Ca\textsuperscript{2+} Sensitivity of Smooth Muscle and Nonmuscle Myosin II: Modulated by G Proteins, Kinases, and Myosin Phosphatase

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Somlyo, Andrew P., and Avril V. Somlyo. Ca\textsuperscript{2+} Sensitivity of Smooth Muscle and Nonmuscle Myosin II: Modulated by G Proteins, Kinases, and Myosin Phosphatase. Physiol Rev 83: 1325–1358, 2003; 10.1152/physrev.00023.2003.—Ca\textsuperscript{2+} sensitivity of smooth muscle and nonmuscle myosin II reflects the ratio of activities of myosin light-chain kinase (MLCK) to myosin light-chain phosphatase (MLCP) and is a major, regulated determinant of numerous cellular processes. We conclude that the majority of phenotypes attributed to the monomeric G protein RhoA and mediated by its effector, Rho-kinase (ROK), reflect Ca\textsuperscript{2+} sensitization: inhibition of myosin II dephosphorylation in the presence of basal (Ca\textsuperscript{2+} dependent or independent) or increased MLCK activity. We outline the pathway from receptors through trimeric G proteins (G\textsubscript{az}, G\textsubscript{al}, G\textsubscript{ar}) to activation, by guanine nucleotide exchange factors (GEFs), from GDP RhoA GDI to GTP RhoA and hence to ROK through a mechanism involving association of GEF, RhoA, and ROK in multimolecular complexes at the lipid cell membrane. Specific domains of GEFs interact with trimeric G proteins, and some GEFs are activated by Tyr kinases whose inhibition can inhibit Rho signaling. Inhibition of MLCP, directly by ROK or by phosphorylation of the phosphatase inhibitor CPI-17, increases phosphorylation of the myosin II regulatory light chain and thus the activity of smooth muscle and nonmuscle actomyosin ATPase and motility. We summarize relevant effects of p21-activated kinase, LIM-kinase, and focal adhesion kinase. Mechanisms of Ca\textsuperscript{2+} desensitization are outlined with emphasis on the antagonism between cGMP-activated kinase and the RhoA/ROK pathway. We suggest that the RhoA/ROK pathway is constitutively active in a number of organs under physiological conditions; its aberrations play major roles in several disease states, particularly impacting on Ca\textsuperscript{2+} sensitization of smooth muscle in hypertension and possibly asthma and on cancer neoangiogenesis and cancer progression. It is a potentially important therapeutic target and a subject for translational research.
I. INTRODUCTION: EVIDENCE OF CALCIUM SENSITIZATION AND DESENSITIZATION

Recognition that calcium is the intracellular messenger that triggers muscle contraction (144) by binding, in striated muscles, to the Ca-binding protein troponin (88) eventually led to the realization that Ca-regulated myosin II plays major roles not only in striated, but also in smooth muscle and in nonmuscle cells. Identification of other Ca-binding proteins, such as the ubiquitous calmodulin, and their effectors revealed complex, interconnected cellular signaling mechanisms, critically regulated by protein kinases (reviewed in Ref. 69) and phosphatases (reviewed in Ref. 70). In the case of smooth muscle and nonmuscle myosin II, their ATPase activity and associated motility (contraction) are activated by actin, but only when Ser-19 of the myosin regulatory light chain (RLC) is phosphorylated, usually by a calcium-calmodulin (Ca2+/CaM)-dependent myosin light-chain kinase (MLCK) (reviewed in Refs. 115, 357; see Fig. 1). This Ca-dependent activation of myosin II plays an essential role in a variety of processes, including, but not limited to, cardiac contractility (Fig. 1) is further modulated by the Ca2+ sensitivity of the Ca2+ sensors and effectors that can also be modified by factors such as the activity (KCaM) of calmodulin for MLCK and the activity of the G protein-regulated myosin phosphatase. Studies with Ca2+-sensitive fluorophores suggested that, as expected (363), force developed at a given global level of [Ca2+]i could vary, depending on the type of excitatory stimulus: agonist-induced force is often higher than depolarization (high K)-induced force at similar, or even lower, [Ca2+]i (39, 145). Studies employing cell permeabilization methods that retained G protein-coupled receptors confirmed that the underlying mechanism is agonist-induced Ca2+ sensitization of the contractile/ regulatory apparatus, and not an artifact of the Ca2+ reporters. When such permeabilized muscles are activated by an agonist or guanosine 5′-O-(3-thiotriphosphate) (GTPγS) while [Ca2+]i is clamped, they respond with increased RLC phosphorylation and force (200). The term Ca2+ sensitization was coined to describe this phenomenon. Interestingly, different agonists can stimulate unequal maximal Ca2+ sensitization (146) through yet to be identified mechanism(s), perhaps qualitatively or quantitatively variable coupling between different agonists and trimeric and monomeric (RhoA) G proteins and guanine nucleotide exchange factors (GEFs) (Fig. 4).

Ca2+ desensitization was also first recognized in similar experiments that showed a decline in force and RLC phosphorylation while [Ca2+]i was unchanged during K+ (depolarization)-induced contractions of intact smooth muscles (147) and was confirmed by the phasic decline in RLC phosphorylation and force in permeabilized phasic smooth muscles maintained at constant [Ca2+]i (202).

Ca2+ sensitization and desensitization are now understood to involve the major physiological mechanisms that regulate myosin II activity: phosphorylation and dephosphorylation. Phosphorylation (at Ser-19) of RLCs of myosin II permits their activation by actin, whereas dephosphorylation (reviewed in Refs. 141, 357) inactivates these actin-activated myosin II ATPases. Consequently, the myosin light-chain kinase (MLCK)-to-myosin phosphatase activity ratio is the major determinant of the Ca2+ sensitivity of myosin II.

Smooth muscle is particularly suitable for identifying, through measurements of force and RLC phosphorylation, mechanisms that regulate the Ca2+ sensitivity of myosin II, and we will use its behavior as our framework for this review. However, because nonmuscle myosin II, MLCK, and myosin light-chain phosphatase (MLCP) are ubiquitously expressed in most, if not all, nonmuscle cells, regulation of (acto)myosin II by phosphorylation/dephosphorylation is a widespread, nearly universal, cellular mechanism. In nonmuscle cells, the development of stress fibers, cell motility, migration, and RLC phosphorylation are often monitored as indices of myosin II activity (reviewed in Ref. 28).

Modulation of Ca2+ sensitivity by posttranslational modification of Ca2+ sensors is not limited to phosphorylation of myosin II but can be mediated by a variety of other mechanisms, as illustrated by the effect of cardiac troponin phosphorylation on the Ca2+ sensitivity of myo-

FIG. 1. Regulation of contraction, stress fiber formation, and cell migration through phosphorylation/dephosphorylation of the regulatory light chain (RLC20) of myosin II. Activation of myosin light-chain kinase (MLCK) by Ca2+-binding to calmodulin (CaM) leads to phosphorylation of the RLCs of myosin II to switch on cross-bridge cycling and force development by actin-activated myosin. The ratio of kinase to phosphatase activities determines the level of RLC phosphorylation and the extent of activation: [Ca2+]i-independent modulation of the activities of MLCK and/or myosin light-chain phosphatase (MLCP) provides additional mechanisms for regulation of RLC20 phosphorylation.
cardiac contractility (53, 247, 284), the variable Ca$^{2+}$ sensitivity of inositol 1,4,5-trisphosphate (InsP$_3$) receptors (159), and the effect of phosphorylation on ryanodine receptors (171).

MLCK and the RhoA-associated Rho-kinase (RhoA/ROK) pathway are two major cellular targets for regulating Ca$^{2+}$ sensitivity of myosin II and, as we shall suggest, they generally operate in parallel. Phenotypes attributable to RhoA/ROK and, more specifically, to ROK require basal [Ca$^{2+}$], levels sufficient for “constitutive” MLCK activity. This is indicated by the inhibition of RhoA/ROK-induced effects by MLCK inhibitors and by inhibition of Ca$^{2+}$ influx (e.g., Ref. 243) and, conversely, by the reduction of myogenic tone by ROK inhibitors in the presence of normal resting [Ca$^{2+}$]$_i$ (34, 394, 436). Therefore, we consider that most phenotypes of RhoA/ROK that reflect myosin II activity are manifestations of Ca$^{2+}$ sensitization to normal, resting levels (~100 nM) of [Ca$^{2+}$], or to increases in [Ca$^{2+}$], often stimulated by the same agonists that induce Ca$^{2+}$ sensitization. The existence of constitutive (Ca$^{2+}$-dependent) MLCK activity in resting cells is indicated by the decreases in resting [Ca$^{2+}$], and reduced RLC phosphorylation and force induced by inhibition of Ca$^{2+}$ influx (e.g., Ref. 148) and the inhibition of RhoA/ROK-induced stress fiber formation by MLCK inhibitors (43, 226, 281). Each of these findings suggests that phenotypes induced by ROK result from Ca$^{2+}$-sensitizing inhibition of myosin phosphatase that also requires some activity of Ca/CaM or of a Ca$^{2+}$-independent MLCK that phosphorylates RLC, because phosphatase inhibition will not increase RLC phosphorylation without kinase activity.

II. MYOSIN LIGHT-CHAIN KINASE ISOFORMS AND TELOKIN

Smooth muscle MLCK (smMLCK) is distinguished by its exquisite substrate selectivity for RLC and its ubiquitous distribution not only in smooth muscle, but also in most, if not all, nonmuscle cells in which smMLCK phosphorlates RLC Ser-19 (or its equivalent) to allow actin to interact with myosin II, not only by Ca$^{2+}$/CaM-activated smMLCK, but also by Ca$^{2+}$-sensitizing and -desensitizing mechanisms.

The smMLCK is the product of a single gene, different from the one giving rise to skeletal muscle MLCK (skMLCK), although both are activated by Ca$^{2+}$/CaM. Splice variant products of the smMLCK gene range from short (130–150 kDa) to long (208–214 kDa) MLCK (116, 181, 222).

A COOH-terminal Ser phosphorylation site of smMLCK provided the earliest evidence that the Ca$^{2+}$ sensitivity of smMLCK could be regulated: phosphorylation of this site reduces the affinity of MLCK for Ca$^{2+}$/CaM (increases $K_{CaM}$) by –10-fold. This inhibitory Ser (Ser-512 in smMLCK) can be phosphorylated by cAMP-dependent PKA (Ref. 72) and also by other kinases (3; reviewed in Refs. 116, 181), but only when Ca/CaM is not bound to MLCK. Increases in cellular cAMP are associated with MLCK phosphorylation (78), but other studies suggested that inhibitory phosphorylation of MLCK in smooth muscle in vivo (Ca$^{2+}$ desensitization) is more likely to be an autoinhibitory mechanism mediated by Ca$^{2+}$-dependent phosphorylation of MLCK by CaM kinase II (250, 383). In nonmuscle cells, however, inhibitory phosphorylation of MLCK may be mediated by PKA or by another kinase. Cyclic nucleotide-activated kinases can also reduce [Ca$^{2+}$], (418) and inhibit the Ca$^{2+}$-sensitizing effect of ROK (see below), complicating the identification of the specific (or major) mechanism of cyclic nucleotide-induced decrease in RLC phosphorylation in intact cells.

cGMPC-dependent kinase (PKG), unlike PKA, does not phosphorylate the inhibitory site of MLCK (277), supporting the conclusion that 8-bromo-cGMP causes Ca$^{2+}$ desensitization by, directly or indirectly, increasing MLCP activity (428, 429) rather than by inhibiting MLCK.

