Regulation of Smooth Muscle Contraction by Small GTPases

Next to changes in cytosolic $[\text{Ca}^{2+}]$, members of the Rho subfamily of small GTPases, in particular Rho and its effector Rho kinase, also known as ROCK or ROCK, emerged as key regulators of smooth muscle function in health and disease. In this review, we will focus on the regulation of the contractile machinery by Rho/ROK signaling and its interaction with PKC and cyclic nucleotide signaling. We will briefly discuss the emerging evidence that remodeling of cortical actin is necessary for contraction.

The small GTPases of the Rho family of monomeric GTPases, Rho, Rac, and Cdc42, emerged as master regulators of actin cytoskeleton remodeling and actomyosin-based contractility of smooth and non-muscle cells. Smooth muscle cells are major constituents of the walls of blood vessels and visceral organs (with the exception of the heart) and are responsible for dynam-ic changes in the diameter and volume of these hollow organs. Being a major player in the regulation of smooth muscle tone, Rho in conjunction with its effec-tor Rho kinase, also known as ROCK or ROCK, is essen-tial for such diverse organ functions like regulation of arterial and pulmonary blood pressure, local organ blood flow, airway resistance, and passage of food through the gut to name a few. By regulating the con-tractile state of endothelial and epithelial cells lining the walls of blood vessels and visceral organs, Rho family proteins are also important determinants of their barrier function, which regulates the controlled transport of fluid and immune cells across the wall of these hollow organs.

Many of these functions involve not only myosin II-based contractility but also the dynamic rearrange-ment of the actin cytoskeleton as well as regulation of gene expression. To fulfill these diverse functions, often several members of the Rho family with their specific regulatory proteins have to cooperate. In this scenario, individual members can be considered as nodal points in complex signaling networks that sort incoming environmental cues, distributing them to a number of specific regulatory proteins have to cooperate. In this scenario, individual members can be considered as nodal points in complex signaling networks that sort incoming environmental cues, distributing them to a subset of distinct intracellular signaling cascades to allow for the appropriate specific cellular answers. Not surprisingly, dysregulation of Rho signaling has been observed in such diverse pathologies like systemic and pulmonary hypertension, asthma, pulmonary fibrosis, and vasospasms following sub-arachnoid hemorrhage.

The focus of this review will be on the regulation of smooth muscle tone by Rho and Rho kinase/ROK in the following called ROK. We will touch the other GTPases when relevant within this context. As regards targets of Rho and ROK, we will concentrate on those directed toward the contractile machinery regulating its activity. The general aspects of signaling by Rho family GTPases has been addressed by several excellent reviews (e.g., Refs. 25, 26, 46, 103, 158, 193, 224, 230). For in-depth coverage of signaling in smooth muscle, the reader is referred to more comprehensive reviews (e.g., Refs. 5, 66, 78, 86, 116, 159, 208, 216, 247). A note on nomenclature used in this review, the term “Rho family proteins” will be used when we refer to Rho, Rac, and Cdc42 in general, whereas “Rho” or “Rho kinase” is reserved for RhoA proper.

### General Principles of Regulation of Smooth Muscle Tone

#### Ca$^{2+}$ Sensitization and Ca$^{2+}$ Desensitization

A rise in intracellular $[\text{Ca}^{2+}]_i$ (12), which triggers phos-phorylation of the regulatory light chains of myosin (MLC$_{20}$) at Ser19 mediated by the Ca$^{2+}$/calmodulin (Ca$^{2+}$/CaM) activated myosin light chain kinase (MLCK) (73, 94, 104), is thought to be the key event in activating smooth muscle contraction in response to a wide spectrum of extracellular signals (216). Relaxation is induced by dephosphorylation of MLC$_{20}$ by a specific type 1 phosphatase, MLCP. MLCP is a heterotrimer consisting of a catalytic subunit, a 20-kDa subunit of myosin phosphatase to center stage as regulators of smooth muscle function. First, there was the pivotal observation of A. P. Ras and A. V. Somlyo and coworkers that G proteins increase Ca$^{2+}$ sensitivity of contraction and that this is due to inhibition of MLCP (112, 215). Inhibition of MLCP was later ascribed to phosphorylation of MYPT1 (90, 178, 232). Second, the field was spurred by the seminal observation of Hirata and coworkers (79) that G-protein-induced Ca$^{2+}$ sensitization is due to inhibition of Rho mediating the inhibition of MLCP (164). Subsequent milestones were the demonstration that, like in not a single study, the GTPases Rho, Rac, and Cdc42 were shown to play multifaceted roles in the regulation of smooth muscle contraction. For an overview of the GTPases Rho, Rac, and Cdc42 in smooth muscle, the reader is referred to more comprehensive reviews (e.g., Refs. 25, 26, 46, 103, 158, 193). All three Rho family proteins are involved in regulation of the actin cytoskeleton, but the mechanisms underlying their role in the control of smooth muscle contraction are less well understood. In contrast, 140s of Ca$^{2+}$ signaling and Ca$^{2+}$ desensitization, the reader is referred to more comprehensive reviews (e.g., Refs. 25, 26, 46, 103, 158, 193). Still, here we will focus on the regulation of MLCP by Rho-ROK and its relationship with PKC and cyclic nucleotide signaling. We will briefly discuss the emerging evidence that remodeling of cortical actin is necessary for contraction.

