrate sequence interacts with the RLC binding site and blocks the phosphotransferase activity. Upon binding of Ca^{2+}/CaM, the autoinhibitory sequence is displaced and the ATP/RLC-binding sites become exposed [50]. The Ca^{2+} sensitivity of MLCK activation by Ca^{2+}/CaM is in the range of 100-400 nM *in vivo* and is to a large extent a combination of CaM affinity to Ca^{2+} and MLCK affinity to Ca^{2+}/CaM.

MLCK binds both actin and myosin *in vitro* [201]. However, only the mutant with deleted KRP domain, but

not the N-terminus, retains the ability to bind actomyosin myofilaments *in vitro* suggesting that MLCK is preferentially bound to actin filaments *in vivo* [222]. It can be hypothesized that myosin phosphorylation *in vivo* occurs through a cyclic solid phase mechanism. Thus, binding of Ca^{2+}/CaM to MLCK bound by the N-terminus to actin filament results in unfolding and deinhibition of MLCK. The molecule of MLCK can span the distance between actin and myosin filaments, which is about 15 nm *in vivo* [222], and binds the opposite myosin molecule by the KRP domain, and this enhances the effectiveness of RLC phosphorylation. Subsequent dissociation of catalytic and KRP [205] domains of MLCK from phosphorylated myosin allows movement of the myosin filament along the actin filament and juxtaposition of the next myosin molecule to MLCK for phosphorylation and activation. Since intracellular concentration of RLC is estimated as 50-80 μM, while MLCK concentration is only 2-4 μM [6, 174], this mechanism might explain rapid agonist-stimulated myosin phosphorylation and smooth muscle force development.

The force and velocity of smooth muscle contraction may be also affected by phosphorylation and change in activity of MLCK. Three principal observations were made in earlier studies *in vitro* and *in vivo*. First, a majority of phosphorylated residues appeared clustered in the regulatory segment and the N-terminal part of KRP domain in MLCK (Fig. 4). Second, the phosphorylation site responsible for inhibition of MLCK was identified in the Ca^{2+}/CaM-binding region of the enzyme (site A). The binding of Ca^{2+}/CaM blocked its phosphorylation and, conversely, phosphorylation at site A decreased MLCK affinity to Ca^{2+}/CaM [27, 70, 91, 178, 179]. The K_{CaM} was increased about 10-fold due to both the 3.5-fold decrease of the Ca^{2+}/CaM association constant and the 6-fold increase in the dissociation constant [106]. Third, the N-terminal sequence of the KRP domain of MLCK was found to contain several closely located phosphorylation sites, including site B (Fig. 4) and most of the phosphate associated with MLCK *in vivo* [223, 239].

Subsequent studies revealed a complex case of MLCK phosphorylation *in vitro*. For example, cAMP-dependent protein kinase (PKA) phosphorylates sites A and B in avian MLCK in the absence of Ca^{2+}/CaM and only site B when Ca^{2+}/CaM is bound [27, 142, 186]. However, in MLCK from sheep myometrium PKA phosphorylates the same sites independently of Ca^{2+}/CaM, but also inhibits its binding [184]. The cGMP-dependent protein kinase (PKG) phosphorylates only site B in bovine MLCK, and this is blocked by Ca^{2+}/CaM binding [71, 178], whilst in human platelet MLCK PKG phosphorylates both site B and a residue distinct from site A [178]. Similar discrepancies were found when PKC and type II Ca^{2+}/calmodulin-dependent protein kinase (CaMK II) were used to phosphorylate MLCK [70, 90, 91, 178, 184]. These data indicate that tissue and species specificity in both MLCK and phosphorylating kinases may be significant. Additionally, as the primary sequence of the MLCK regulatory region is quite conservative (see Fig. 4), a flexibility of MLCK conformation in solution may impact the accessibility of phosphorylation sites and effect of Ca^{2+}/CaM binding. This may contribute to specificity in a mosaic pattern of MLCK phosphorylation in various tissues stimulated by different stimuli, for several closely located residues, including sites A and B, are phosphorylated in MLCK *in vivo* [223, 239].

Whereas the mechanism of MLCK inhibition by phosphorylation of site A is all but apparent, a role of site B phosphorylation remains quite unclear. Selective phosphorylation of site B did not affect the interaction of avian MLCK with Ca^{2+}/CaM, yet it enhanced the effect of site A phosphorylation on Ca^{2+}/CaM binding [179]. Subsequent identification of the myosin-binding site within the KRP domain of MLCK [205] and its suggested role in MLCK oligomerization [175] allow somewhat to reconcile the significance of site B phosphorylation. An altered affinity of MLCK to myosin has been observed upon phosphorylation of MLCK [195], which may affect the kinetics of myosin phosphorylation in contrast to phosphorylation of isolated RLC as has been studied before [27, 179]. It can be also hypothesized that MLCK phosphorylation in the site B region may affect intramolecular conformation of the MLCK molecule. According to the pseudosubstrate hypothesis, the C-terminal domain of MLCK is folded back towards the N-terminus enabling the binding of an autoinhibitory sequence to the catalytic cleft of the enzyme and bringing into vicinity the homologous KRP domain and second Ig-like repeat (Fig. 4). Susceptibility of these structures to aggregation as shown by the KRP domain mediated oligomerization of MLCK [175] suggests the possibility of their intramolecular interaction in MLCK, which would restrain binding of Ca^{2+}/CaM and enzyme activation. Perhaps phosphorylation at site B and/or adjacent residues inhibits this interaction and facilitates MLCK activation. Such activating effect of phosphorylation by MAP-kinase has been recently shown for MLCK *in vitro* [122, 164], consistent with localization of the major phosphorylation site in the KRP domain close to site B [164, 239] (Fig. 4).