The range of Ca$^{2+}$ sensitivity that can be determined by $K_{CaM}$ is constrained, at least in smooth muscles, by the high cellular concentrations of CaM and MLCK, considering the very high affinity (~1 nM $K_{CaM}$) for MLCK (116) and the very large bound fraction of cellular CaM (116, 444). Ca$^{2+}$ sensitivity of MLCK may also be affected selectively by its localization relative to the relevant protein kinases, and, in nonmuscle cells, its effect on phenotype will also depend on the variable subcellular localization of myosin II heavy chain isoforms (190, 207). Binding of MLCK to native thin filaments could also affect its Ca$^{2+}$ sensitivity and reminds one of the still unanswered question of how the low micromolar cellular MLCK, bound to thin filaments, can phosphorylate nearly 100% of the ~80 μM RLC in smooth muscle (368). MLCK can also be regulated independently of $K_{CaM}$ by proline-directed phosphorylation by mitogen-activated protein kinase (MAPK) which increases its $V_{max}$ without affecting $K_{CaM}$ (205, 257). The Ca$^{2+}$ sensitivity of MLCK may also be modulated by a G protein: GTPγS increases the level of MLCK phosphorylation, possibly because a G protein-regulated phosphatase also dephosphorylates MLCK (382). However, the specific phosphatase (perhaps MLCP) that dephosphory-
lates MLCK has not been identified. Phosphorylation (probably at Ser-439 and Ser-991) of MLCK by p21-activated kinase (PAK) inhibits it and desensitizes nonmuscle cell myosin II to Ca\(^{2+}\) (123, 326).

Long smMLCK isoforms (208–214 kDa; Ref. 181) are alternatively spliced products of the same gene as the short smMLCK and telokin. The NH\(_2\)-terminal extension of long MLCKs contributes to its strong actin binding with higher affinity than that of short MLCK (353). Several smooth muscles contain both short and long MLCK (29). Long MLCK is prominently expressed in embryonic smooth and nonmuscle cells (181), particularly in endothelium (402), where both forms are present in mature cells, although the long MLCK tends to predominate in cultured endothelial or smooth muscle cells (29), and its expression increases with passage in culture. NH\(_2\)-terminal Tyr phosphorylation of endothelial long smMLCK by p60\(_{src}\) has been suggested to be regulatory, possibly by affecting its subcellular localization (26, 27), but it remains to be determined whether it occurs in vivo and affects Ca\(^{2+}\) sensitivity. Localization of enzymes relative to their substrates, such as different localization of, respectively, long and smMLCK (29) and different subcellular Ca\(^{2+}\) domains are likely to result in spatially selective modulation of Ca\(^{2+}\) signaling.

Downregulation of short smMLCK in cultured smooth muscle cells does not reduce basal or stimulated RLC phosphorylation (19), suggesting that either long smMLCK can substitute for the small isoform in these cells or that the <10% remaining short MLCK is sufficient to carry out normal RLC phosphorylation. This possibility cannot be excluded, considering that <1% of total CaM in smooth muscle is required to support maximal force (444), and the two isoforms have comparable kinase functions (29). The potential of other kinase to compensate, at least partially, for Ca\(^{2+}/CaM\)-MLCK is indicated by contraction of smooth muscles in which the gene has been deleted and each isoform is absent (H. Wang, A. P. Somlyo, and A. V. Somlyo, unpublished data).

Telokin, a 17-kDa acidic peptide whose sequence is identical to the COOH terminus of MLCK, is expressed independently through a promoter located in an intron of the MLCK gene (74, 167). It is a PKG substrate in vivo implicated in Ca\(^{2+}\) desensitization (see below).

skMLCK, a somewhat smaller (77–90 kDa) product of a different gene than smMLCK (181), is also regulated by Ca\(^{2+}/CaM\) and can be Ser-phosphorylated by PKA, but this phosphorylation does not affect its Ca\(^{2+}/CaM\) sensitivity (27, 89).

### III. TOOLS AND TRAPS OF THE TRADE

Much has been learned about the RhoA/ROK pathway by relatively new research tools and methods, only some of which can be discussed here.

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### A. Toxins and Enzyme Inhibitors

Bacterial toxins and exoenzymes that affect Rho subfamily small GTPases (reviewed in Refs. 36, 37, 44) have been valuable not only to the pathogens utilizing them, but to investigators of the small GTPases targeted. The C3-exoenzyme (reviewed in Ref. 36) and the *Escherichia coli* protein EDIN (130, 369) are ADP-ribose transferases that ADP-ribosylate the Asn-41 residue of Rho, but not Rac or Cdc42. ADP-ribosylation inactivates RhoA and inhibits Ca\(^{2+}\) sensitization (108, 130). The high specificity of C3 led to its extensive use for inhibiting the RhoA/ROK pathway upstream without directly affecting Rac or Cdc42. Experimental use of the relatively cell impermeant C3 is facilitated by the chimeric protein DC3B that utilizes the B fragment of diphtheria toxin to allow intracellular penetration of C3 in whole tissues (15, 108). *Clostridium difficile* toxin B, a major cause of severe diarrhea, also inactivates RhoA (233), but is less specific, as it glycosylates not only Thr-37 of RhoA, but also the equivalent (Thr-35) residues of Rac and Cdc42. Toxin B inactivates these proteins by ‘‘immobilizing’’ the glycosylated residues that contribute to Mg\(^{2+}\) coordination required for nucleotide binding (417) and so prevents the GTP/GDP exchange necessary for activity. The *Yersinia enterocolitica* toxin (YopT) uncouples RhoA from its effectors, detaches it from membranes, and prevents its interaction with the Rho-binding domain of rhotekin (364, 446). YopT is a Cys proteinase that cleaves RhoA by removing the prenylated COOH-terminal methyl between Gly-189 and Cys-190 of RhoA (344). This finding, combined with the inability of cleaved RhoA to interact with the Rho-binding domain of its effector, also suggests that the interaction between RhoA and its effectors normally occurs at a lipid membrane. The observation that Rho-guanine nucleotide dissociation inhibitor (GDI) interacts with RhoA even after treatment with YopT (364) is surprising, because insertion of the prenylated RhoA COOH terminus into the hydrophobic cavity of GDI provides the strong binding for the complex. It may be reconciled by the possibility that weak binding of the NH\(_2\)-terminus of GDI to RhoA (231) is sufficient for coimmunoprecipitation, because YopT can extract endogenous RhoA from its complex with GDI (4), and GDI can interact with nonprenylated RhoA in a yeast two-hybrid screen (99).

In contrast to inhibitory toxins, the cytotoxic necrotizing factor 1 (CNF-1) activates Rho, Rac, and Cdc42 by deamidating Gln-63 (of Rho) or Gln-61 (Rac and Cdc42), converting a Gln to a glutamate. This ‘‘mutation’’ blocks the GTase activity of the Rho proteins, rendering them constitutively active, causing not only major changes in cytoskeletal architecture, but also disturbance of cell to cell interactions and epithelial barrier function; *E. coli* producing this toxin are implicated in urinary tract infections (37, 154).
B. ROK Inhibitors

Highly selective inhibitors against ROK, in particular, Y-27632, discovered by Narumiya and co-workers (107, 393), have been extremely useful in assessing specific phenotypes and consequences of ROK inhibition. In addition to Y-27632 and another pyridine derivative, Wf-536, two other chemically unrelated compounds, HA-1077 and H-1152P (328), show high selectivity against ROK. The inhibitory constant (K_i) of Y-27632 is 0.2–0.3 μM for ROK and 10 μM for protein kinase C (PKC)-δ; an important property of Y-27632 is that, although it competes with ATP for binding, it is highly effective in cells (containing high ATP levels), with the same IC_50 as its K_i (165). The most recent compound, H-1152P, has a K_i of 0.0016 μM for ROK and 9.27 μM for PKC (328). Although ROK inhibitors are not perfectly selective, their significantly greater (100-fold) activity against ROK than against conventional PKCs is sufficient, in most cases, for evaluating specific ROK functions. Minor caveats are that against the novel PKC, PKC-δ, the IC_50 of Y-27632 is 14 μM in the presence of 2 mM ATP (94), whereas 10 μM Y-27632 inhibits very significantly ROK-mediated Ca^{2+} sensitization in the presence of 4.5 mM MgATP. The extent to which inhibition of novel PKCs by Y-27632 could interfere with the interpretation of its effects may also depend on the expression levels, in different smooth muscles, of novel PKCs and CPI-17, activated by PKCs (see below). The second caveat is that, at concentrations (e.g., 100 μM) usually higher than required to inhibit Ca^{2+} sensitization of force, Y-27632 reduces the fura 2 signal used to measure agonist-induced increases in the cytoplasmic Ca^{2+} signal (168). In most cases Y-27632 at 10 μM concentrations or less is a sufficiently specific inhibitor of ROK, and the few uncertainties about its specificity can be resolved by upstream inhibition of RhoA itself with C3 or EDIN.

Inhibition of geranyl-geranyl transferase is another in vivo, albeit nonspecific (51), method for inhibiting RhoA upstream (224), because COOH-terminal geranyl-geranylation is required for membrane localization involved in Ca^{2+}-sensitizing Rho activity (108, 127, 130).

C. Proteins Expressed, Overexpressed, Constitutively Active, and Phosphorylations: Estimating Active RhoA

The valuable tools of molecular biology can also lay traps for the unwary. The activity and target specificity of constitutively active kinases may differ from those of the endogenous forms, and expression of transfected proteins and, even more so, overexpression may result in subcellular distributions and activities that are not representative of physiological pathways. Unexpected cross-reactivity of antibodies (e.g., Ref. 132), unless validated by protein sequencing when first used, could also mislead. We suggest that criteria, like those developed by Krebs and Beavo (216), should be extended to include stringent controls for these powerful, newer methods. Their criteria (216) for verifying that an enzyme undergoes physiologically significant phosphorylation/dephosphorylation are still valid, and it is particularly important in the case of signaling mechanisms to demonstrate that a kinase substrate is phosphorylated in an intact cell system with a time course consistent with the anticipated functional change. We also suggest that localization of an enzyme relative to both its substrate and its activating mechanism should be consistent with its effect on integrated cell function. If the function of a given G protein, kinase, or phosphatase requires translocation to its substrate (or vice versa), such as the case of RhoA and ROK translocation to the plasma membrane (108, 127, 254, 375) and the cellular traffic of MLCP (34, 349) or CPI-17 between ROK and myosin filaments, the time course of this relocalization should precede and parallel the change in Ca^{2+} sensitivity.