#### Ca$^{2+}$ Desensitization

G-protein-induced Ca$^{2+}$ sensitization is due to activa-tion of the MLCP, which regulates the activity of the actin cytoskeleton as well as regulation of gene expression. To fulfill these diverse functions, often several members of the Rho family with their specific regulatory proteins have to cooperate. In this scenario, individual members can be considered as nodal points in complex signaling networks that sort incoming environmental cues, distributing them to a subset of distinct intracellular signaling cascades to allow for the appropriate specific cellular answers. Not surprisingly, dysregulation of Rho signaling has been observed in such diverse pathologies like systemic and pulmonary hypertension, asthma, pulmonary fibrosis, and vasospasms following sub-arachnoid hemorrhage. The focus of this review will be on the regulation of smooth muscle tone by Rho and Rho kinase/ROK in the following called ROK. We will touch the other GTPases when relevant within this context. As regards targets of Rho and ROK, we will concentrate on those directed toward the contractile machinery regulating its activity. The general aspects of signaling by Rho family GTPases has been addressed by several excellent reviews (e.g., Refs. 25, 26, 46, 103, 158, 193, 224, 230). For in-depth coverage of signaling in smooth muscle, the reader is referred to more comprehensive reviews (e.g., Refs. 5, 66, 78, 86, 116, 159, 208, 216, 247). A note on nomenclature used in this review, the term “Rho family proteins” will be used when we refer to Rho, Rac, and Cdc42 in general, whereas “Rho” or “Rho kinase” is reserved for RhoA proper.

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Identification of Rho Proteins as Key Regulators of Smooth Muscle Contraction

The involvement of Rho in smooth muscle contraction has been unequivocally demonstrated by the following experiments: First, the bacterial toxins, EDIN, function because it has been shown that h-Ras increases Ca\textsuperscript{2+} sensitivity in permeabilized arteries (202) and regulates the expression of smooth muscle myosin light chain kinase (70).

Based on their effects on the actin organization, Rho family proteins are divided into five groups: the Rho-, Cdc42-, Rac-, Rnd-, and RhottiB subfamilies (6, 25). Rho-, Rac-, and Cdc42-like proteins assume specific functions in the regulation of actin filament remodeling, such as formation of stress fibers, lamellipodia, and filopodial protrusions (25, 46, 103, 158, 162, 184). Rnd proteins are endogenous negative regulators of Rho in smooth muscle (29, 129). They are unique because they exist in a constitutively active GTP-bound form (53, 103). Their expression level is high in the pregnant uterus but declines dramatically at the time of delivery, suggesting that Rnd proteins contribute to silencing of the uterus during pregnancy (27).

Members of Small GTPases Involved in Smooth Muscle Contraction

The Rho (rat sarcoma) superfamily of monomeric GTPases consists of more than 100 members (21, 224, 244), which are master regulators of many aspects of cell behavior (46). Based on their primary sequence and function, they fall into six subfamilies: Ras, Ran, Rad, Rab, Arf/Sub1, and finally Rho (244). Rho family proteins emerged as key regulators of a variety of effects associated with changes in actin cytoskeleton, such as cell migration, adhesion, and contraction (Refs. 184, 186; reviewed in Refs. 25, 103). Still, h-Ras may play a role in smooth muscle that, like in non-muscle cells (184, 185), Rho is activated by contractile agonists that signal through heterotrimeric G proteins (56, 62, 69, 95, 115, 171), 2) the demonstration that ROK is responsible for the inhibitory phosphorylation of MYPT1 (50, 110). It was the availability of rather specific ROK inhibitors (223, 233) that in the years to follow overwhelmingly demonstrated the importance of Rho/ROK signaling in the regulation of contraction of every single smooth muscle under physiological and pathological conditions.

Taken together, these findings provided a mechanistic explanation for the observation made already in the mid 1980s by Morgan and coworkers (147) who found that, in the presence of phorbol ester, [Ca\textsuperscript{2+}]i more compre- sively, whereby the thin filament-myosin light chain kinase (MLCK) is the key event in the response to a contractile agonist (2). Relaxation can be ascribed to an increased sensitivity of force for Ca\textsuperscript{2+} phosphorylation, carbachol treatment produced much smaller increases in Ca\textsuperscript{2+} and MLCK activation, implicating inhibition of MLCP.

This overview of myosin-linked regulation of smooth muscle contraction would be incomplete without mentioning that actomyosin interaction is much smaller increases in Ca\textsuperscript{2+} and MLCK activation, implicating inhibition of MLCP.