An involvement of site A phosphorylation in regulation of MLCK was investigated *in vivo*. Stimulation of β-adrenoreceptors and direct activation of adenylate cyclase by forskolin to increase intracellular cAMP and activate PKA led to phosphorylation of MLCK and relaxation of tracheal smooth muscle [32]. However, it later appeared that the receptor-dependent activation of PKA and PKC in this tissue results in phosphorylation at site B, but not at site A [221]. In contrast, site A phosphorylation, inactivation of MLCK, and subsequent relaxation in intact and skinned tracheal smooth muscle were a result of increase in $[Ca^{2+}]_i$ [221, 223, 236]. CaMK II was found to mediate the Ca^{2+}-dependent phosphorylation of site A

and alteration of K_{CaM} of MLCK [227, 229, 230]. It also appeared that $[Ca^{2+}]_i$ required for the half-maximum activation of CaMK II equals 500 nM, whereas that for MLCK activation is only 250 nM, suggesting a role for CaMK II in inactivation of MLCK when smooth muscle is hyperactivated and $[Ca^{2+}]_i$ rises above some critical level. Treatments of tracheal cells by CaMK II inhibitors decreased the level of MLCK phosphorylation achieved upon increase in $[Ca^{2+}]_i$ and elevated the RLC phosphorylation level due to decrease in half-maximum $[Ca^{2+}]_i$ required for RLC phosphorylation to 170 nM [229, 254].

The idea that CaMK II directly phosphorylates site A *in vivo* is somewhat controversial since at elevated $[Ca^{2+}]_i$ this sequence must be blocked by bound Ca^{2+}/CaM. It was suggested that availability of Ca^{2+}/CaM for these protein kinases is restricted due to compartmentalization in the cell and MLCK competes with CaMK II for Ca^{2+}/CaM [223, 229]; however, it is difficult to prove this hypothesis experimentally. Still, phosphorylation and dephosphorylation of MLCK correlate with the contraction–relaxation cycle and changes in Ca^{2+} sensitivity of RLC phosphorylation in smooth muscle [254]. Thus, Ca^{2+}-dependent phosphorylation of MLCK may serve as a feedback mechanism to decrease the level of RLC phosphorylation and ATP consumption by cycling actomyosin cross-bridges [168].

The p21-activated protein kinase (PAK) isoforms have been recently shown to phosphorylate and inhibit MLCK in non-muscle cells [57, 196, 202]. PAK1 incorporates 1 mol of phosphate per mol of MLCK *in vitro* and this is not inhibited by Ca^{2+}/CaM. Phosphorylated MLCK has a lower V_{max} but unaltered K_{CaM} [196]. At the same time, PAK2 phosphorylates the serine residue adjacent to the regulatory site A (Fig. 4) and a serine located between the second Ig-like and FN-like repeats N-terminal to the catalytic domain [57]. This also results in MLCK inhibition; however, due to increase in K_{CaM}, while individual role of phosphorylated residues is uncertain. These observations suggest possible involvement of PAK and Rho-activated regulatory cascades in MLCK phosphorylation and modulation of Ca^{2+} sensitivity of RLC phosphorylation in smooth muscle if PAK has intracellular access to the contractile machinery.

MAP-kinase also phosphorylates a site located in the KRP domain and a site in the N-terminal part of MLCK [164]; however, stimulation of MAP-kinases by phorbol ester in intact smooth muscle results in preferential phosphorylation of the KRP domain [239]. This phosphorylation has been reported to either activate MLCK directly without a change in K_{CaM} [164], or to increase the affinity of MLCK for Ca^{2+}/CaM while not affecting V_{max} [122]. In non-muscle cells expressing constitutively active MEK, i.e., MAP-kinase-kinase that activates ERK-MAP-kinases, the level of MLCK phosphorylation was higher than in control cells. Pharmacological inhibition of MEK activation as well as transfection of cells with the dominant negative MEK led to decreased phosphorylation of MLCK, RLC and cell motility [122]. Thus, ERK-MAP-kinases may phosphorylate and activate MLCK in smooth muscle too, sensitizing the phosphorylation of myosin RLC to Ca^{2+}.

MLCK undergoes an autophosphorylation [41]. At least two sites are located in the regulatory domain and one is in proximity of the N-terminal actin-binding site of MLCK. Autophosphorylation within the regulatory domain resulted in decreased affinity to Ca^{2+}/CaM and, *vice versa*, binding of Ca^{2+}/CaM greatly inhibited the autophosphorylation [2, 31]. According to others, autophosphorylation may activate MLCK [242]. However, the autophosphorylation is substantial only at Mg^{2+} concentration around 30 mM that is much higher than physiological, thus leaving its meaning for MLCK regulation *in vivo* rather illusive.

Thus, phosphorylation by various kinases may affect the activity of MLCK and the balance of MLCK and MLCP activities *in vivo*, mirrored by changes in the dependence of RLC phosphorylation level on $[Ca^{2+}]_i$ and Ca^{2+} sensitivity of contraction. On the other hand, alteration of this activity ratio is significantly determined by mechanisms that control the MLCP activity.

4.1.2. Modulation of MLCP activity. The existence of a regulatory mechanism for Ca^{2+} sensitization of smooth muscle contraction coupled to a specific RLC phosphatase was proposed more than 10 years ago [120]. In 1992 such a phosphatase was purified from avian smooth muscle [9], and later it was isolated from a mammalian tissue [203]. MLCP belongs to a group of type 1 protein phosphatases (PP-1) and is a heterotrimeric complex composed of the regulatory (110-130 kD, $M_{110\text{-}130}$ or MYPT), catalytic (37 kD, PP-1c), and 20 kD (M20) subunits (Fig. 5). The catalytic subunit is common to all the type 1 phosphatases, whereas MYPT determines the binding and specificity of PP-1c to myosin, while the function of M20 is yet unknown [67]. MYPT interacts with myosin and the N-terminal domain of PP-1c, activates dephosphorylation of RLC, and accelerates the PP-1c induced relaxation of skinned smooth muscle strips [9, 203]. The C-terminal domain of MYPT containing an additional myosin binding site and an M20 binding site is also required for full activation of PP-1c [101] (Fig. 5). However, the interaction of MYPT with M20 does not affect the MLCP activity, while direct binding of M20 to PP-1c was not detected [101]. It is suggested that M20 additionally ties MLCP to the shaft of myosin filaments [101] and thus can provide for a mobility of MLCP within the contractile apparatus [68]. The latter may be important for regulation of MLCP activity *in vivo* since intracellular concentration of the phosphatase is 20-30 times less than that of its substrate, myosin RLC [9]. The compartmentalization and the activity of MLCP may also depend on the interaction of the C-terminal MYPT domain with phospholipids [93], Rho [118], and arachi-

donic acid [47]. Phospholipids and arachidonic acid inhibit MLCP, probably by dissociating the holoenzyme [59], whereas the binding of Rho does not change the MLCP activity. It is likely that these interactions serve to localize MYPT outside actomyosin in non-muscle cells and are not executed in smooth muscle cells where MLCP colocalizes with the contractile units providing effective dephosphorylation of myosin. The latter is corroborated by a specific binding of MLCP to phosphorylated myosin, while dephosphorylation of RLC significantly decreases the affinity of MLCP to myosin [67, 68].