Failure to identify the specific phosphorylation site(s) in a kinase or phosphatase can complicate interpretation of findings. For example, results obtained with, respectively, 32P incorporation and site-specific antibodies to phosphopeptides may lead to differing conclusions. Radioisotopic assay showing increased radioactivity in a protein may reflect turnover or incorporation into a non-functional site rather than stoichiometric change in a functional residue, whereas a site-specific antiphosphopeptide antibody will not detect potentially relevant phosphorylation of a residue other than the one against which the antibody was generated. This problem can arise when evaluating Thr-696 phosphorylation of myosin phosphatase inhibitor (MYPT1) that contains more than one ROK phosphorylation site (10). In case of negative results, such as contraction without detectable RLC phosphorylation (reviewed in Ref. 337), the time course of phosphorylation should be determined using rapid kinetic methods: RLC phosphorylation in smooth muscle can peak within 3 s of stimulation and return close to resting levels within 30 s while significant force is maintained (147).

Active GTP·RhoA is the major determinant of Ca^{2+}-sensitizing ROK activity, rather than the total cellular RhoA that in nonstimulated cells exists largely as the inactive GDP·RhoA·GDI complex (Fig. 2). Methods of estimating the cellular concentration of GTP·RhoA are based on the binding of GTP·RhoA, but not GDP·RhoA, to specific regions of Rho effectors: Rho binding domains (RBDs) that can precipitate GTP·RhoA from cell homogenates while leaving GDP·RhoA (complexed with GDI) in solution. The RBDs of rhotekin (308) and of p140 mDia (194) have been valuable in detecting changes in RhoA activity induced by Ca^{2+}-sensitizing agonists and inhibitors.
Ideally, the time course of RhoA activation and of the downstream effect of phosphorylation by activated ROK should be determined. This would not only validate the role of RhoA/ROK in a given process, but would also provide information about intervening steps. For example, the kinetics that include a long lag phase (~6 s) between photolysis of caged GTP in the nucleotide binding pocket of the RhoA/GDI complex and the onset of contraction suggests the intervention of a multistep, slow process preceding RLC phosphorylation (Fig. 3).

IV. REGULATION UPSTREAM

A. Agonists, Receptors, Trimeric and Monomeric G Proteins, Ligands, Lipid Messengers, and Microtubules

A variety of agonists can induce Ca\(^{2+}\) sensitization through RhoA and its downstream effectors (Fig. 4). RhoA, a small (~20 kDa) monomeric GTPase (reviewed in Ref. 28), was identified as a major messenger of Ca\(^{2+}\) sensitization in two studies initiated by us that showed that activated RhoA (GTP\(\rightarrow\)RhoA) Ca\(^{2+}\)-sensitized force (130, 149) and RLC phosphorylation (130) in permeabilized smooth muscle. Agonists can activate RhoA through numerous G protein-coupled receptors: \(\alpha\)-adrenergic, muscarinic, prostanoid (75, 146, 166, 325), purinergic (332), endothelin (412), thrombin (90, 241, 398), vasopressin (220, 376), oxytocin, epidermal growth factor, purinergic, ephrin, semaphorin; angiotensin and EDG lysophospholipid (365, 386) receptors (e.g., Refs. 9, 16, 68, 75, 87, 90, 114, 130, 146, 150, 153, 155, 166, 241, 263, 293, 332, 379, 406, 411, 437 and reviewed in Refs. 321, 338, 357, 358). Most G\(\alpha\)-coupled receptors activate both RhoA and phospholipase C (PLC) that induces InsP\(_3\) production and Ca\(^{2+}\) release from the sarcoplasmic/endoplasmic reticu-
lum, increase Ca\(^{2+}\) influx through receptor-operated or voltage-gated channels (reviewed in Refs. 184, 359) and inhibit maxiK channels resulting in depolarization (8). Therefore, under physiological conditions, the three mechanisms, Ca\(^{2+}\) release, influx, and sensitization, often act in concert (1, 357, 358). However, InsP\(_3\) does not affect Ca\(^{2+}\) sensitivity and to what extent diacylglycerol, the other product of PLC, has a significant physiological role in Ca\(^{2+}\) sensitization is still under examination (see sect. VI). Furthermore, agonists acting on thromboxane A\(_2\) receptors and linked through G\(_{q}\) family trimeric G proteins are upstream initiators of RhoA/ROK-mediated Ca\(^{2+}\) sensitization through complex downstream mechanisms that are only now being unraveled (see below and Refs. 124, 204).

Trimeric G protein(s) have been implicated in RhoA/ROK-mediated Ca\(^{2+}\) sensitization by the demonstration that transfected α-subunits of G\(_{12,13}\) activate RhoA (140, 186, 240) and by the Ca\(^{2+}\)-sensitizing effect of aluminum fluoride (AlF\(_4^-\)) (130, 189, 438). Because early work (177) did not show direct interaction of AlF\(_4^-\) with monomeric GTPases, whereas a G\(_{q}\)GDP adduct interacts with GEFs that activate RhoA (e.g., Refs. 35, 45, 60, 111, 140), it had been thought that AlF\(_4^-\) cannot directly activate RhoA. However, recently observed structural interactions between AlF\(_4^-\) and small G proteins (56, 152, 313) revive the possibility, entertained earlier (130), of a direct functional interaction (activation) between AlF\(_4^-\) and RhoA.

**FIG. 4.** Signaling pathways for Ca\(^{2+}\) sensitization in smooth muscle. Different smooth muscles respond to a large number of different agonists including catecholamines, muscarinic agonists, thromboxane, histamine, serotonin, as well as the sphingolipids: sphingosine 1-phosphate and sphingosylphosphorylcholine. Activation of their receptors initiates signaling through the illustrated cascades that inhibit myosin phosphatase (MLCP), increase RLC\(_{20}\) phosphorylation and contraction, stress fiber formation, and/or cell migration. The Rho/ROK pathway can also lead to activation of smooth muscle differentiation marker gene expression. GPCR, G protein-coupled receptors; GEF, guanine nucleotide exchange factor(s); RhoGD; GDP dissociation inhibitor; RhoGAP, GTPase activating protein; ROK, Rho-kinase, ROKα/ROCK I, ROKβ/ROCK II; C3, clostridial C3 exoenzyme ADP ribosylates and inactivates RhoA; iPLA\(_2\), Ca\(^{2+}\)-independent phospholipase A\(_2\); CPI-17, PKC-potentiated inhibitor protein of 17 kDa; MYPT1 and PP1c, myosin phosphatase regulator and catalytic subunits; MLCK, myosin light-chain kinase; DAG, diacylglycerol; PKC, protein kinase C; AA, arachidonic acid; SR, sarcoplasmic reticulum; InsP\(_3\), inositol 1,4,5-trisphosphate; PLC, phospholipase C; PIP\(_2\), phosphatidylinositol 4,5-bisphosphate.
Sphingosine 1-phosphate (SIP), sphingosylphosphorylcholine (SPC), and lysophosphatidic acid (LPA) are lipid Ca\(^{2+}\)-sensitizing agonists that activate the RhoA/ROK pathway (9, 270, 386; reviewed in Refs. 234, 320, 360, 364). SPC increases the force-to-[Ca\(^{2+}\)]\(_i\) ratio in intact and force at constant [Ca\(^{2+}\)] in permeabilized coronary artery smooth muscle (386). In rat microvessels the effect of SPC is partially inhibited by PLC inhibitors (9). However, it is difficult to identify the mechanisms through which the PLC inhibitor acts without evaluating changes in Ca\(^{2+}\) in nonpermeabilized preparations. It is likely that Ca\(^{2+}\) sensitization by SPC involves Tyr phosphorylation, most likely of a RhoGEF. This is suggested by the ability of SPC to induce translocation of the Tyr kinase Fyn from the cytosol to the cell membrane and the inhibition of the SPC-induced phosphorylation of PLC-\(\delta\) (in coronary artery smooth muscle) by the Tyr kinase inhibitor PP1 (270). The Tyr kinase inhibitor also inhibits translocation of ROK to the membrane and relaxes the Ca\(^{2+}\)-sensitized contraction (270). SPC-induced localization of ROK and possibly RhoGEF may be related to its ability to induce Tyr phosphorylation of focal adhesion proteins: p125\text{FAK}, p\(_{130}\text{CAS}\), and paxillin (reviewed in Ref. 318). SIP-induced Tyr phosphorylation of FAK may also be a downstream effect of RhoA/ROK: it is inhibited by C3 in 3T3 fibroblasts (411). C3 does not inhibit Tyr phosphorylation of another focal adhesion protein, paxillin in permeabilized smooth muscle (249), or in neuroblastoma cells in which C3 inhibits phosphorylation of FAK (260).

Phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) and products of phosphatidylinositol 3-kinase (PI3K) have also been implicated in interactions with RhoA/ROK (282, 442), but a clear picture has yet to emerge to show whether PIP\(_2\) and/or PI3K products contribute to physiological regulation of RhoA/ROK. In Swiss 3T3 cells, the PI3K inhibitor wortmannin inhibits activation of Rac by platelet-derived growth factor (PDGF) and insulin receptors, but does not affect the induction of stress fibers by RhoA/ROK (282), whereas the latter effect is inhibited by the Tyr kinase inhibitor tyrphostin. Similarly, inhibition of PI3K does not affect cytoskeletal changes induced by the GEF Vav3, whereas PI3K-dependent stimulation of cell motility is mediated by Rac1 and CDC42, not by Rho (320, 380). Although PIP\(_2\) can stimulate GDP release from both CDC42 and Rho in vitro (442), it is uncertain whether this effect of PIP\(_2\) is physiological. Integrin-mediated activation of nuclear factor \(\kappa B\) (NF-\(\kappa B\)) is inhibited by wortmannin, but this mechanism is also mediated by Rac, not RhoA (310). The available evidence suggests that PI3K products are more likely to be involved in interactions with Rac and CDC42 than with the RhoA/ROK pathway. Given the hydrophobicity of phosphoinositides, the suggestion that their stimulation of GEFs, at least for Rac, occurs when both are localized to the lipid membrane (354) is consistent with the converging evidence that RhoA, RhoGEFs, and ROK are recruited to the plasma membrane during activation of the RhoA/ROK pathway. RhoA, Rac, and CDC42 bind to the same GDI isoform, and it is yet to be determined how phosphoinositides can act selectively on Rac · GDI, presumably sensing subtle differences between the GDP · Rac · GDI and the GDP · RhoA · GDI interface. It may be the result of utilization of different GEFs activated by, respectively, phosphoinositides or Tyr phosphorylation. Alternatively, different Tyr kinases (or in different cellular localizations) may be employed.