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The tissue, and perhaps also on the species indicating that Rho proteins are both necessary and sufficient to increase Ca\(^{2+}\) sensitivity under these experimental conditions. However, we also note that whereas the increase in F-actin during stimulation of smooth muscle by recombiant RhoA-augmented agonist induced Ca\(^{2+}\) sensitization and delayed its rundown (171). Third, in intact smooth muscle, membrane-permeable toxins inhibited the tonic but not the initial phase of contractions elicited by muscarinic and adrenergic agonists, demonstrating that it is the late phase of a contraction that is Rho dependent (55, 134, 171). These findings indicate that Rho proteins are both necessary and sufficient to increase Ca\(^{2+}\) sensitivity under these experimental conditions. However, we also note that whether and to what extent Ca\(^{2+}\) sensitization is inhibited by the bacterial toxins depends on the agonist, on the tissue, and perhaps also on the species indicating that other pathways can lead to Ca\(^{2+}\) sensitization as well (e.g., Refs. 62, 200, 226; reviewed in Ref. 176).

Interestingly, Rho/ROK not only regulates Ca\(^{2+}\) sensitivity but also expression of smooth muscle differentiation genes that define the contractile phenotype of smooth muscle (172, 239). These genes include smooth muscle \(\alpha\)-actin, smooth muscle myosin heavy chain, and calponin, i.e., proteins of the contractile machinery. In the so-called synthetic phenotype, which predominates during development and after injury expression of these genes is downregulated (172, 254). Transcription of the contractile proteins is activated by serum response factor (SRF) and is dependent on RhoA, whereby the effect of RhoA may be secondary to Rho-mediated increase in actin polymerization (76, 136, 238). In keeping with these ideas, maintenance of smooth muscle phenotype of blood vessels in organ culture depends on activation of Rho and actin polymerization and appears to critically require mechanical loading by stretching them (2). It is therefore tempting to speculate that Rho is a nodal point that orchestrates downstream effectors that regulate the short-term mechanical output of smooth muscle coincident with the long-term maintenance of their contractile phenotype (239).

### Rac and Cdc42:
**Functional Antagonism to Rho and the Emerging Importance of Actin Cytoskeleton Dynamics for Smooth Muscle Contraction**

The function of Rac and Cdc42 in differentiated smooth muscle is less well understood. Under certain conditions, Rac is antagonistic to Rho (Refs. 190, 197; reviewed in Ref. 25). Rac is part of the NADPH oxidases NOX1 and NOX2, which play important roles in the development of vascular smooth muscle hypertrophy and migration (85, 135) as well as in endothelial barrier function (14, 113). The Rac effector PAK3 has been suggested to induce a Ca\(^{2+}\)- and MLCK phosphorylation-independent contraction by phosphorlylating and disassembling the thin-filament-linked protein caldesmon (235). In contrast, others found that PAK inhibits Ca\(^{2+}\)-dependent activation of MLCK, thereby inhibiting contraction (152, 250).

A novel interesting but still poorly understood concept suggests that the actin-cytoskeleton is remodeled during agonist-induced contraction and that this remodeling process is important for tension generation in addition to the canonical actomyosin interaction (86, 109). Actin exists as monomers (G-actin) and filaments (F-actin). Coincident with activation of contraction, the proportion of F-actin at the cell cortex increases. This increased polymerization is orchestrated by Cdc42, which recruits WASp family proteins to the cell membrane resulting in activation of the Arp2/3 complex (227, 259). Actin polymerization is also promoted by ROK-mediated activation of LIM kinase, leading to inactivation of the actin-severing protein cofilin (130) (FIGURE 2B).

Of note, the increase in F-actin during stimulation of smooth muscle tissue is small (from ~80 to ~90%), and it is not clear at present how such a small increase is important for tension generation (109). One intriguing model suggests that the increase in F-actin at the cell cortex provides a rigid structure for force transmission to the extracellular matrix and between smooth muscle cells (66). Tight coupling between smooth muscle cells is obviously necessary for effectively altering the diameter of hollow organs.

### Regulation of the Activity of Rho, GEFS, GAPs, and GDIs

Like all small GTPases, Rho proteins are molecular switches that cycle between a GDP-bound “off” and a GTP-bound “on” state (23). The “on” fraction of Rho depends on the concerted action of three classes of regulators (FIGURE 2A), namely guanine nucleotide exchange factors (GEFs) (21, 132, 191, 207), GTPase activating proteins (GAPs) (12, 21, 145, 206), and GDP dissociation inhibitors (GDIs) (37). These proteins are tightly regulated by protein-protein and protein-lipid interaction, posttranslational modifications (PTMs) and other regulating mechanisms (1). For example, these regulators are phosphorylated on several residues, which may lead to allosteric changes that affect the activity of Rho proteins (14, 113). The function of Rac and Cdc42 is reviewed in Ref. 25. LARG (57, 222), Cdc42, and Rac are involved in the regulation of the cytoskeleton and appear to critically require mechanical loading by stretching them (2). It is therefore tempting to speculate that Rho is a nodal point that orchestrates downstream effectors that regulate the short-term mechanical output of smooth muscle coincident with the long-term maintenance of their contractile phenotype (239)
interaction, posttranslational modification, and second messengers (11, 21). We note that information about these regulators of the exchange/hydrolysis cycle in differentiated smooth muscle is still very limited.