Phosphorylation of MYPT is thought to be a general mechanism for G-protein dependent elevation of RLC phosphorylation level at constant $[Ca^{2+}]_i$ [187, 212, 213]. It has been quite conclusively shown that Rho-kinase mediates this effect in smooth muscle, as well as during remodeling of cytoskeleton [102] and cell migration [214]. However, at present there is no full description of the signaling cascade that mediates phosphorylation and regulation of MLCP in smooth muscle. Rho-kinase has been shown to directly phosphorylate MYPT at Thr^{697} and Ser^{854} and to inhibit MLCP [109, 118]. Phosphorylation of Ser^{854} increased in an epithelial cell line treated with phorbol ester or growth factor, correlated with membrane ruffling and enhanced cell motility [109], and was increased in vasospasm *in vivo* [197]. However, the *in vitro* inhibition of MLCP by the phosphorylation of Ser^{854} has not been established, while it was demonstrated for that of Thr^{697} [39] achieved in smooth muscle by an endogenous actomyosin-bound protein kinase distinct from Rho-kinase [88]. This MLCP kinase was recently identified as a homolog of zipper-interacting protein (ZIP) kinase [145]. Thus, while Rho-kinase directly phosphorylates MYPT in non-muscle cells, in smooth muscle this is unlikely due to membrane localization of activated Rho-kinase and actomyosin-bound MLCP. In this case the ZIP-like kinase may be the downstream effector responsible for phosphorylation and inhibition of MLCP. Nonetheless, it remains vague how these protein kinases are coupled within one signaling cascade *in vivo*. Rho-kinase is incapable of phosphorylating ZIP-like kinase *in vitro* [145], but still is involved in agonist-induced Ca^{2+} sensitization [225] and its inhibition reduces the ZIP-like kinase activation by carbachol and subsequent phosphorylation of MYPT [145]. Thus, the discovery of a coupling intermediate such as a kinase of ZIP-like kinase is soon anticipated.

How does the phosphorylation of Thr^{697} in the C-terminal MYPT domain affect the MLCP activity? The critical sites determining the PP-1c binding and MYPT positioning toward RLC are located in the N-terminal domain, whereas the C-terminal part of MYPT contains only an additional myosin-binding site [101]. It has been suggested that phosphorylated Thr^{697} occupies the catalytic cleft of PP-1c and competes for RLC binding. A restoration of the MLCP activity occurs upon autodephosphorylation of Thr^{697} [7] (Fig. 5).

Another way of inhibiting MLCP is to obstruct interaction between the N-terminal MYPT domain with PP-1c and/or myosin RLC. *In vitro*, this is achieved by phosphorylation of two residues in the N-terminal MYPT domain by PKC [232], but this phosphorylation has not been demonstrated *in vivo*.

Activation of PKC causes Ca^{2+} sensitization of RLC phosphorylation and contraction via a mechanism that does not involve the Rho protein activation [44, 60], but also leads to MLCP inhibition *in vivo*. PKC phosphorylates a protein called CPI-17 (a 17 kD C-kinase potentiated inhibitor of MLCP), thereby activating it [38, 119, 121, 138]. Moreover, the phosphorylation of CPI-17 is a convergence point for PKC- and Rho-mediated signaling pathways to inhibition of MLCP *in vivo*. Phosphorylation of CPI-17 increases when smooth muscle is stimulated by either phorbol esters, direct activators of PKC [253], or GTPγS, an activator of Rho [119], while specific inhibitors of PKC and Rho-kinase block agonist-induced phosphorylation of CPI-17 and contraction at low $[Ca^{2+}]_i$ [119]. PKC and Rho-kinase have been shown to phosphorylate CPI-17 at the single Thr^{38} [126]. The same residue is also phosphorylated *in vitro* by other members of Rho-activated cascades, such as PKN [65], myotonic dystrophy protein kinase [165] and ZIP-kinase [146], once again indicating the important role of MLCP regulation in smooth muscle contraction.

MLCP activation that leads to decrease of endogenous level of RLC phosphorylation at given $[Ca^{2+}]_i$ (Fig. 3) is likely important for smooth muscle relaxation. An interaction of MYPT with PKG has been revealed by the yeast two hybrid screening, and uncoupling of this interaction impaired the cGMP-mediated dephosphorylation of RLC [224]. Although PKG has not been demonstrated to phosphorylate MLCP directly, the relaxing activity of nitrovasodilators that activate guanylate cyclase and elevate intracellular cGMP may be suggested to involve, at least partially, the activation of PKG-mediated phosphorylation of MLCP.

4.1.3. A role for KRP. The level of myosin RLC phosphorylation and force of smooth muscle contraction not only depend on the activity ratio of MLCK and MLCP, but also on the availability of the critical RLC phosphorylation site for these enzymes in a particular tissue context. This could be controlled by proteins that bind to myosin, MLCK, or MLCP but do not directly affect their enzyme activity. Recent experimental advance have revealed that KRP, also known as telokin, may execute such a function [237].

KRP was originally isolated from chicken gizzard smooth muscle as a bypass product of CaM purification [29, 92]. Later, KRP primary structure was found to match the C-terminal sequence of smooth muscle MLCK, and consequently KRP was identified as an

autonomous protein product of the MLCK gene locus that is independently expressed due to its own promotor located within an intron of the MLCK gene [16, 25, 48]. Because of the genetic and structural relationship with MLCK, this protein was independently given two names: KRP, i.e., the *k*inase-*r*elated *p*rotein [25] and telokin (from *telo*s of the *kin*ase [92]).