Microtubule depolymerizing agents, nocodazole or colchicine, induce contractions of smooth muscle that are relaxed by Y-27632 (64, 441). Disassembly of microtubules in neutrophil granulocytes also increases ROK activity and RLC phosphorylation inhibited by Y-27632 (274). The release of tubulin or of a microtubule-associated protein (possibly a microtubule-bound GEF; Refs. 122, 217, 309, 396) during disassembly may activate RhoA, consistent with the interpretation that microtubular depolymerization induces Ca\(^{2+}\) sensitization by activating RhoA/ROK. These findings provide a mechanistic explanation of why nocodazole and colchicines induce an, albeit, small (4–6% of maximal contractile responses) contraction of mature smooth muscles (441) that contain few microtubules. If this mechanism has a physiological role it is more likely to be in dividing and proliferating cells.

**B. GEFs, GDIs, GTPase-activating Proteins, RhoA, and ROK**

1. GEFs

Activation of RhoA, the Ca\(^{2+}\)-sensitizing step that follows engagement of the trimeric G protein with its coupled receptor, requires active GEFs (reviewed in Ref. 336) that catalyze the exchange of cytoplasmic GDP · RhoA complexed with GDI (reviewed in Ref. 289) into the active GTP · RhoA that associates with the plasma membrane and activates ROK (108, 127) (Fig. 2). Numerous GEFs have been identified in the human genome (reviewed in Ref. 178), with the most information available about PDZ-RhoGEF (PDG), LARG, and p115 RhoGEF (35, 60, 62, 140, 212, 215, 219, 411, 420). A common feature of these GEFs is the presence of a DBL homology (DH) domain responsible for GEF activity, followed in tandem by a pleckstrin homology (PH) domain involved in protein-phosphoinositide lipid, and protein-protein interactions (317; reviewed in Ref. 151). Thus the PH domain also directs subcellular localization to regions other than the lipid membrane: it is required for localizing the RhoGEF, Lbc to actin stress fibers as well as for transformation of NIH 3T3 cells, although the DH domain of Lbc is sufficient for stimulating DNA synthesis (290). Trimeric G proteins,
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\( \alpha_{12,13} \) (60, 110) and \( \alpha_q \) (35), are coupled to GEF(s), LARG, PDZ-RhoGEF and p115 RhoGEF, through an RGS (regulators of G protein signaling) like domain (RGS1) of the GEF. This domain has GTPIase-activating protein (GAP) activity toward the associated \( \alpha \) (214, 215) and strong binding of p115 RhoGEF to \( \alpha_{13} \) requires both the RGS domain and the DH and PH domains, although the RGS domain is sufficient for GAP activity (420). Association of PDZ-GEF with \( \alpha_{12} \) family proteins occurs also through an Lsc homology (LH; the murine homolog of the RGSL; Refs. 56, 230) domain (111).

The mechanism and/or protein domains involved in activation of RhoA by \( \alpha_{13} \) (186), but not the effect of \( \alpha_{12} \) on neuronal morphology (372; reviewed in Ref. 110; see below). In addition to p115 RhoGEF, three other RhoGEFs, Vav, LARG, and PDZ-RhoGEF, may be activated by Tyr phosphorylation that appears to be a common, although probably not the only, mechanism activating of PDZ-RhoGEF, LARG (372, reviewed in Ref. 110), Vav (reviewed in Refs. 45, 336), and vascular smooth muscle RhoGEF (285). The Tyr kinase(s) that Tyr phosphorylates PDZ-RhoGEF and LARG in response to stimulation with thrombin (60) has been identified in some instances as c-Src (45, 336, 405) and in others FAK. The variable inhibition of RhoA activation by different Tyr kinase inhibitors suggests that more than one Tyr kinase (and phosphorylation that is involved in RhoGEF regulation, depending on cell type and Ca\textsuperscript{2+} sensitizing agonist.

The ability of \( \alpha_q \) to activate the RhoA/ROK pathway was considered likely, because agonists activating receptors coupled to \( \alpha_q \) usually also cause Ca\textsuperscript{2+} sensitization (358) that can be dissociated from the Ca\textsuperscript{2+}-releasing activity of InsP\textsubscript{3} (206), and the upstream role of \( \alpha_q \) was directly established in cells lacking \( \alpha_{12,13} \) (62, 403). The \( \alpha_q \)-coupled mechanism is inhibited by a dominant-negative mutant of LARG, further indicating that this GEF can be coupled to \( \alpha_q \) although in these cells (HEK 293T) \( \alpha_{12,13} \) activates RhoA still more effectively than \( \alpha_q \) does. The mechanism and/or protein domains involved in activation of RhoGEFs by, respectively, \( \alpha_{13} \) and \( \alpha_q \), however, are different. Activated \( \alpha_q \) and stimulation of \( \alpha_q \)-coupled receptors can strongly activate endogenous RhoA in HEK 293T cells, but whereas PDZ-RhoGEF, LARG, and p115 RhoGEF coimmunoprecipitate with \( \alpha_{12,13} \), they do not coimmunoprecipitate with \( \alpha_q \) (62). Furthermore, in the same type of cell, \( \alpha_{13} \) but not \( \alpha_q \) recruits p115 RhoGEF to the plasma membrane and can activate it both directly and as the result of upstream activation of a thromboxane A\textsubscript{2} receptor (25). Strong plasma membrane-recruiting activity requires both the RGS and PH domains (25). GEF activity of LARG in NIH 3T3 cells is stimulated by active \( \alpha_q \) as well as by \( \alpha_{12,13} \) (35). Although it may be early to conclude, but these studies, performed on transfected cells, suggest that whereas LARG and PDZ-RhoGEF can be activated by both \( \alpha_{11} \) and by \( \alpha_{12,13} \), activation of p115 RhoGEF is more limited to the \( \alpha_{12,13} \) family. It remains to be seen whether these implications will be found valid about the functions of the endogenous proteins in nontransfected and noncultured cells and tissues. The Ca\textsuperscript{2+}-sensitizing effectiveness of a given agonist probably depends on the cellular location of different receptors, trimeric G proteins, and RhoGEFs. The very high Ca\textsuperscript{2+}-sensitizing activity of thromboxane A\textsubscript{2} receptors (146) that are coupled to \( \alpha_{12,13} \) does suggest that in other systems, as in the transfected cell (403), this family of trimeric G proteins mediates Ca\textsuperscript{2+} sensitization most potently.

Stimulation of thromboxane A\textsubscript{2} receptors linked to \( \alpha_{13} \) and stimulation of lysophosphatidic acid (LPA) receptors recruit cytoplasmic p115-RhoGEF to the plasma membrane of, respectively, HEK 293T and NIH 3T3 and COS cells (25, 419). Such observations and the translocation of both RhoA and ROK to the cell membrane during Ca\textsuperscript{2+} sensitization (108, 127) suggest that Ca\textsuperscript{2+} sensitization-signal complexes are assembled at (and/or form) docking sites on the plasma membrane where RhoA, ROK, and GEFs are translocated (25, 108, 127, 419) and, probably, activated.

The multiplicity of possible permutations between the many agonists, receptors, trimeric G proteins, GEFs and, not to mention, cell types, complicates and, so far, eludes identification of the agonist- and cell-specific GEFs mediating Ca\textsuperscript{2+} sensitization of a given cell type in vivo. GEF activity in solution is usually assessed by measuring GEF-catalyzed acceleration of nucleotide exchange on nonprenylated Rho-family G proteins, rather than on the endogenous form: the prenylated GDP•RhoA•GDI complex. Expression (often overexpression) of these GEFs in vivo is often assayed by determining their ability to induce malignant transformation of cells. The pathway mediating this response is not necessarily identical, or even utilizes the same domains as induction of stress fibers (336), and does not identify (with the possible exception of an integrin-related process with Rac GDI, see Ref. 79) the mechanism that dissociates, at least temporarily, the tightly bound GDP•RhoA•GDI complex to release GDP (231, 304; reviewed in Ref. 289) and allow the exchange of GTP for GDP.

It is likely that nucleotide exchange on RhoA, and probably also Rac (314) as well as ROK activation are facilitated by lipid-protein (or protein-protein) interactions at the plasma membrane, because a preformed GTP•RhoA in a GTP•RhoA•GDI complex can Ca\textsuperscript{2+} sensitize smooth muscle and “spontaneously” translocates in vitro to liposomes (129, 304). Furthermore, prenylated RhoA is a much better substrate for p115 RhoGEF than nonprenylated RhoA (420).

The importance of cellular specificity and localization of RhoGEFs is indicated by recent reports showing expression of an ephrin-receptor coupled GEF that is
selectively expressed in vascular smooth muscle, Vsm-
RhoGEP (285), another GEF specifically at epithelial tight
junctions (23) and the human ECT2 GEF localized to
nuclei (384). It will be interesting to find out if Vsm-
RhoGEP docks preferentially on the smooth muscle se-
lective protein LPP (132).

Rnd1 is a recently discovered small GTP binding
protein, related to RhoA, that inhibits RhoA-mediated
stress fiber formation (283) and Ca2+- sensitization of
smooth muscle (229). Rnd1 shares 45–49% identity with
Rho but has no significant GTPase activity and appears to
be in a permanently GTP-bound form (283). It is farnesyl-
ated and associated with the membrane, hence its ability
to inhibit RhoA also supports the notion that some step(s)
of RhoA/ROK activation occurs at the cell membrane. The
absence of RhoA/ROK-mediated Ca2+ sensitization in
midterm myometrium correlates well with the concurrent
increase in Rnd3 expression. Ca2+ sensitization can be
restored by farnesyl transferase inhibitors that prevent
membrane localization of Rnd3 (47). These results are
consistent with a physiological role of Rnd proteins in
regulating the RhoA/ROK pathway either negatively, as in
this instance, or positively, when Rnd1 associates with
plexin (288).

Prenylated GTP · RhoAVal14 Ca2+ sensitizes smooth
muscle permeabilized with β-escin, but not with Triton
(130), whereas nonprenylated GTP · RhoAVal14 fails to
Ca2+-sensitize the more mildly (β-escin)-permeabilized
preparations. The unexpected observation that when it
associates with the cytoplasmic tail of plexin, an in-
tramembrane protein, Rnd1 activates RhoA/ROK, instead
of inhibiting it (288) suggests catalytic activity to occur at
membranes. These findings are consistent with the con-
clusion that association with a relatively intact (not Tri-
ton-treated) membrane is required for Ca2+ sensitization
by RhoA.