GEFs increase the intrinsically low dissociation rate of GDP (half time > 1 h; reviewed in Ref. 21) by several orders of magnitude. This allows the more abundant GTP to bind, which in turn lowers the affinity of Rho for GEFs (21, 45, 191). Five Rho-GEFs, namely LARG (57, 222, 249, 257), PDZ-RhoGEF (36, 75), p115 RhoGEF (13, 83, 118, 243), Vsm-RhoGEF (167), and SmgGDS (231), have hitherto been found in smooth muscle compared with more than 70 Rho-GEFs encoded in the human genome (21, 132, 191). Rho-GEFs are multidomain proteins that typically contain a catalytic DH homology (DH) domain and an adjacent PH domain that directs GEFs to membrane surfaces (28, 45, 82, 132, 191). Outside of this DH-PH tandem, GEFs are quite variable and contain additional distinct sequence motifs important for specific functions (191). Thus LARG, PDZ-RhoGEF, and p115 RhoGEF contain a regulator of G-protein

![FIGURE 2. Regulation of Rho and downstream targets at the contractile machinery](http://physiologyonline.physiology.org/)
...it is important to keep in mind that high K+ can induce spurious release of neurotransmitters from nerve terminals within the smooth muscle preparation.

and p73RhoGAP (222), whereby p190RhoGAP appears to be the most important one (23, 149). Inhibition of GAP activity is sufficient to induce activation of Rho in the absence of extracellular stimuli, demonstrating that GAPs are necessary to maintain Rho/ROK activity low under basal conditions (11, 23). Furthermore, it was reported recently that angiotensin II (23) and endothelin (149) can increase GTP-loaded Rho by decreasing p190RhoGAP activity.

Some GAPs are bifunctional because they contain a GEF domain for another GTPase. In this way, one GTPase can be turned on to facilitate the activation of another GTPase. In this way, the RhoGEF domain of p140RhoGEF (58) has been shown to be activated by PKC in endothelial cells (141). We note that most agonists activate PKC via a RhoGEF domain for another GTPase. In this way, one GTPase can be turned on to facilitate the activation of another GTPase. For example, PKC activation by low but not by high acetylcholine concentrations in coronary smooth muscle cells: Graf (228), p190RhoGAP (23, 149).

Since activation of G-protein-coupled receptors (GPCRs) by extracellular stimuli can lead to activation of RhoA, the GTPase that controls the monomeric GTPase exchange factor (GEF) and the GTPase activating protein (GAP) for RhoA are also activated. This leads to activation of PKC and to the formation of a complex between PKC and RhoA, which results in increased activity of RhoA. PKC activation by low but not by high acetylcholine concentrations in coronary smooth muscle cells: Graf (228), p190RhoGAP (23, 149).
Since activation of Rho by high K⁺ is absolutely dependent on Ca²⁺ (196), it may be the KCl-induced increase in cytosolic [Ca²⁺] rather than the membrane depolarization per se that activates Rho. Possible mechanisms of Ca²⁺-induced activation of Rho include class II PK kinases (240, 258). Alternatively, Ca²⁺ may activate sphingosine kinase leading to release of sphingosine-1-phosphate and parasite activation of S1P receptors (242).

**Coupling of heterotrimeric G proteins of the Gαq and Gα12 families to Rho activation**

Activation of Rho has been demonstrated in different types of smooth muscle in response to a large number of contractile agonists (125, 127, 195, 216). With few exceptions, most of these agonists activate through their specific G-protein-coupled receptors (GPCRs) more than one G protein of the Gαq, Gα12, or Gα13 family (FIGURE 2B) (60, 188). Examples include the endothelin ET₂, the α₁-adrenoceptor, the muscarinic M₁ and M₃, the 5-HT₂C, the angiotensin AT₁, lysophosphatidic acid LPA, and the sphingosine-1-phosphate SIP₁ and SIP₂ receptors, which activate both Gα₁₁q, and Gα₁₂/₁₃ (see tables in Refs. 139, 150, 246). Hence, on the one hand, a limited number of G proteins integrates the information from a large number of environmental stimuli and distributes it to distinct intracellular signaling cascades. On the other hand, a high level of complexity exists. This is because there is extensive crosstalk between these signaling cascades including feed-forward and feedback regulation.

Of the different pathways that mediate GPCR-induced activation of Rho, signaling through Gα₁₁q is best understood. Thus activated Gα₁₁q binds the RGS-like domain containing Rho-GEFs, PDZ-RhoGEF, LARG, and p115 RhoGEF (for reviews, see Refs. 188, 193, 216), thereby positively regulating their Rho-GEF activity, resulting in activation of Rho (Refs. 57, 71; reviewed in Ref. 58). At the same time, these GEFs switch off the upstream signal. This is because their RGS-like domain acts as a GAP for Gα₁₁q (Ref. 118; for review, see Ref. 58). It was proposed that this mechanism limits activation of Rho to rapid and robust activation of Gα₁₁q, which is thought to increase the signal-to-noise ratio (191). As will be detailed below, these GEFs can also be activated by tyrosine phosphorylation (e.g., Ref. 31).