KRP is an acidic protein (p*I* 4.5) with calculated molecular mass of 17.7 kD. Crystal structure of KRP revealed by an X-ray analysis at the 2.8 Å resolution [81] showed seven antiparallel β-sheets that form a barrel-like structure (Fig. 4). Positions of 103 from 154 residues of KRP were identified whereas the 32 N-terminal and 19 C-terminal residues were not visible on the electron density maps suggesting that these sequences are highly flexible. Interestingly, an individual exon encodes for the first 28-residue N-terminal sequence of KRP that includes Ser^{12} phosphorylated by PKA and PKG, equivalent to Ser^{828} in MLCK (i.e., the site B) as well as several other phosphorylatable sites (see below). The primary structure of the crystallized region of KRP is homologous to sequences found in some muscle proteins such as titin, twitchin, and nebulin [81]. All of these proteins contain Ig-like repeats of type I or type II, which are involved in interaction with myosin [140]. KRP and the KRP domain of MLCK contain such a repeat of type II and serve the same function [205, 209]. In addition, the very C-terminal sequence of KRP significantly contributes to myosin binding [207].

In tissues, KRP is expressed as several isoforms heterogeneous both in the N-terminal and in the C-terminal regions [194]. Three N-terminal variants are produced by alternative transcription initiation of the KRP gene from one of the three methionine codons. Six C-terminal variants contain different number (from 0 to 5) of adjacent Glu residues. The C-terminal heterogeneity of KRP may possibly have a role in differential regulation of myosin filament assembly *in vivo* [194].

KRP interacts with the sequence connecting the rod and globular head portions of smooth and non-muscle myosins, close to the RLC binding region [207]. This interaction prevents the transition of the myosin molecule from an extended (6S) to folded (10S) conformation in ATP and physiological salt containing solutions, thus mimicking the effect of RLC phosphorylation [154, 205]. As only the 6S myosins form filaments, KRP stabilizes the filamentous structure of unphosphorylated myosin and thus may keep the contractile apparatus structured in resting cells [237]. At the same time, KRP does not interact with striated muscle myosins, consistent with their incapability of the ATP-dependent 6S ↔ 10S conformational transition. The specificity of KRP for binding and regulation of certain myosins is probably reflected by its tissue specific expression restricted to smooth muscles. Moreover, its intracellular concentration approximates that of myosin [205] and appears dramatically higher in phasic than in tonic smooth muscles [48, 75, 239, 255], probably because in the latter the level of unstimulated phosphorylation of RLC is sufficiently high to keep myosin arranged in filaments.

Phosphorylation of RLC prevents the interaction of KRP with myosin and binding of KRP inhibits myosin phosphorylation by MLCK [205]. KRP probably blocks the MLCK binding site on myosin decreasing MLCK affinity to myosin, and also may shield RLC, all this inhibiting RLC phosphorylation by MLCK [175, 205, 207]. This inhibition does not take place *in vitro* when myosin subfragment 1 (an individual head), isolated RLC, or synthetic RLC peptide are used as MLCK substrates, indicating that KRP does not directly inhibit the catalytic activity of MLCK. Thus, *in vitro*, KRP is a competitive inhibitor of MLCK binding to its physiological substrate, myosin. On that account it has been suggested that, in smooth muscle, KRP may shift the balance of myosin phosphorylation and dephosphorylation in favor of the latter and thus slow down tension development or accelerate relaxation [237].

Indeed, exogenous KRP decreased the level of RLC phosphorylation and caused relaxation in skinned smooth muscle strips depleted of KRP [210, 243, 255]. Using ATPγS to block the endogenous dephosphorylation of RLC, the effect of KRP on the kinetics of RLC thiophosphorylation has been assessed in permeabilized tissue [255]. KRP has been concluded to stimulate dephosphorylation of RLC rather than to inhibit the phosphorylation process [255]. However, it is worthwhile to note that the experimental conditions and obtained results may not have a unique interpretation, taking into account the lack of the direct effect of KRP on MLCP activity [147, 243] and extremely fast phosphorylation of RLC. Under the conditions studied [243, 255], the phosphorylation of RLC is nearly completed, whereas the concentration of the exogenous KRP (10-20 μM) was notably less than that of myosin in the tissue [175, 255]. Thus, the effect of KRP could have not been detected even assuming its effective infusion and complete binding to myosin. Under similar conditions *in vitro*, a clear effect has been achieved only by a molar excess of KRP over myosin [175].

However, the fact that both KRP and $KRP_{1\text{-}143}$, a mutant with deleted C-terminal sequence which is important for myosin binding, had similar relaxing activities [243], while KRP mutant devoid of the N-terminal region did not relax the contraction [255], suggests the existence of KRP targets distinct from myosin. Moreover, a stable analog of cGMP enhanced relaxation induced by KRP [255] and $KRP_{1\text{-}143}$ [243], and this was accompanied by phosphorylation of the N-terminal Ser^{12} of KRP equivalent to the site B of MLCK (see Fig. 4). Consistent with this hypothesis, phosphorylation of this site had no effect on KRP interaction with myosin *in vitro* [241], further suggesting that the N-terminal region of KRP and its phosphorylation may have a role in Ca^{2+} desensitization

and smooth muscle relaxation through a mechanism different from inhibition of myosin RLC phosphorylation by MLCK [215, 255].

In intact smooth muscle, KRP is profoundly phosphorylated at site B (Ser^{12}) and at least at two additional sites within the N-terminal sequence [129, 239, 241]. Ser^{18} was identified as a MAP-kinase substrate and its phosphorylation increased upon treatment of tissue with phorbol ester [129] or GTPγS [141]. Although the third of the *in vivo* phosphorylation sites has not been exactly identified, it is probably Ser^{15}, which is phosphorylated *in vitro* by acidotropic glycogen synthase kinase 3 after the Ser^{18} is phosphorylated by MAP-kinase [129, 239]. The mammalian KRP contains Thr^{27} and Ser^{32} additional to the avian counterpart and one of them is phosphorylated by CaMK II *in vitro*, although *in vivo* this has not been demonstrated [255].