2. GDI and GAPs

GDI has the important role of maintaining the other-
wise hydrophobic RhoA in an inactive cytosolic, ternary
GDP · RhoA · GDI complex by capturing the prenylated
COOH terminus of RhoA in its hydrophobic pocket while
its NH2-terminal region interacts with the switch-1,
switch-2 regions of RhoA and inhibits nucleotide ex-
change (231; reviewed in Ref. 289) until activated by
GEFs. High concentrations of GDI relax force Ca2+ sen-
sitized by GTPγS, recombinant GTP · G14V RhoA or by
α-adrenergic and muscarinic agonists and can extract
GTP · RhoA translocated to membranes (129). The very
high concentration of GDI required for these effects sug-
ests that the likely in vivo function of GDI is not to
interact directly with active GTP · RhoA, but to prevent
perpetual recycling of GDP · RhoA, following hydrolysis
of GTP · RhoA, to GDP · RhoA, in the presence of signif-
antly higher cytosolic GTP than GDP. ADP-ribosylation
of cytosolic RhoA occurs in vivo (108, 118) and increases
its binding to GDI (118), contributing to the inhibition of
RhoA activity by C3. However, the lack of activity of GTP ·
RhoA that was ADP-ribosylated in vitro (130) suggests
that ADP-ribosylation inhibits RhoA through more than
one mechanism.

Hydrolysis of RhoA-bound GTP (and other Rho fam-
ily proteins) is catalyzed by GAPs (reviewed in Refs. 255,
297, 338; see Fig. 2). GAPs are required to accelerate the
otherwise slow rate of hydrolysis by Rho family proteins,
but information about their mechanism of activation or
constituitive activity is limited. Interestingly, Tyr phos-
phorylation of p190 GAP increases its GAP activity that
decreases the active GTP · RhoA (142), whereas Src-
mediated Tyr phosphorylation also activates GEFs to in-
crease RhoA/ROK activity. Given that there are 53 Rho ·
GAP domain-containing proteins encoded in the human
genome and GAP specificity for their Rho family proteins
is different in vitro and in vivo (297), identification of
which GAP functions under specific physiological con-
ditions is going to be challenging.

3. ROK

Activation of the Ser/Thr kinase ROK by GTP · RhoA
is the next step of Ca2+ sensitization. The two major
isoforms of ROK are Rho-kinase α/ROK II and Rho-kinase
β/ROK 1 (10, 163, 225, 244). Binding of GTP · RhoA to the
RBD of ROK leads, through a conformational change, to
autophosphorylation and activation of the kinase (55;
reviewed in Ref. 178) that is the major effector of Ca2+
sensitization of myosin II (reviewed in Ref. 358). The
physiological importance of regulation of myosin II by
RhoA/ROK is shown by numerous studies indicating that
Ca2+ sensitization can be inhibited both upstream,
through ADP ribosylation by the bacterial exoenzymes C3
and EDIN (108, 130) or glycosylation with C. difficile
toxin B (233) to inhibit RhoA, as well as downstream with
the selective ROK inhibitors Y-27632 (107, 165, 393) or
HA-1077 (265, 328). Point mutations of RhoA that cause
loss of its GTPase activity (e.g., G14V, Q63L) yield constit-
tutively active proteins, albeit with variable GDI binding
properties (reviewed in Ref. 232). We note that ROK
mediates Ca2+ sensitization (MLCP inhibition) and in-
duces focal adhesions, but other RhoA effectors mediate
actin polymerization (164; but see sect. viUC).

Not only RhoA, but arachidonic acid can also activate
ROK in solution (101) and possibly also in vivo, as indi-
cated by the reversal of arachidonic acid-induced Ca2+
sensitization by Y-27632 (14, 107). Agonists can increase
cellular arachidonic acid (131, 137) that may contribute to
Ca2+ sensitization by activating ROK (14, 107) and/or by
dissociating the regulatory from the catalytic subunit of
myosin phosphatase (14, 126, 401) and/or by activating an atypical PKC (112).

Direct phosphorylation of the myosin RLC by ROK is not a physiologically significant mechanism, at least in smooth muscle, although ROK can phosphorylate both smooth muscle and nonmuscle myosin RLC in solution, and exogenous ROK can contract isolated stress fibers (187). However, activation of RhoA/ROK by GTPγS in the absence of Ca\(^{2+}\) and MLCK activity fails to cause RLC phosphorylation or significant force development by smooth muscle (161, 356, 373) and the specificity constant \(k_{cat}/K_m\) of MLCK for RLC is much higher than that of ROK (40). It is possible that ROK is concentrated to specific sites such as the cleavage furrow, if, activated by RhoA or by some yet unrecognized mechanism, directly phosphorylate RLC. Cytokinesis is accompanied by RLC phosphorylation and accumulation of ROK and citron kinase at the cleavage furrow (165, 210, 245). However, inhibition of ROK with Y-27632 does not block cytokinesis, implicating another kinase (165, 210, 245), such as another Rho effector, citron kinase (433).

Spatial differences in RLC phosphorylation between, respectively, cortical and central regions of a cell (254), may reflect different levels of myosin phosphatase inhibition influenced by localization of the RhoA/ROK system, including MYPT1 (34, 389) or recruitment of MLCK (58), rather than direct phosphorylation of RLC by ROK. Differences in spatial control by MLCK, ROK, and other kinases and myosin phosphatase may also be influenced by localized sources of Ca\(^{2+}\), inhomogeneous influx through Ca\(^{2+}\) channels, and release from the sarcoplasmic reticulum (32, 171, 211, 359, 400).

Spatial control of function in cultured nonmuscle cells may also be affected by the different subcellular distributions, activities, and turnover of nonmuscle myosin II isoforms (207). Related to whether ROK directly phosphorylates RLC is the issue of diphosphorylation of RLC. In mature, contracting smooth muscle, RLC diphosphorylation (Thr-18 in addition to Ser-19 of RLC) by MLCK is observed only when phosphorylation levels exceed 40% (199). In vitro, ROK is more effective than MLCK in diphosphorylating RLC and may be responsible for localized accumulations of diphosphorylated RLC (245) that is also increased in cells transfected with ROK and colocalizes with the latter (392). The extent and in vivo functional consequences of RLC diphosphorylation (41) by ROK, by citron kinase (433), or by other kinases (261, 275) remain to be further explored.

C. Ephrins and Plexins: RhoA/ROK in Axonal Guidance and Angiogenesis

Ephrins are a special class of receptor Tyr kinases whose ligands are ephrins bound to cell membranes by GPI glycosylphosphatidylinositol (GPI) anchoring (A-ephrrins) or having a membrane-spanning region (ephrin B); they are important upstream regulators of RhoA/ROK and play major roles in morphogenesis (reviewed in Ref. 153). The ligands, ephrins, are obligatory membrane dwellers and, therefore, act on their receptors, ephrins, on adjacent contacting cells. The cytoplasmic domains of ephrins allow for bidirectional signaling.

Ephrin signaling plays important roles in both axon guidance and morphogenesis. In neuronal cells activation of RhoA/ROK causes repulsion and growth cone collapse, whereas in endothelial cells ephrins induce endothelial de-adhesion and migration. Activation of RhoA/ROK provides a repulsive signal that results in collapse of growth cones seeking the appropriate dendrite, whereas activation of Rac provides attractive cues by activating PAK and inhibiting MLCK (295). Ephrin signaling through Rho-GTPases can be mediated by a GEF; a specific GEF, ephexin, directly interacts with the cytoplasmic tail of the Eph-A4 receptor (reviewed in Ref. 295). Given the opposing roles of, respectively, Rac and RhoA on neuronal growth cones, it is interesting that activated ephexin mediates converging signals; it reduces the activation of Rac while it activates RhoA. Activation of the ephrin-A5 pathway in retinal ganglion cells results in increased concentration of active GTP · RhoA, active ROK, and increased RLC phosphorylation, each inhibited by C3 or Y-27632 (406), clearly revealing cytoplasmic myosin II as the target effector of RhoA/ROK.

During early angiogenesis, ephrin-B2 ligand is a marker of the arterial endothelium (114, 348), whereas Eph-B4 is expressed in venous endothelium. The adult ephrin-B2, in addition to continuing to be a marker of arterial endothelium, also becomes expressed in the smooth muscles cells and pericytes investing the endothelium (114, 348). The persistence of arterial expression of ephrin-B2 in adult blood vessels and during tumor angiogenesis suggests the continued importance of ephrin signaling in the adult (114, 348) and, given expression of an appropriate receptor, ephrin-B2, may even mediate signaling between endothelium and smooth muscle.

The importance of the persistence of ephrin signaling into adulthood is indicated by the ephrin-B2 expression during neovascularization and tumor angiogenesis (114, 348). Signaling to the RhoA/ROK pathway is indicated not only by the ephrin-induced activation of RhoA/ROK in neuronal cells (406), but also by the inhibition of the earliest stage of angiogenesis, endothelial vacuole formations, as well as in vivo tumor angiogenesis by ROK inhibitors (361, 362). Because ephrin-B2 deficiency of null (−/−) mutants (413, reviewed in Ref. 435) is lethal (348), it would be interesting to determine the effect of targeting this gene product to inhibit tumor angiogenesis.

Plexins are large transmembrane protein receptors whose ligands are transmembrane or secreted semaphor-
ins (reviewed in Ref. 379); like ephrins, they participate in axon guidance and angiogenesis. Plexins associate with neuropilins, another class of semaphorin receptors (reviewed in Ref. 271). Plexins, like ephrins, play a repulsive role in axon guidance. The COOH terminus of activated Plexin-B1 associates with PDZ-RhoGEF in 3T3 cells (87) and with LARG and PDZ-RhoGEF in HEK293 cells and activates RhoA (16). The interaction with the cytoplasmic tail of plexin-B1 is thought to contribute to recruitment of LARG and PDZ-RhoGEF to the plasma membrane during semaphorin 4D-plexin-B1-mediated activation of RhoA (150), similar to the interactions of the COOH terminus of the insulin-like growth factor I with PDZ domains (385). These membrane-associated interactions involve both the RGSL and PDZ domains of the GEFs (374). Mutation of the COOH-terminal amino acids of plexin-B1 that are required for interaction with the PDZ domain or expression of a dominant negative PDZ-RhoGEF blocks activation of RhoA by plexin-B1 and inhibit the RhoA-mediated neurite contraction and retraction. Activation of RhoA by endogenous plexin-B1 activated by semaphorin 4D is also inhibited by overexpression of the PDZ domain of RhoGEF, although not by expression of the RGSL domain (298).

A surprising, recently reported aspect of the activation of RhoA by plexin-B1 has all the earmarks of RhoA/ROK activation, including contraction of COS-7 cells that is inhibited by Y-27632. However, robust activation of RhoA/ROK occurs not by solely activating plexin with the semaphorin ligand, but also requires the association of plexin-B1 with Rnd1 (288), the Rho family small GTPases that hitherto has been only known as an inhibitor or RhoA/ROK (see above). The question of whether plexin-B1 deinhibits constitutive negative regulation of RhoA by Rnd1 is yet to be answered.