Although it was shown that expression of constitutive active Gα₁₁q, contracts VSMC in a Rho-dependent manner (60), mice deficient for Gα₁₁q or LARG had a normal basal blood pressure (249). Interestingly, development of salt-sensitive hypertension was abrogated in both the Gα₁₁q and LARG-deficient mice. This suggests that Rho signaling is required for the development of hypertension (249).

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development of hypertension but not for the regulation of blood pressure under basal conditions, a conclusion supported by earlier experiments with ROCK inhibitors (233). This finding may not be surprising because the contractile response to stimulation with the α-adrenergic agonist phenylephrine was not affected in aortas isolated from the knockout mice. In contrast, the contractile response to endothelin, U46619, and angiotensin II was attenuated, albeit to different extents (248). This is consistent with earlier observations that these agonists differ in their ability to increase the amount of GTP-bound Rho (195) and to induce Ca²⁺ sensitization (77).

Since the contractile response to phenylephrine and serotonin was completely abrogated in Gq-deficient fibroblasts (257). Second, interventions that interfered with Gq signaling blocked ROCK activation (81, 87, 217, 280). Little is known in smooth muscle about the mechanisms that functionally link Gq to Rho activation. In model cell systems, it may occur through specific Rho-GEFs like LARG (26, 32, 237) and/or tyrosine phosphorylation (51, 107). However, it is not clear whether Gq is sufficient to induce Rho activation (32, 45, 107, 192) or whether signal transmission requires additional factors (9, 225). Since in smooth muscle activation of Gq leads to an IP₃-mediated increase in intracellular [Ca²⁺], it is possible that membrane depolarization and activation of Gq converge on Ca²⁺ with subsequent activation of Rho through the above-described mechanisms.

FIGURE 4
Putative mechanisms by which cyclic nucleotides antagonize Rho activation

The canonical pathway of cAMP leads to activation of PKA, which, like PKG, can phosphorylate GDI as well as Rho. Phosphorylation of Rho and/or GDI enhances the affinity of Rho for GDI-GTP and sequesters Rho-GTP into the cytosol. In addition, PKG can block Rho activation through a non-phosphorylation-based mechanism (195) and/or tyrosine phosphorylation (51, 107). However, it is not clear whether Gq is sufficient to induce Rho activation (32, 45, 107, 192) or whether signal transmission requires additional factors (9, 225). Since in smooth muscle activation of Gq leads to an IP₃-mediated increase in intracellular [Ca²⁺], it is possible that membrane depolarization and activation of Gq converge on Ca²⁺ with subsequent activation of Rho through the above-described mechanisms.
sensitivity permeabilized smooth muscle (4, 54) and is raised in response to certain contractile agonists (62, 67). Another lipid, sphingosylphosphorylcholine (SPC) has also been suggested to directly activate ROK (214) and is likely responsible for long-term vasoconstrictions of cerebral arteries following subarachnoid hemorrhage (122).

ROK phosphorylates several targets at the contractile machinery (FIGURE 2B). The most important effect of ROK is the inhibition of MLCP activity (see below), which leads to an increase in MLC20 phosphorylation (117, 118) and inactivation of MLCP (121). However, this appears to be of minor importance in smooth muscle. ROK phosphorylates calponin (105), and this may account for the MLC20 phosphorylation-independent force enhancement (137). Further targets include LIM kinase, which phosphorlates cofilin, leading to inhibition of its actin filament severing function (138). Thus the overall effect is to enhance actomyosin interaction and to prevent actin depolymerization.

Much of the current understanding of ROK as a key regulator of smooth muscle contraction is based on pharmacological tools, i.e., the ROK inhibitors Y-27632 (93, 233) and H1077, also called fasudil (154). These compounds have been shown to inhibit force in vascular (16, 156, 233), respiratory (30, 92), and ileal (178, 223) smooth muscle. They are considered to be rather specific because their Ki values of 0.14 μM (Y-27632 and 0.33 μM [H1077]) are much lower than those for PKA, PKC, and MLCK (233). Furthermore, of a large panel of kinases tested by Cohen and coworkers (7, 144), only two other kinases were inhibited by Y-27632 with similar potency as ROK. These kinases are PKR1 and 2, also called PRKNI and 2, distinct Rho-dependent kinases that phosphorylate CPI-17 (see below and Ref. 69). Since Y-27632 inhibits ROK by competing for ATP (93), it is not surprising that the IC50 value for inhibiting vascular smooth muscle contraction is higher (~1 μM) with maximal inhibition at ~10 μM (233). However, when drawing conclusions about involvement of ROK, one should be aware that, at 10 μM, Y-27632 also inhibits PKCβ in vascular smooth muscle (47, 151). Hence, even though the ROK inhibitors have a much better specificity compared with other inhibitors of serine/threonine kinases (7), firm proof of the involvement of ROK requires supplementation by, e.g., molecular analysis (174).