At present, it is not fully understood how the site-specific phosphorylation of KRP (and of the equivalent residues in MLCK) changes in intact smooth muscle stimulated by various agonists and relaxants, as well as how it affects the contractile response. Largely this is because of the multiplicity and close location of phosphorylation sites that hampers and makes less effective their analysis by standard techniques such as the phosphopeptide mapping [129, 239], mass-spectrometry [255], sequencing [147], and point mutation [243]. In this regard, phospho- and site-specific antibodies become now widely used to monitor phosphorylation of muscle proteins in living cells and tissues [30, 127, 197]. Such antibodies have been produced to equivalent residues of KRP and MLCK phosphorylated by PKA/PKG and MAP-kinase[1]. Pilot experiments show that site B phosphorylation in KRP and MLCK similarly increases in intact muscle stimulated to contract by phorbol ester or treated by the relaxant forskolin, while only phorbol ester enhances phosphorylation of the MAP-kinase phosphorylated Ser^{18}. Since the phorbol ester induced phosphorylation of KRP-domain of MLCK correlates with tonic contraction[1] [239], a role for the MAP-kinase mediated phosphorylation and activation of MLCK might be suggested, as it has been shown in other models [122, 164]. At the same time, the physiological significance of multiple and site-specific phosphorylation of KRP remains subject for future experimentation.

4.1.4. Myosin RLC phosphorylation by Ca^{2+}-independent kinases. There is a growing body of evidence that inhibition of MLCP and dephosphorylation of RLC is the mechanism for altering the relationship of RLC phosphorylation and $[Ca^{2+}]_i$ *in vivo*. Clearly, smooth muscle has to possess a Ca^{2+}-independent RLC kinase different from MLCK to provide the functionality of this mechanism at low $[Ca^{2+}]_i$. Several of such kinases have been recently described and shown to phosphorylate myosin at $[Ca^{2+}]_i$ values less than those required for MLCK activation. However, they may be more important for myosin phosphorylation in non-muscle or cultured cells rather than in differentiated smooth muscle cells where they probably have a modulatory function.

Integrin-linked kinase (ILK) phosphorylates Ser^{19} and Thr^{18} of myosin RLC in chicken gizzard muscle in the absence of Ca^{2+} [33, 247]. ILK binds and co-purifies with myofilaments, suggesting a major, in addition to already known, function of this kinase in Ca^{2+}-independent myosin phosphorylation in smooth muscle. However, the mechanisms of regulation the ILK activity *in vivo* remain undefined.

Rho-kinase directly phosphorylates Ser^{19} of RLC *in vitro* [10] and, possibly, *in vivo*, causing contraction of skinned strips of rabbit portal vein in the absence of Ca^{2+} [130]. However, the role of this direct phosphorylation in Ca^{2+} sensitization of RLC phosphorylation in intact cells has been questioned [213, 225].

PAK phosphorylates endothelial myosin II *in vitro* and activates contractility and migration of endothelial cells [23, 258]. Addition of constitutively active PAK fragment to skinned smooth muscle fibers resulted in Ca^{2+}-independent contraction, but the level of myosin RLC was unchanged comparatively to that in relaxed fibers [235]. This suggests that, in contrast to endothelium, PAK does not directly activate myosin in smooth muscle and employs a rather different mechanism to stimulate contraction, for instance, the described below regulation of thin filaments through phosphorylation of actin bound caldesmon.

Myosin RLC can be also phosphorylated at Ser^{19} by MAP-kinase activated protein (MAPKAP) kinase-2 *in vitro* [125], $p34^{cdc2}$ kinase [126], and ZIP-kinase [166, 176]. These events are now considered as probably important for cytokinesis of non-muscle cells, but this is no more than a hypothesis until such phosphorylation is proven to happen *in vivo*. The role of these kinases in smooth muscle is even more obscure.

4.2. Dependence of force on the level of RLC phosphorylation. As noted above (Fig. 3), an increase in force at constant level of RLC phosphorylation is another mechanism of Ca^{2+} sensitization in smooth muscle [198]. This is achieved by altering the activity of thin filament associated proteins that affect the interaction of actin with myosin and activation of myosin ATPase [20, 84].

4.2.1. Caldesmon. Caldesmon is expressed as two isoforms with molecular masses of about 90 kD (*h*-caldesmon) and 60 kD (*l*-caldesmon) characteristic for smooth muscle and non-muscle cells, respectively [1]. Both isoforms contain sites of interaction with other contractile proteins distributed along the elongated caldesmon molecule. The functionally important actin-, tropomyosin-, and calmodulin-binding sites are located

[1] Krymsky, M. A., Khapchaev, A. Yu., Sidorova, M. A., Bespalova, Zh. D., Shirinsky, V. P., and Vorotnikov, A. V., in preparation.

in the C-terminal caldesmon domain that is responsible for inhibition of myosin ATPase activation by actin [1, 152]. The N-terminal domain contains the major myosin-binding site [139, 240], while the central region of *h*-caldesmon, absent in the non-muscle isoform, has an additional, low affinity tropomyosin binding site and may serve to separate the actin- and myosin-binding sequences.

Caldesmon is bound to actin filaments within the contractile domain of smooth muscle cells [181]. It is located alongside tropomyosin in the groove formed by the dumb-bell shaped actin monomers. The N-terminal myosin-binding site enables caldesmon to interact simultaneously and cross-link actin and myosin filaments, but this was demonstrated for *l*-caldesmon in cultured cells [58] and only suggested for *h*-caldesmon in smooth muscle [133].

Caldesmon to actin ratio is about 1 : 16 in phasic but can be lower in tonic smooth muscles [62, 151]. One caldesmon has been demonstrated to inhibit cooperatively 14-16 actin monomers *in vitro*, and it is tropomyosin that provides for the cooperativity [152]. Functioning similarly to striated muscle troponin, caldesmon keeps tropomyosin in position blocking the strong myosin binding sites on actin and thus prevents actin filament sliding over myosin in an *in vitro* motility assay [43, 204]. Upon elevation of $[Ca^{2+}]_i$, Ca^{2+}/CaM binds caldesmon and relieves block of tropomyosin allowing the cooperative binding of myosin heads and activation of their ATPase by actin [1, 152].