V. MYOSIN PHOSPHATASE

The physiologically relevant myosin phosphatase (MLCP) responsible for inactivating smooth muscle and nonmuscle II by dephosphorylating its highly specific substrate, RLC bound to myosin heavy chain, is a trimeric enzyme in both avian (6, 345) and mammalian (351) smooth muscle. It consists of a catalytic 37- to 38-kDa PPIc, an associated 110- to 130-kDa regulatory targeting subunit (MYPT1), and a tightly bound 20-kDa subunit of unknown function (reviewed in Refs. 42, 141, 358; Fig. 5). Several other protein phosphatases can dephosphorylate isolated, but not the heavy chain-bound, RLC.

Long thought to be an unregulated “housekeeping enzyme,” we suggested, based on the Ca2+-independent
contractile effect of GTPγS on permeabilized smooth muscle, that smooth muscle MLCP is a G protein-regulated enzyme (356) and verified it by demonstrating that GTPγS and agonists increase RLC phosphorylation independently of changes in [Ca\(^{2+}\)]\(_i\), by inhibiting dephosphorylation of RLC (199, 201). Recognition of MLCP inhibition as the major mechanism of Ca\(^{2+}\)- sensitization (199, 201, 356), combined with the demonstration that pretreatment of smooth muscle with adenosine 5'-O-(3-thiotriophosphate) (ATPγS) under Ca\(^{2+}\)-free conditions caused thio-phosphorylation of MYPT1 and increased the Ca\(^{2+}\) sensitivity of force (391) focused attention on MYPT1 as a ROK for myosin II (113, 143). The inhibitory site phosphorylated by ROK was identified as Thr-696 (133-kDa isoform; Thr-654 of the 130-kDa isoform) in the human sequence (102, 158), although ROK can also phosphorylate other NH₂-terminal threonines (102; reviewed in Ref. 10).

Phosphorylation (or thio-phosphorylation) of Thr-696 MYPT1 through the RhoA/ROK pathway has been detected in a variety of cells, including platelets (271), smooth muscle (166, 301, 373), and human endothelial and prostate cancer cells (362) but is not invariably detectable in intact Ca\(^{2+}\)-sensitized tissues and may be developmentally regulated (reviewed in Ref. 42). MYPT1 undergoes a developmental isoform switch in chicken gizzard smooth muscle (83). The adult gizzard contains an isoform (M130) from which a central region (exon 13) that is present in the embryonic form (M133) is spliced out. The mature aortic smooth muscle retains this sequence (286) that is near to, but does not include, the regulatory phosphorylation site. GTPγS does not Ca\(^{2+}\)- sensitize adult gizzard (311, but cf. Ref. 12), in spite of detectable MYPT1 thio-phosphorylation (although its site has not been determined). In contrast, the tonic chicken aortic smooth muscle that contains the spliced in (M133) MYPT1 isoform is Ca\(^{2+}\)-sensitized by GTPγS without a detectable increase in phosphorylation (311). We have also been unable to detect changes in MYPT1/Thr-696 phosphorylation in chicken amnion smooth muscle Ca\(^{2+}\)-sensitized with GTPγS, although the same site-specific antibody against phospho-Thr-696 readily detected increased MYPT1 phosphorylation in cultured amnion smooth muscle (A. Stevenson, M. Eto, J. D. Matthew, A. P. Somlyo, and, A. V. Somlyo, unpublished observation) and other cells (362). More recently agonist-induced increases in phosphorylation of Thr-853, but not the regulatory Thr-696 site, as well as Thr-38 of CPI-17 was detected in several smooth muscles (198). Thr-853 is not an inhibitory site in vitro (102), but its phosphorylation state could affect localization of MLCP and its catalytic subunit on myosin (401), and thereby, in conjunction with Thr-696 phosphorylation, may be responsible for Ca\(^{2+}\)-sensitizing inhibition of MLCP when Thr-696 is constitutively phosphorylated. It remains to be determined whether the difficulty in detecting phosphorylation of MYPT1 Thr-696 reflects its rapid dephosphorylation (contrary to its resistance to dephosphorylation in vitro; Ref. 378) or operation of another RhoA/ROK-mediated mechanism that does not require Thr-696 phosphorylation or is targeted to a different inhibitory residue not detected by the Thr-696-specific antibody. The functional importance of MYPT1, which binds both PP1c and myosin II (113, 141), is also indicated by the observation that an NH₂-terminal fragment of M130 inhibits dephosphorylation of RLC (443) presumably by competing with the endogenous enzyme.

In contrast to the inhibitory phosphorylation of Thr-696, phosphorylation of another MYPT1 site (probably Ser-430) during mitosis increases myosin phosphatase activity (245, 390).

Protein kinases other than ROK can also phosphorylate MYPT1. They include the zilpikine associated with myosin phosphatase (38, 235) and integrin-linked kinase (81, 196, 275), but not PKN, another RhoA effector that can phosphorylate CPI-17 (139). It is not known whether these kinases have an in vivo function as downstream effectors in a ROK cascade, as suggested by the universal inhibition of G protein-coupled Ca\(^{2+}\)-sensitization by ROK inhibitors. Some of the Ca\(^{2+}\)-independent kinases can also directly phosphorylate and diphosphorylate RLC (261, 416, 433), and they or other constitutively active protein kinase (s) may be responsible for the Ca\(^{2+}\)-independent thio-phosphorylation (with ATPγS) of MYPT1 (301, 391) and for the RLC phosphorylation induced, in the absence of Ca\(^{2+}\), by the phosphatase inhibitors okadaic acid, calyculin A, taumotycin, and microcystin (126, 127, 262, 416 and references therein). Some Ca\(^{2+}\)-independent kinases can be inhibited by staurospsorine, although not by ROK inhibitors (221). Surprisingly, RLC is phosphorylated by a yet to be identified kinase in contracting smooth muscles that are completely Ca/CaM MLCK deficient (H. Wang, A. P. Somlyo, and A. V. Somlyo, unpublished data).

Because the activity of the catalytic subunit PP1c of MLCP is "targeted" to myosin (its specificity increased) by MYPT1 (6, 113, 143), dissociation of these two subunits of MLCP by arachidonic acid (126) or by phosphorylation of a site other than Thr-696 (349) could inhibit MLCP activity without requiring MYPT1 Thr-696 phosphorylation by ROK. In vitro phosphorylation of MYPT1 within residues 40–296 by PKC (388) or Thr-853 by ROK (401) reduces its affinity for, respectively, PP1c and myosin.

Two additional MYPT gene products have been identified in addition to the splice variants of MYPT1 (reviewed in Refs. 42, 141). MYPT2 is 61% identical to MYPT1 and is expressed exclusively in heart and brain (109). Protein expression of MYPT2 in skeletal muscle is not detectable in spite of the presence of abundant message (109), but what are thought to be NH₂-terminal fragments
of MYPT2 are present and accelerate dephosphorylation of skeletal muscle myosin (256). MYPT2 also contains a Thr equivalent to the inhibitory site of MYPT1, suggesting that it may be regulated by phosphorylation. MYPT3, identified as a PP1 partner in a yeast two-hybrid screen, has a prenylatable COOH terminus of still unknown significance (352). Another, p85 protein-related to MYPT1 has been isolated from brain cytosol (381). It binds PP1c and contains regulatory phosphorylation site equivalent to Thr-696 of MYPT1.

Phosphatase inhibitor I, an endogenous phosphopeptide inhibitor of PP1, does not inhibit MLCP (48, 125).

VI. CPI-17 AND PROTEIN KINASE C

CPI-17 is another potential mediator of Ca\(^{2+}\) sensitization that is not dependent on phosphorylation of MYPT1. CPI-17 is a 17-kDa peptide first isolated from porcine aorta (95), whose phosphorylation (on Thr-38) enhances its potency for inhibiting myosin phosphatase. It can be phosphorylated by several kinases (see below) and dephosphorylated by PP2A and PP2C (378). Phospho-CPI-17 inhibits PP1c at nanomolar concentration in solution (96) and at micromolar concentrations in permeabilized smooth muscle. Thiophosphorylated CPI-17 inhibits PP1c ~7,000-fold more efficiently than the nonphosphorylated form (96). CPI-17 was initially recognized as a PKC substrate (96), and introduction of PKC-\(\alpha\) with unphosphorylated CPI-17 into smooth muscle increases RLC phosphorylation and histamine- and GTP\(\gamma\)S-induced Ca\(^{2+}\) sensitization, accompanied by phosphorylation of CPI-17 (197). However, agonist-induced CPI-17 phosphorylation and Ca\(^{2+}\) sensitization are inhibited by Y-27632 (198), consistent with Thr-38 of CPI-17 also being a substrate of ROK (213). Another RhoA effector protein kinase N (PKN) that is also activated by arachidonic acid (reviewed in Ref. 258) can phosphorylate CPI-17 with affinity similar to that of ROK (139), but is not sensitive to inhibition by Y-27632 (165) and hence unlikely to be the kinase phosphorylating CPI-17 in vivo. The MYPT-associated, ziplike kinase (235) can also phosphorylate CPI-17 (94), but the physiologically relevant kinase(s) is yet to be identified. The extent to which Thr-38 phosphorylation of CPI-17 contributes to Ca\(^{2+}\) sensitization of smooth muscle is thought to depend on its expression level: highest in tonic smooth muscle and low, for example, in vas deferens (198). Transfected CPI-17 inhibits MLCP in nonmuscle cells (97), and CPI-17 is endogenous to and functional in cerebral Purkinje cells where its activation modulates long-term synaptic depression (97). Thus CPI-17 or a related phosphorylatable PP1c inhibitor may also Ca\(^{2+}\)-sensitize nonmuscle myosin II (93).

The contribution of PKCs to physiological regulation of smooth muscle myosin II, although the subject of numerous publications (reviewed in Refs. 223, 319, 409) is still uncertain. We will consider the mechanisms of experimentally manipulated PKC activity and attempt to assess them in a physiological context.

Known activators of conventional and novel PKCs (diacylglycerol analogs and phorbol esters) Ca\(^{2+}\)-sensitize force and increase RLC phosphorylation in smooth muscle, but it is not clear whether agonists can increase endogenous diacylglycerol to sufficiently high levels for this effect.

Activation of conventional or novel PKCs by phorbol esters increases RLC phosphorylation independently of a change in [Ca\(^{2+}\)], (112, 253, 316, 407), consistent with a mechanism that inhibits MLCP. Ca\(^{2+}\) sensitization by phorbol ester-stimulated PKCs is not through the RhoA pathway, as it is not inhibited by GDI or by Y-27632 (129) nor is agonist- (155, 173) or GTP\(\gamma\)S-induced Ca\(^{2+}\) sensitization abolished by downregulation of conventional PKCs (407). Conversely, phorbol ester-induced Ca\(^{2+}\) sensitization persists when GTP\(\gamma\)S-induced sensitization is downregulated (128). Angiotensin-induced CPI-17 phosphorylation in cultured smooth muscle cells is inhibited by a PKC inhibitor, but not by Y-27632, whereas the opposite is the case for MYPT1 phosphorylation (341). Whether the cells in this study were contractile or synthetic is not stated, and in cultured smooth muscle angiotensin is a mitogen (291) that can activate noncontractile pathways.