Myosin Phosphatase as Target of ROK

ROK inhibits MLCP primarily by phosphorylation of its regulatory subunit, MYPT1 (97) (cf. FIGURE 3). MYPT1 is phosphorylated at several serine and threonine residues by a number of kinases, including ROK and ZIP kinase, whereby Thr686 and Thr683 seem to be the major phosphorylation sites for ROK (97) (FIGURE 3). More recently, it was proposed that ROK indirectly phosphorylates Thr696 by activating ZIP kinase (68). Phosphorylation of Thr686 inhibits enzymatic activity, whereas phosphorylation of Thr683 lowers the affinity for its substrate myosin, resulting in an apparent decrease in MLCP activity (97). In smooth muscle, both sites are significantly phosphorylated already under resting conditions in a ROK-independent manner (e.g., Refs. 111, 133, 160). Although most investigators found that phosphorylation of Thr683 is ROK sensitive, the correlation between increase in force and Thr686 phosphorylation is much more variable (96, 101, 111, 133, 144, 248). MYPT1 is also phosphorylated by PKA/PKG at Ser695, which has no effect on MLCP activity (FIGURE 3) but prevents subsequent phosphorylation of Thr686, thereby possibly regulating the availability of MYPT1 for ROK-mediated inhibitory phosphorylation (155, 159, 217, 252).

Under certain conditions (e.g., Ref. 174), ROK inhibits MLCP also by phosphorylating the endogenous inhibitory peptide of MLCP, called CPI-17 (FIGURE 3). CPI-17 is predominantly expressed in vascular smooth muscle and was initially discovered as a protein kinase C (PKC) substrate (48). It is now known that CPI-17 can be phosphorylated in vitro by a number of kinases including ROK (117), PKN (69), and integrin-linked kinase (35), however, in smooth muscle, phosphorylation of PKCα appears to predominate (47).

Temporal and Spatial Aspects of Ca2+- and Rho-Signaling

As already mentioned, most contractile agonists activate both Goq and Go12/13. The canonical downstream events initiated by Goq signaling are generation of IP3 and diacylglycerol followed by release of intracellular Ca2+ and activation of PKC, respectively, whereas Go12/13 activates Rho. In this way, signaling events that lead to activation of the Ca2+/CaM-dependent MLCK and those that lead to inhibition of MLCP are activated in parallel, however, possibly with different kinetics. In the tonic vascular smooth muscle, the increase in [Ca2+] is typically rapid followed by a decline to lower but still suprabasal levels, whereas force is maintained (e.g., Refs. 41, 147, 161). In other words, force is sensitized to the activator Ca2+. The activation of MLCK follows this Ca2+ transient (94), whereas activation of Rho/ROK is thought to occur slower than the rate of tension rise and is sustained (41, 143, 233). This sustained activation of Rho, which predominantly occurs at the cell membrane where Rho meets its effectors, ROK (61, 143), is considered to be responsible for the sustained increase in MLCP phosphorylation and Ca2+ sensitization (143). The notion that activation of Rho/ROK occurs at a slow rate was deduced from the findings that...
REVIEWS

PHYSIOLOGY

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Maintenance of force rather than the initial rise in tension was inhibited by chimeric D3CB toxin (55) or Clostridium difficile toxin B (134) or by the ROK inhibitors Y-27632 and fasudil (223). Several observations challenge this view. First, ROK inhibitors inhibit rhythmic contractions of rat uterine smooth muscle induced by electrical field stimulation (212). Second, Clostridium difficile toxin B inhibited the initial rise in MLC20 phosphorylation in ideal smooth muscle, interestingly without significant inhibition of force (134). Third, direct determinations of MLCK activation in the phasic bladder smooth muscle indicated that inhibition of MLCP significantly contributes to the carbachol-induced rise in force (144).

Despite this, a large body of evidence suggests that the early phase of the increase in MLCP phosphorylation is governed by activation of MLCK, whereas activation of Rho/ROK governs the late phase. The lifetime of the "on" state of Rho, i.e., the rate of GTP hydrolysis, will determine the duration and amplification of downstream signal propagation (22). As mentioned, the intrinsic GTPase activity of Rho is significantly accelerated by GAPs. Nevertheless, little is known about the regulation of kinetics of inactivation of Rho in smooth muscle, which is of great biological importance. For instance, since Rho/ROK signaling is not only involved in the tonic contraction of blood vessels as already shown but also in the phasic contractions of urethral smooth muscle (212), it will be interesting to see whether the lifetime of GTP-loaded Rho differs between these different types of smooth muscles. Furthermore, augmented Rho signaling has been implicated in several vascular diseases such as hypertension and vasospasm of cerebral arteries (209). One possible mechanism might involve increasing the lifetime of GTP-loaded Rho by downregulation of GAP activity (149).