Phosphorylation may be an alternative mechanism to relieve the caldesmon inhibition at low $[Ca^{2+}]_i$. Caldesmon is phosphorylated by several protein kinases *in vitro* (reviewed in [1, 206]), but in smooth muscle its phosphorylation is mediated mostly by MAP-kinases [3-5, 128, 239]. Although some recent reports suggest possible involvement of other kinases [128, 235], their contribution to phosphorylation of caldesmon *in vivo* is probably minor.

Caldesmon phosphorylation increases during smooth muscle contraction induced by various agonists [4, 11, 28, 34, 55] and identical residues are modified [4]. Two of those have been identified as Ser^{759} and Ser^{789} phosphorylated by MAP-kinases in porcine carotid artery muscle [3] (Fig. 6). Inhibition of MAP-kinase activation reduced caldesmon phosphorylation and tonic contraction [34]. Phosphospecific antibody analysis showed that level of Ser^{759} is low and slightly changes upon stimulation of this tissue [30], consistent with localization of this residue in the actin-binding loop of caldesmon and its likely hindrance by actin. However, phosphorylation of Ser^{759} increased during phenylephrine-induced contraction in ferret aorta [34] and in rat aorta where it was further decreased upon forskolin-induced relaxation[1]. In contrast, the level of Ser^{789} phosphorylation was high in resting smooth muscle and did not change upon stimulation [30]. These observations of limited site specific reactivity are contradictory to the 2-3-fold increase in total caldesmon phosphorylation level induced by agonists, and the presence of additional *in vivo* phosphorylation site(s) in caldesmon could be suggested. Indeed, several tryptic phosphopeptides were found in caldesmon isolated from chicken smooth muscles [128, 239], even though avian caldesmon does not contain an equivalent of Ser^{789}. *In vitro*, MAP-kinase phosphorylated avian caldesmon at serine and threonine [23a, 206] that have been identified as Ser^{702} and Thr^{673} equivalent to mammalian Ser^{759} and Thr^{730}, respectively[2] (Fig. 6). Finally, phosphorylation by MAP-kinase has been demonstrated for Thr^{673} in avian caldesmon *in vivo* and the equivalent Thr^{730} in mammalian caldesmon *in vitro*, indicating that it is an additional, earlier missed, site of caldesmon phosphorylation *in vivo*[2]. It is possible that phosphorylation of this particular threonine located in the functional region of caldesmon is highly dynamic and is responsible for regulation of caldesmon activity in contracting muscle. Indirect support for this possibility is that this site is preferentially phosphorylated in *l*-caldesmon by $p34^{cdc2}$ kinase, another member of the proline-directed kinase family, and this results in reduction of *l*-caldesmon affinity to actin and dissociation from cytoskeleton during mitosis of non-muscle cells [148, 256].

Phosphorylation of avian caldesmon by MAP-kinase or point mutation of Ser^{702} (equivalent to Ser^{759} in mammals) to mimic the phosphorylation, led to reduction in caldesmon inhibition of actin-activated myosin ATPase [189] and actin filament sliding over myosin in *in vitro* motility assay [55, 128]. However, it has been noted that the effect of phosphorylation is manifested at high caldesmon/actin ratios and almost undetectable at their physiological ratio and in the presence of tropomyosin [55, 128]. This suggests that phosphorylation by MAP-kinase affects direct caldesmon interaction with actin [58], rather than tropomyosin-dependent regulation of thin filaments by caldesmon. Concordantly, an increase in caldesmon phosphorylation following an addition of exogenous MAP-kinase did not affect the contraction of skinned rabbit smooth muscle [180]. On the other hand, addition of MAP-kinase to skinned canine smooth muscle stimulated contraction [54]. Because no detailed analysis of phosphorylation stoichiometry and that of Thr^{730} was performed, the role of caldesmon phosphorylation by MAP-kinases in smooth muscle contraction remains undefined.

The data exist that a protein kinase distinct from MAP-kinase may phosphorylate caldesmon *in vivo*. The minor impact of PKC and involvement of novel caldesmon-kinase have been observed in phasic smooth muscle [128]. Addition of p21-activated protein kinase

[2] Krymsky, M. A., Shirinsky, V. P., and Vorotnikov, A. V., in preparation.

(PAK) to skinned *taenia coli* smooth muscle caused Ca^{2+}-independent contraction correlated with phosphorylation of caldesmon [235]. *In vitro*, PAK phosphorylates avian caldesmon at Ser^{657} and Ser^{687} located within actin- and Ca^{2+}/CaM-binding sequences (Fig. 6a) and reduces Ca^{2+}/CaM binding and inhibition of actin–tropomyosin-activated myosin ATPase by caldesmon at its physiological ratio to actin [40]. Thus an important objective for future studies is to reveal whether phosphorylation of Ser^{657} and Ser^{687} takes place in intact tissue.

Analysis of the distribution of phosphorylation sites in the C-terminal domain of caldesmon reveals their interesting regularity (Fig. 6a). The distance between MAP-kinase phosphorylated sites (mammalian Ser^{789}, Ser^{759}, and Thr^{730}) and PAK phosphorylated sites (Ser^{714} and Ser^{744} in mammals) is 29-30 amino acids. The distance between all phosphorylation sites, excluding $Ser^{773/774}$, whose phosphorylation has not been demonstrated, is 14-15 residues. This reflects similar periodicity in binding sequences for Ca^{2+}/CaM [82, 152, 156a] and actin [42, 152], suggesting a possible role of phosphorylation in modulation of these interactions.

A model of multiple sited interaction of caldesmon with actin has been developed [152], reporting that loss of one of the contacts B, B′, or C (see Fig. 6b) would hardly affect the affinity, maximum binding, and inhibition of actin [42]. Experiments with caldesmon deletion mutants showed that most essential is the central site B while an involvement of any other binding site is required for strong actin binding and tropomyosin-dependent inhibition of actomyosin ATPase [152]. Thus, phosphorylation of one or more residues may affect the affinity of an elementary contact leading to structural changes in the protein complex without significant dissociation. Changes in the spectrum of phosphorylation sites due to involvement of different kinases may result in fine regulation of the complex topography. In this line, the maximum phosphorylation of only the MAP-kinase or PAK sites in caldesmon decreases, but does not completely prevent the actin binding [23a, 128, 235]. On the contrary, phosphorylation of avian caldesmon by a constitutively active PKC fragment at multiple sites located within or in the vicinity of binding sequences and equivalent to mammalian Ser^{656}, Ser^{714}, Ser^{743}, and Ser^{783} (see Fig. 6a), abates actin–tropomyosin binding and the caldesmon inhibitory activity *in vitro* [238]. It can be hypothesized that regulation of caldesmon activity *in vivo* is achieved by mosaic phosphorylation through combination of protein kinases and small changes in phosphorylation stoichiometry of a particular site [206]. Inasmuch as MAP-kinases are the primary caldesmon-kinases *in vivo*, the respective caldesmon phosphorylation sites may serve as template and determine the effect of subsequent phosphorylation by other kinases.