A constitutively active, proteolytic fragment of PKC can Ca\(^{2+}\)-sensitize force and inhibits relaxation (71, 162), but because constitutively active enzymes can also directly phosphorylate RLC (13, 266), the physiological relevance of this effect is uncertain.

Therefore, it was plausible to consider that PKC physiologically mediates Ca\(^{2+}\) sensitization by phosphorylating CPI-17 (94, 203, 426). Phosphorylated CPI-17, a PKC substrate, is a very potent myosin phosphatase inhibitor. However, other results (see above) suggest that PKC is not a necessary component of the RhoA/ROK pathway. Y-27632 inhibits only Ca\(^{2+}\) sensitization induced by very low concentrations of phorbol ester (94) but not the effect of higher concentrations (107), and the highly specific RhoA “buffer” GDI does not relax force induced by stimulation of PKCs with phorbol ester (129). These experiments argue against a major role of PKCs in myosin II regulation by RhoA/ROK, although both mechanisms (ROK and PKC) converge to inhibit dephosphorylation of myosin II (173). Inhibition of agonist-induced Ca\(^{2+}\) sensitization by an inhibitory PKC pseudopeptide suggests a possible role of PKC-\(\zeta\), an atypical PKC activated by arachidonic acid (112), and the inhibition of CPI-17 Thr-38 phosphorylation by a less specific PKC inhibitor suggests that other PKC isoforms may be contributors to Ca\(^{2+}\) sensitization, possibly depending on the expression levels of CPI-17 in different smooth muscles (198, 276).
Protein kinases other than PKC can also phosphorylate the inhibitory (Thr-38) site of CPI-17: ZIP-like kinase (236), PKN (130), integrin-linked kinase (80), PAK (377), and, most importantly, ROK (213). It remains to be shown which of these kinases, particularly ROK, is spatially and kinetically appropriate for phosphorylating CPI-17. With the assumption that arachidonic acid activates the RhoA pathway, inhibition of arachidonic acid-induced Ca$^{2+}$ sensitization by Y-27632 (14, 107), if also accompanied by inhibition of CPI-17 phosphorylation, would support this mechanism.

VII. TYROSINE KINASES: FOCAL ADHESION KINASE

Tyr kinases (reviewed in Ref. 318) were first implicated in inhibitory signaling to smooth muscle myosin II by the finding that three structurally different Tyr kinase inhibitors reduced the amplitudes of contractions of intact smooth muscle stimulated by muscarinic or $\alpha$-adrenergic agonists (85; reviewed in Ref. 84). However, mechanisms that can connect Tyr kinase activity with Ser-19 RLC phosphorylation, such as GEF-activation, are only now beginning to be recognized. For example, isoprostane-induced contractions of vascular smooth muscle are inhibited by both Tyr kinase and ROK inhibitors (Y-27632 and HA1077), but not by inhibitors of PKC (121, 172). A connection between increased Ser-19 RLC phosphorylation and Tyr phosphorylation is also indicated by the effect of the Tyr phosphatase inhibitor diperoxoy vanadate in endothelial cells (117).

Upstream Tyr kinases can mediate, by activating RhoGEFs, Ca$^{2+}$ sensitization of myosin II by RhoA/ROK. The increase in active GTP $\cdot$ RhoA (detected by Rho binding assay) induced by the thromboxane A$_2$ mimetic U-46619 is inhibited by a Tyr kinase inhibitor (325), and both ROK and Tyr kinase inhibitors can inhibit agonist-induced contractions of smooth muscle (172). Similarly, genistein inhibits Ca$^{2+}$ sensitization of force and RLC phosphorylation in $\alpha$-toxin- (366) or $\beta$-escin-permeabilized vascular smooth muscle. The lack of effect of genistein in Triton-skinned preparations indicates that it does not inhibit MLCK or cross-bridge cycling directly, but through a Rho-mediated mechanism that is inactive in Triton-permeabilized preparations (130). In $\alpha$-toxin-permeabilized muscles, genistein reduced force without proportionally inhibiting RLC phosphorylation (366). However, a site other than Ser-19 of RLC may have been phosphorylated in these experiments. In endothelial cells genistein did not inhibit the thrombin-stimulated increase in activated RhoA $\cdot$ GTP, indicating involvement of a genistein-insensitive Tyr kinase or another GEF mechanism (398).

Stimulation of Tyr kinase by $G_q$ and $G_{12,13}$ $\alpha$-subunits and activation of GEFs by Tyr phosphorylation (24, 45, 68, 174) can connect the upstream, G protein-coupled receptors and their related trimeric G proteins to Tyr phosphorylated GEFs that activate RhoA and ROK leading to Ca$^{2+}$ sensitization. It remains to be determined how many different Tyr kinases, such as FAK and p60c-src, are coupled to different receptors and G proteins and activate different GEFs, and whether Tyr phosphorylation plays a role in the recruitment of certain GEFs from the cytoplasm to the plasma membrane (25, 419). Characterization of the kinetics of Tyr kinase activity and RhoA activation should help our understanding of these processes, as suggested by the observation that Tyr kinase inhibitors inhibit the delayed, but not the early, phase of RhoA activation in HEK293T cells (60), raising the possibility that more than one GEF is involved or more than one mechanism transduces the effect of the same GEF. The complexities of upstream RhoA/ROK signaling are also illustrated by a recent report that shows that, although $G_{o13}$ stimulates the unphosphorylated Rho-GEF, LARG, $G_{a12}$ activates it only when it is Tyr phosphorylated (372).

In intact (nonpermeabilized) cells signaling through Tyr kinase, activation and MLCP inhibition may involve both Ca$^{2+}$ sensitization and increases in cytoplasmic Ca$^{2+}$ mediated by FAK (reviewed in Ref. 119). The c-Src Tyr kinase inhibits MaxiK channels causing depolarization and opening of voltage-gated Ca$^{2+}$ channels (8), whereas Ca$^{2+}$ is released from the sarcoplasmic reticulum by InsP$_3$ generated by Tyr-phosphorylated PLC-1 activity (reviewed in Refs. 84, 439).

FAK is a nonreceptor Tyr kinase localized to focal adhesions in cultured cells (reviewed in Ref. 43). RhoA appears to function both upstream and downstream of FAK, perhaps depending on cell type and type of stimulus. Inactivation of RhoA with C3 inhibits Tyr phosphorylation of FAK in some cells (260), whereas in other cells, such as tracheal smooth muscle, Tyr phosphorylation of neither FAK nor of another focal adhesion protein, paxillin, is inhibited by Y-27632 (249). Downregulation of FAK expression by antisense peptides reduces the increase in $[Ca^{2+}]_i$ induced by acetylcholine or KCl, consistent with other evidence that Tyr phosphorylation in smooth muscle contributes to myosin II regulation, in part, by altering $[Ca^{2+}]_i$ (8). FAK Tyr phosphorylates and activates one or more Rho $\cdot$ GEFs (see above; Ref. 43) and could thereby increase Ca$^{2+}$ sensitivity of myosin, at least locally, by activating Rho/ROK. Anchoring of receptor-Tyr kinase (RTKs) to the cell membrane and localization of nonreceptor Tyr kinases to its proximity (52) provide further support of a membrane-associated receptor-trimeric G protein RhoGEF-RhoA-ROK sequence activation mechanism.
VIII. MITOGEN-ACTIVATED PROTEIN KINASE, P21-ACTIVATED KINASE, AND LIM-KINASE

A. MAPK

MAPKs and their activating kinases form complex, interconnected cascades whose nuclear activity is involved in transcriptional regulation (reviewed in Ref. 54). It has been suggested that MAPK regulates myosin II indirectly through caldesmon. This thin filament-associated protein inhibits actomyosin ATPase activity in vitro, and this inhibition is removed when caldesmon is phosphorylated by MAPK (306). Several studies, however, failed to show operation of this mechanism in vivo. Although MAPK can stoichiometrically phosphorylate caldesmon in permeabilized smooth muscle, it does not Ca\(^{2+}\)-sensitize force (279), and inhibitors of p42 and p44 MAPK that prevent its activation by Ca\(^{2+}\) (185) and by histamine do not affect the Ca\(^{2+}\) sensitivity of maximal force (133). The time course of caldesmon phosphorylation is also not well correlated with its anticipated effect in modulating contractile activity (2). PAK1, the predominant isoform in smooth muscle, inhibits Ca\(^{2+}\) sensitivity of permeabilized smooth muscle (422) by phosphorylating MLCK, without phosphorylating caldesmon. MAPK is activated by [Ca\(^{2+}\)\_i] and by agonists (73), as well as by stretch (105), generally applied during measurement of isometric contractions and complicating the interpretation of experiments performed on intact cells and tissues. Ca\(^{2+}\) sensitization of tracheal, but not colonic, smooth muscle by a commercial, preactivated MAPK (120) may have resulted, like Ca\(^{2+}\)-independent contraction stimulated by PAK3 (248), from altered substrate specificity of the enzyme. The majority of evidence suggests that phosphorylation of caldesmon by MAPK does not mediate Ca\(^{2+}\) sensitization (279, 434). Similarly lacking is evidence of Ca\(^{2+}\)-sensitizing activity of another smooth muscle specific protein, calponin (246), although this does not preclude effects of these actin-associated proteins on actomyosin ATPase activity, similar to the enhancement of tonic force by MgADP at constant RLC phosphorylation (192).

Activation of MAPK (ERK1) accelerates cancer cell migration associated with RLC phosphorylation that has been attributed to phosphorylation of MLCK by MAPK (205, 273). Such stimulated migration is integrin-species dependent (273), also requires ROK activity (175), and is not readily ascribed to a single mechanism. The long-term effects of MAPK in nonmuscle cells may be due to slow activation of MLCK by MAPK and enhanced by ROK-mediated inhibition of myosin phosphatase or to a less direct, slow mechanism. The lack of a detectable effect of MAPK on contractility (279) argues against its contributing to a fast and direct mechanism of myosin II regulation.