There is also some evidence for spatial regulation of Rho signaling. For instance, the initial, MLCK-dependant MLCP phosphorylation occurs at the central region, whereas the sustained, Rho-dependent MLCP phosphorylation occurs at the cell cortex (143). In addition, MYPT1 is translocated from the cytosol to the cell membrane during stimulation of smooth muscle (17, 159, 213); however, the functional relevance has yet to be determined.

Different Roads Lead to Ca\(^{2+}\) Sensitization: Cross Talk Between PKC and Rho/ROK Signaling

Activation of the PKC-dependent pathway through G\(_\text{q}\)α-mediated activation of phospholipase C\(_\beta\), generation of diacylglycerol (DAG), and activation of PKC increases Ca\(^{2+}\) sensitivity in a pathway parallel to the Rho/ROK signaling cascade. Both cascades converge at inhibition of MLCP, albeit typically through different targets, namely CPI-17 and MYPT1 (41, 87, 144, 160). However, the phosphorylation of these two proteins does not necessarily rise in parallel. Thus, in arterial smooth muscle, only CPI-17 is phosphorylated during the rising phase of the contraction, whereas, during the sustained phase, both proteins are phosphorylated (41). In contrast, in the phasic bladder smooth muscle, the initial phase of the carbachol-induced contraction was associated with a PKC-dependent phosphorylation of CPI-17 as well as a PKC- and/or ROK-dependant phosphorylation of MYPT1, whereas during the later phase only MYPT1 phosphorylation persisted (144). Furthermore, CPI-17 phosphorylation occurred at lower carbachol concentrations than MYPT1 phosphorylation (144).

PKC and Rho signaling cascades not only represent parallel pathways, they also interact. For instance, Rho activates PLD (49), which results in a prolonged activation of PKC (151), whereas vice versa PKC can activate Rho by a not yet defined mechanism (6, 173). Furthermore, it was suggested that, in intestinal smooth muscle, PKC indirectly phosphorylates MYPT1 at the ROK sites by activation of integrin-linked kinase ILK (91), whereas in turn, in vascular and bronchial smooth muscle, ROK can phosphorylate the PKC substrate CPI-17 (160, 174, 194).

To conclude, whether PKC and Rho/ROK cooperate or act individually to increase Ca\(^{2+}\) sensitivity depends on the type of smooth muscle (200, 226), partly because the expression level of CPI-17 differs between tonic and phasic smooth muscle (251). It also depends on the time in the course of a contraction (41, 144, 161), the applied agonist (74, 200, 260), the concentration of a given agonist (144), and for a given mode of stimulation on the species (42, 200).

PKA/PKG decrease Ca\(^{2+}\) Sensitivity Through Inhibition of Rho Signaling

It has been known for more than 20 years that cyclic nucleotides relax smooth muscle by decreasing Ca\(^{2+}\) sensitivity (146), whereby the underlying mechanisms are still not fully understood. The initial proposal that cAMP decreases MLCK activity was later questioned (116). Both cAMP and cGMP converge on MLCP to increase or rather disinhbit its activity (124, 133, 155) both in a Rho-independent manner (88, 108, 251, 252) and/or by antagonizing Rho/ROK signaling (18, 153, 159, 200). Antagonizing the Rho pathway can occur at several steps along the pathway from G\(_\text{q}\)\(_{11/13}\) to Rho itself (FIGURE 4). As described in the chapter "Regulation of the Rho activity," an attractive mechanism to inactivate Rho in vascular smooth muscle cells is the PKG-mediated phosphorylation of Rho resulting in an increased Rho-GDI complex formation and extraction of Rho from the plasma membrane (203). However, the NO-mediated inactivation of Rho in hamster microarteries was not blunted by overexpression of a non-phosphorylatable mutant of RhoA, RhoAN174Val (18).

Unfortunately, this review is limited in this section on Rho.

Recently, it has been demonstrated that CAMP mediates a downregulation of PKA by activation of AMP-activated protein kinases (AMPK). Furthermore, PKA and cGMP are part of a feedback loop to increase the activity of cGMP and, hence, decrease the level of PKA. Recently, it was demonstrated that PKA activates EPAC2, which further stimulates cGMP by activating the G-protein-regulated AMP-activated protein kinase (GAPkinase). Hence, the PKA/PKG-Rho/ROK signaling pathway may represent a feedback loop to inhibit Rho signaling.