How does phosphorylation by MAP-kinases decrease the affinity of caldesmon to actin at the molecular level? Spatial changes in conformation of complexes between actin and caldesmon synthetic peptide LW30 or isolated domain 4b that include two C-terminal binding sites B and B′ and previously assigned residues [51] have been assessed by NMR spectroscopy [153] (Fig. 6b). The $Ser^{702/759}$ of caldesmon has been located on the side of a polypeptide loop flanked by the B and B′ sequences. The glycine-containing turn segment at the top of the loop (shown in green in Fig. 6b) constrains relative disposition of the binding sites enabling their effective interaction with actin in solution. Phosphorylation of $Ser^{702/759}$ by MAP-kinase caused the loop to unfold and the binding sites to segregate, leading to loss of the dual sited interaction. As a result, the interaction of domain 4b with actin was reduced and the binding of LW30 was completely blocked [153] (Fig. 6b). It may be expected that concurrent phosphorylation of two or more residues by MAP-kinase and/or PAK would affect actin binding and the inhibitory activity of caldesmon to a greater extent.

4.2.2. Calponin. Calponin is another actin- and calmodulin-binding protein that is relatively specific for smooth muscles. It is suggested to be also involved in Ca^{2+}-dependent regulation of smooth muscle contraction, and its phosphorylation by PKC is thought to contribute to Ca^{2+}-sensitization [1, 249]. Calponin was localized to actin filaments *in vivo* [17, 245] and shown to inhibit the actomyosin ATPase [82, 251], actin filament motility in an *in vitro* assay [204], and to reduce isometric force and shortening in skinned muscle fibers [182]. PKC and CaMK II phosphorylate Ser^{175} of calponin *in vitro* and phosphorylation of Ser^{175} has been reported to increase in tissue upon stimulation [250]. This phosphorylation greatly decreases affinity of calponin to actin and, consequently, its ability to inhibit actomyosin ATPase [251]. Based on these results, calponin phosphorylation has been suggested as a possible mechanism of smooth muscle force modulation at constant level of myosin RLC phosphorylation [249].

However, in contrast to a remarkable similarity of calponin to caldesmon, some evidence argues against a key role of calponin in regulation of smooth muscle contraction. First, calponin is a steric rather than allosteric (as caldesmon) actin inhibitor [80]; it modulates the maximum ATPase rate but hardly affects the myosin binding to actin [82]. The full inhibition of actomyosin ATPase is achieved *in vitro* at 1 : (2-3) molar ratio of calponin to actin, respectively, and it is not potentiated by tropomyosin [82, 251]. However, the stoichiometry of calponin to actin is as low as from 1 : 14 to 1 : 7 in smooth muscle [249]. Second, Ca^{2+}/CaM is ineffective in alleviation of this inhibition *in vitro*, while the reports on agonist-induced changes of calponin phosphorylation in various smooth muscles are rather controversial [84, 249]. Third, calponin was localized to cytoskeletal and not to contractile compartments of cells [181]. Finally, knockout of the calponin gene neither resulted in significant

changes of isometric force, nor altered the Ca^{2+} sensitivity of phasic smooth muscle contraction [155].

It is nonetheless possible that in tonic smooth muscle calponin mediates signal transduction from plasmalemma to the contractile proteins. Calponin redistributed from cytosol to membrane fraction following agonist-, but not Ca^{2+}-dependent stimulation of cells [183]. Temporarily, this redistribution coincided with translocation to the membrane of Ca^{2+}-independent PKC isoform and ERK-MAP-kinases, and was prevented by PKC inhibitors [158, 183]. Furthermore, calponin was shown to interact with MAP-kinases [135] and with regulatory domain of PKC, facilitating the autophosphorylation of PKC [136]. This led to the hypothesis that calponin participates in PKC-mediated perimembrane activation of MAP-kinases, that are translocated to contractile apparatus at the second phase of agonist-induced contraction, bind actin, and colocalize with caldesmon [114]. Calponin translocation to non-muscle β-actin in stimulated cells was also observed, consistent with calponin localization in cytoskeletal compartment [181] and in the vicinity to the dense bodies, where it may associate with desmin in a Ca^{2+}/CaM-dependent manner and possibly integrate into intermediate filaments [144, 246].

4.2.3. Small heat shock proteins. There is considerable evidence that small heat shock proteins (sHSP) mediate signal transduction from membrane receptors to contractile machinery and regulate actin filaments [53, 61, 132]. The sHSP family consists of the 15-40 kD proteins homologous to αB-crystallin of eye lens. The most studied to date is human sHSP27 and its mouse and chicken homolog sHSP25. sHSP27 forms large oligomers, interacts with actin, and inhibits actin polymerization. Phosphorylation is thought to dissociate sHSP27 and reduce its inhibitory activity [14, 131]. *In vivo*, sHSP27 is phosphorylated and activated in response to stress stimuli, including heat and oxidative stresses, and upon activation of receptors to inflammatory cytokines. Thus, sHSP27 was suggested to play major role in cytoskeleton protection and non-muscle cell tolerance to the stress stimuli. In addition, current evidence indicates that sHSP27 is involved in regulation of cell contractile activity and mediates the responses to mechanical stimuli. A high level of sHSP27 expression is detected in smooth muscle [159] and phosphorylation of sHSP27 was implicated in focal contact stabilization [199] and smooth muscle cell migration [72]. Moreover, blocking the sHSP27 phosphorylation or introduction of anti-sHSP27 antibody diminished smooth muscle cell contractile responses to certain agonists [18, 157, 257].

The signaling mechanisms leading to sHSP27 phosphorylation in cells require involvement of p38 stress-activated MAP-kinases [61, 132]. It is suggested that activation of p38 MAP-kinase pathway is mediated by phosphatidylinositol-3-kinase (PI3K) and PAK downstream of Rho [53]. The p38-activated MAPKAP-kinase 2 phosphorylates several sites in sHSP27 and affects its ability to bind actin *in vitro* [21], the effect that can be reverted by phosphorylation with PKG [21]. This pattern of phosphorylation implicated in regulation of sHSP27 activity is somewhat reminiscent of a suggested involvement of MAP-kinases in tonic contraction and PKG in relaxation of smooth muscle contraction.

Indeed, the nitric oxide and forskolin induced relaxations occur without gross changes in the level of RLC phosphorylation, but follow the PKA/PKG activation and phosphorylation of sHSP20, the other sHPS family member abundant in smooth muscle [12, 191]. A similar dissociation between force and RLC phosphorylation has been observed in intact smooth muscle treated with okadaic acid, an inhibitor of endogenous protein phosphatase [228]. This further suggests a role for phosphorylation of sHSP20 and other thin filament associated proteins in blocking the interaction between actin and phosphorylated myosin in contracting smooth muscle [193]. Comparative analysis of the smooth muscles capable and incapable of the forskolin-induced relaxation allowed to dissect a specific, MLCK-independent role of sHSP20 phosphorylation [252] at Ser^{16} [12]. A sequence homology to the inhibitory region of troponin I has been found in sHSP20, and the corresponding synthetic peptide of sHSP20 inhibited actomyosin ATPase *in vitro* and contraction of skinned muscle strips [191]. The phosphorylation at Ser^{16} has been hypothesized to enhance interaction of sHSP20 with actin and to block binding of actin to myosin *in vivo* [191].

An intriguing question is how activity of different sHSP proteins is coordinated in smooth muscle? An interaction of sHSP27 with sHSP20 has been detected by the yeast two hybrid system, whereas the mutant of sHSP27 mimicking its triple phosphorylation bound to sHSP22, the other member of this protein family [15]. sHSP22 also appeared to be a phosphoprotein *in vivo*, and its phosphorylation by casein kinase CK2, PKC, and ERK-MAP-kinase, but not MAPKAP-kinase 2 has been observed *in vitro* [15]. It is likely that site-specific phosphorylation of sHSP20, sHSP22, and sHSP27 may determine the mode of their mutual interactions and binding to contractile proteins. Clearly, if this mechanism takes place *in vivo*, an activation of specific signaling cascades by different agonists would result in differential activation of sHSPs, their binding to actin, and diverse contractile responses. As a model, sHSP22 may exert a switch function, providing reduction in sHSP20 phosphorylation at Ser^{16} when p38 MAP-kinase mediated phosphorylation of sHSP27 is stimulated [124], and this would support the contraction [18, 257]. In turn, phosphorylation of sHSP22 following activation of other agonist-stimulated cascades may affect the distributing function and dissociate the effects of sHSP27 and sHSP20, permitting the sHSP20-mediated relaxation [12, 191]. Thus, an upcoming appearance of novel and exciting information con-

cerning sHPS function in regulation of smooth muscle contractility can be anticipated.

Thus, binding of agonists to smooth muscle cell surface receptors switches on and activates diverse signaling pathways that culminate in modulation of sensitivity of either contractile proteins, or the contractile response itself to $[Ca^{2+}]_i$. Phosphorylation of the contractile and associated regulatory proteins plays a critical role both in the tonic contraction and in relaxation. Despite some examples of a sustained contraction dependent only on myosin phosphorylation level, these contractions appear to be energetically unfavorable and most probably exceptional. In contrast, the mechanisms of Ca^{2+}-sensitization determine the ability of smooth muscles, such as of blood vessels, to long maintain the contraction at low energy costs.

Although we presently lack exact knowledge of to what extent the described mechanisms are manifested during an integral contractile response of smooth muscle, attempts are already in progress to apply the available information for a search of new therapeutics. A design of inhibitors of protein kinases involved in regulation of smooth muscle Ca^{2+} sensitivity is one of the approaches contemplated now in practice. Most of the compounds that are being produced and screened interact with the ATP-binding pocket of protein kinases. For instance, compound HA1077 (Eril) effectively inhibits Rho-dependent kinases rather than PKC, PKA, and MLCK, and exerts vasodilating and antispastic effects in both animal models and clinical trials [77]. Another Rho-kinase inhibitor, Y-27632, is reported to have an antihypertensive activity [234]. Novel MLCK inhibitors are being developed that are more selective than existing ML-7 and ML-9 and they also show the smooth muscle relaxing activity *in vivo*.

The clinical use of the MAP-kinase inhibitors has an uncertain perspective since these protein kinases phosphorylate multiple substrates in smooth muscle as well as in non-muscle cells, and their inhibition may considerably affect the homeostasis. An inability to alter specifically the phosphorylation of particular protein targets of the multi-substrate kinases such as MAP-kinases, PKC, PAK, and CaMK II by means of inhibiting their enzyme activity would likely urge the development of some substrate decoys. These might be peptides containing the functionally important phosphorylation sites of smooth muscle regulatory proteins. By competing with substrates for protein kinases the substrate mimetics would reduce site-specific phosphorylation of certain proteins and, therefore, block the phosphorylation effects on contractile response. Undoubtedly, achievement of this objective will require novel advances such as the specific delivery of drugs to smooth muscle cells and their protection from intracellular degradation.

Finally, the extensive studies of MLCP and other phosphatases of regulatory proteins may succeed in discovery of new regulators for these enzymes that become valuable tools to control the phosphorylation level of key regulatory proteins of the smooth muscle contractile machinery.

Thus, a research priority in the field of pharmacological correction of circulatory and visceral smooth muscle contraction is the design of compounds efficient in modulating activity of particular regulatory proteins in order to replace drugs with a broad specificity, for example, the Ca^{2+} antagonists. Taking into account the complexity of the smooth muscle regulatory cascades, the practical fruitlessness of an empirical approach, i.e., nonspecific drug factoring and their screening in physiological models, is rather apparent. Instead, drug discovery is to be based on the knowledge of molecular mechanisms that regulate the smooth muscle contraction.

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