Potential Dysfunction

Since the initial discovery of the role of Rho in blood pressure regulation (233), a large body of evidence has demonstrated a variety of physiological functions of Rho and its downstream effectors (249) as well as a wide variety of pathological processes that antagonize Rho (256). reviewed in this review to cover the large number of diseases. Here, we just mention that most of these diseases are ascribed to altered function of Rho and they involve a large number of smooth muscles, e.g., vascular, bronchial and cerebral arteries, cardiac muscle, platelets, and diabetes mellitus. Hence, they involve a large number of smooth muscles, e.g., vascular, bronchial and cerebral arteries, cardiac muscle, platelets, and diabetes mellitus. Hence, they involve a large number of smooth muscles, e.g., vascular, bronchial and cerebral arteries, cardiac muscle, platelets, and diabetes mellitus. Hence, they involve a large number of smooth muscles, e.g., vascular, bronchial and cerebral arteries, cardiac muscle, platelets, and diabetes mellitus. Hence, they involve a large number of smooth muscles, e.g., vascular, bronchial and cerebral arteries, cardiac muscle, platelets, and diabetes mellitus. Hence, they involve a large number of smooth muscles, e.g., vascular, bronchial and cerebral arteries, cardiac muscle, platelets, and diabetes mellitus. Hence, they involve a large number of smooth muscles, e.g., vascular, bronchial and cerebral arteries, cardiac muscle, platelets, and diabetes mellitus. Hence, they involve a large number of smooth muscles, e.g., vascular, bronchial and cerebral arteries, cardiac muscle, platelets, and diabetes mellitus.
However, the NO-Rho-ROK cooperate signaling due to the phospholipase-D2 (PLD2)-dependent contraction in arterial smooth muscle (8, 173). The inhibition persisted for 24 h, which was the duration of MYP1R phospholipase-D2 gene expression in vitro (18). The Rho-ROK cooperation strongly represents the enzymes, namely, the non-phosphorylated versus PKC can synergize with Rho-ROK-dependent phosphorylated RhoA expression (84), resulted in a decrease in Ca²⁺ sensitivity of contraction (177). Whether this is due to Rap1-mediated activation of the RhoA-ROK, ARAP3 (as demonstrated in Refs. 119, 218) remains to be seen.

### Potential Role of Rho/ROK Dysfunction Causing Disease

Since the initial report that inhibition of ROK lowers blood pressure in different animal models of hypertension (233), an ever increasing number of reports described dysregulation of Rho/ROK signaling in a wide variety of disorders (e.g., Refs. 15, 30, 198, 210, 249) as well as the beneficial effects of interventions that antagonize Rho/ROK signaling (Ref. 65, 169, 204, 256, reviewed in Ref. 86). It is beyond the scope of this review to cover this exciting aspect and the reader is referred to the literature (33, 130, 165, 165, 209, 245). Here, we just want to emphasize one aspect, namely that most of these pathologies cannot be simply ascribed to altered smooth muscle function. Rather they involve a complex interaction between smooth muscle cells, the endothelial/epithelial lining, activated platelets, and mast cells within the walls of the visceral organs or blood vessels (e.g., Ref. 24; reviewed in Refs. 209, 242). For instance, diseases like hypertension and diabetes are associated with endothelial dysfunction and a decrease in NO bioavailability. This likely leads to a shift from the rapidly activatable cytosolic GDI-Rho-GTP complex to the active membrane-bound Rho (31). At the same time, the increased Rho/ROK activity causes a decreased endothelial eNOS expression (Ref. 15; for review, see Ref. 33) and mRNA stability (100, 123), thus creating a vicious cycle. Furthermore, by antagonizing Rho signaling, PKA not only ameliorates pulmonary hypertension (89) but also has a protective effect against pulmonary barrier dysfunction seen under many inflammatory conditions (180). Hence, the beneficial effects of Rho/ROK inhibitors are likely not simply due to normalization of smooth muscle tone but also involve inhibition of proliferation and cell migration, i.e., reversing the remodeling processes (for reviews, see Refs. 169, 181, 209).

### Concluding Remarks

The mechanical output of smooth muscle and also non-muscle cells is the summation of these stimulants and relaxing signals, which are integrated at the level of the contractile machinery mainly through MLCP. This also means that it is misleading to investigate these signals in a linear way, often necessary to reduce complexity for the time being, without taking into consideration that a highly complex network of signaling cascades regulates the contractile machinery. These pathways exhibit properties of positive and negative feedback and interact extensively at the level of the GTPases (25) but also further downstream. Despite the fact that the major pathways have been outlined, there are still significant gaps in our knowledge. For example, information is limited regarding the regulation of Rho activity and its cross talk with other GTPases in differentiated smooth muscle.

The mechanical output of smooth muscle itself is rather complex, exhibiting widely different contractile patterns with graded responses and different temporal profiles to environmental stimuli. Such a complex mechanical pattern requires signaling modules that can encode both the signal intensity and also the duration of the environmental signal (10). We still do not fully understand how the different modules are put together to obtain a specific contractile response both in amplitude and duration. It also appears likely that some of these modules can be exchanged for each other without affecting the overall effect, e.g., Ca²⁺ sensitization not always relies on Rho/ROK (e.g., Refs. 42, 200). Given the fact that Rho and ROK regulate a vast number of cell functions in addition to regulation of smooth muscle tone acting over different time scales (102), understanding the precise temporal and spatial aspects of signaling to the contractile machinery should possibly lead to better-tailored therapeutic strategies.

Because of space limitations, the reference list is by no means comprehensive and we do apologize to the many authors who significantly contributed to our understanding of Rho signaling and whose work is not cited here. We thank R. Stähle for stimulating discussions and D. Metzler for the artwork. The helpful comments of the anonymous reviewers are gratefully acknowledged.

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### References


REVIEWS