B. PAK

PAK Ser/Thr-kinase isoforms are effectors of the Rac1 and Cdc42 members of the Rho subfamily (18, 226). Phenotypes induced by Rac and Cdc42 are characterized by, respectively, lamellopodia and filopodia, in contrast to the stress fibers and focal adhesions induced by the RhoA/ROK pathway (312). PAK family kinases are activated upon binding GTP \cdot Cdc42 or GTP \cdot Rac followed by autophosphorylation. The effects of PAK on myosin II are the subject of somewhat contradictory reports. Phosphorylation of myosin II by PAK1 has been inferred from finding phosphorylated RLC communoprecipitated with activated PAK1 (343) and from in vitro phosphorylation of RLC by proteolytically activated PAK3 (395). In the latter study, contractions induced in permeabilized smooth muscle by PAK3 were not accompanied by RLC phosphorylation, possibly as a result of dephosphorylation during storage at -20°C. A constitutively active PAK3 slowly contracted permeabilized smooth muscle, but the phosphorylation state of RLC was not determined in this study (248), whereas PAK1 can phosphorylate Ser-19 in endothelial cells (440).

In contrast to the above studies suggesting that PAK phosphorylates RLC, other results indicate that PAK controls myosin negatively, by phosphorylation of MLCK (263, 422). Phosphorylation of MLCK by PAK, like phosphorylation by PKA, is inhibited by bound CaM (123). Inhibition of MLCK by PAK would not have been detectable in experiments (395) in which PAK acted in the absence of Ca\(^{2+}\) and, therefore, not allowing the detection of its effect on Ca/CaM-activated MLCK.

PAK negatively regulates myosin II in several cell types by inhibiting RhoA/ROK, as indicated by loss of stress fibers and dissolution of focal adhesions by Cdc42 (reviewed in Ref. 226). Activators of PAK are also antagonistic to ROK upstream: constitutively active Cdc42 and Rac (G12VCdc42 and Q61LRac1) inhibit Ca\(^{2+}\) sensitization by constitutively active (GTP \cdot G14V) RhoA (129). Rac and its GEF Tiam1 may also affect RhoA/ROK activity by reducing the availability of active GTP \cdot RhoA through inhibition of nucleotide exchange or enhancement of Rho GAP activity (326), but these mechanisms do not explain the antagonism of Cdc42 and Rac against GTP \cdot RhoA Val-14 that is already activated and stably complexed with GTP (129).

PAK inhibits migration of endothelial (195) and tracheal smooth muscle (77) cells, and although (exogenous) PAK phosphorylates RLC (57, 195), this may be related to overexpression and use of constitutively active enzymes. PAK1 can also phosphorylate MLCK and reduce its V\(_{\text{max}}\) and (constitutively active) PAK1 inhibits RLC phosphorylation, indicative of negative regulation of myosin II (327), and PAK2 inhibits tension development by endothelial monolayers (123).
Evaluation of the respective roles of ROK and PAK in endothelial cell function is further complicated by the qualitatively, quantitatively, and temporally different effects of activated thrombin receptors, compared with activation of EDG receptors by SIP (293, 405). Although both thrombin and SIP activate the RhoA-ROK pathway in a variety of cells, more detailed, time-resolved examination of their effects on human umbilical vascular endothelial cells (HUVECs) reveals that thrombin induces rapid and robust RhoA activation but inhibits Rac1, whereas SIP causes only weak and delayed activation of RhoA but activates Rac1 and, presumably, PAK in endothelial cells (405).

In general, we did not find direct evidence that wild-type (not preactivated and/or overexpressed) PAK increases RLC phosphorylation of myosin II in vivo either in smooth muscle or in nonmuscle cells. We consider it likely that, under physiological conditions, Cdc42 and Rac inhibit myosin II activity either through phosphorylation of MLCK by PAK, as shown recently for smooth muscle (263, 422), and/or by upstream antagonism to Rho itself.

C. LIM-Kinase

LIM-kinase (LIMK), a LIM-domain containing Ser/Thr/Tyr/protein-kinase is a downstream effector of Rho family GTPases (reviewed in Ref. 17) that phosphorylates the actin depolymerizing factors coflin (on Ser-3) and ADF and inactivates their depolymerizing activity (287, 370). Regulation of cytoskeletal organization through actin depolymerization does not directly affect myosin II ATPase but has the sometimes overlooked effect of a basic property of actomyosin; only polymerized F-actin, but not G-actin, activates myosin II ATPase. ROK phosphorylates and activates LIMK (239), which decreases coflin activity to increase the availability of polymerized F-actin to activate myosin II ATPase. Although stabilization of F-actin by LIMK may be sufficient for polymerization of actin into stress fibers (11), both phosphorylated RLC and F-actin are required for developing significant force and motility through actomyosin ATPase activity. RhoA/ROK contributes to both: it inhibits MLCP to increase RLC phosphorylation and promotes polymerization of G- to F-actin through LIMK activity. Conversely, regions containing significant concentrations of active, nonphosphorylated coflin can be expected to be deficient in F-actin and have low myosin II ATPase activity. An additional object lesson learned from these experiments with constitutively active recombinant enzymes is that whereas a truncated, constitutively active ROK activates both LIMK1 and LIMK2, full-length ROK selectively activates LIMK2, but not LIMK1 (370).

IX. CALCIUM DESENSITIZATION: GUANOSINE 3',5'-CYCLIC MONOPHOSPHATE-MEDIATED PATHWAY AND ANTAGONISM TO RHOA

cAMP and cGMP reduce [Ca\textsuperscript{2+}], and relax smooth muscle through their cAMP- (PKA) and cGMP-activated (PKG) kinases (50, 410, 418). Several effects of cAMP are mediated by cross activation of PKG (50, 104), and significant components of the relaxant effects of cGMP are mediated through Ca\textsuperscript{2+} desensitization (188) (Fig. 6). Inhibition of cAMP-induced Ca\textsuperscript{2+} desensitization by a relatively selective PKG inhibitor also suggests that major effects of both cAMP and cGMP on the Ca\textsuperscript{2+} sensitivity of myosin II are mediated by PKG, rather than PKA, whereas the nitric oxide (NO)-induced decrease in [Ca\textsuperscript{2+}] is independent of PKG (418), and perhaps mediated by PKA cross-activated by cGMP.

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**Table: Ca\textsuperscript{2+}-desensitization**

<table>
<thead>
<tr>
<th>Inhibition of Ca\textsuperscript{2+}-sensitization</th>
<th>Activate MLCP</th>
<th>Inhibit MLCK</th>
</tr>
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<tbody>
<tr>
<td>Inhibition of RhoA</td>
<td>? cAMP-PKA</td>
<td>CAM-Kil</td>
</tr>
<tr>
<td>- C3, EDN, Toxin B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- PKG phosphorylation of S188 of GTP•RhoA</td>
<td>cGMP-PKG</td>
<td>PAK</td>
</tr>
<tr>
<td>- PKA phosphorylation of G\textsubscript{a13}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibition of ROK</td>
<td>? Telokin</td>
<td>? PKA non-muscle</td>
</tr>
<tr>
<td>- RND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Y-27632, HA-1077 and H-1152P</td>
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<td></td>
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<tr>
<td>Dephosphorylation of CPI-17</td>
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Fig. 6. Potential upstream signaling pathways (described in text) that lead to Ca\textsuperscript{2+} desensitization. The Rnd subfamily of Rho proteins is membrane-attached by their farnesylated COOH terminus and binds, but does not hydrolyze, GTP. They inhibit RhoA signaling (47, 229; but cf. Ref. 288). Cyclic nucleotides have the potential to inhibit Ca\textsuperscript{2+} sensitization through PKG phosphorylation of Ser-188 RhoA•GTP (331, 335), through PKA phosphorylation of Go\textsubscript{13} and PKA or PKG phosphorylation of telokin (237, 408, 428), phosphorylation of MYPT1 by PKG or its other interactions with MYPT1 (269, 371), and through PKA phosphorylation of MLCK (72). The latter may play a role in phosphorylation of nonmuscle myosin II, but less likely in the case of smooth muscle myosin (367).
Desensitization to Ca\(^{2+}\) is mediated by PKG that is activated by cGMP produced by a soluble guanylate cyclase activated by NO or by a particulate (membrane-associated) receptor cyclase activated by atrial natriuretic factors (ANFs). The existence of a cAMP/PKA-specific pathway, separate from PKG in some species or cells, is indicated by the relaxation of PKGα-deficient (null) smooth muscle by a cAMP analog, although in nonpermeabilized cells this may result from decreased [Ca\(^{2+}\)]\(_i\) (299). The PKG knock-out animals are hypertensive and suffer intestinal dysfunctions, demonstrating the physiological importance of the PKG and also the RhoA/ROK pathway. The Ca\(^{2+}\)-regulatory defect of PKGα-deficient mice is rescued by transfection of PKGα (100). As in the case of Ca\(^{2+}\)-sensitizing agents that increase the sensitivity of myosin II to Ca\(^{2+}\) while also increasing [Ca\(^{2+}\)]\(_i\), Ca\(^{2+}\) sensitization by PKG operates in parallel with mechanisms that reduce [Ca\(^{2+}\)]\(_i\). Inhibitory phosphorylation of PLC-β2 by PKG contributes to relaxation of intact (nonpermeabilized) smooth muscle by NO-cGMP (431), as does inhibitory phosphorylation of the InsP\(_3\) receptor, Ca\(^{2+}\)-release channel by reducing [Ca\(^{2+}\)]\(_i\) (208).

PKG causes a modest shift in Ca\(^{2+}\) sensitivity in some (taenia coli) but not other (chicken gizzard) permeabilized smooth muscles (278, 300). Desensitization of Triton X-100 detergent-skinned preparations (428), in which the Rho/ROK mechanism is inactive (130), suggests that PKG can also increase the activity of MLCP independently of antagonizing ROK. Direct stimulation of MLCP may not require phosphorylation of an activating site of MYP1, although this has not been excluded, but may occur through interaction between the leucine zipper motifs of PKGIα and MYP1 (371). The specific sites of phosphorylation under these conditions are not known nor is it known whether phosphorylation of either protein affects their leucine zipper interaction. A role of the leucine zipper interaction in PKG-mediated Ca\(^{2+}\) desensitization has also been suggested by a correlation between the developmental switch when chicken gizzard loses its leucine zipper and its resistance to 8-bromo-cGMP-induced dephosphorylation of myosin II and relaxation (42, 191). Because PKG inhibitors inhibit Ca\(^{2+}\) desensitization (188), it would be of interest to know whether they also inhibit heterodimerization of PKG with MYP1 (371).

Inhibition of the RhoA/ROK pathway is perhaps the most important mechanism of Ca\(^{2+}\) desensitization by cGMP, as first shown by the dramatic reversal of GTPγS (and agonist)-induced Ca\(^{2+}\) sensitization of force and RLC phosphorylation in smooth muscle (176, 429) and recently by the effect of upstream stimulation of PKG by NO (65). Antagonism between ROK and PKG pathways is also shown by the enhanced α-adrenergic activation of RhoA/ROK when NO production is inhibited (49).

Although it has been suggested that PKG inhibits RhoA/ROK by phosphorylating Ser-188 of RhoA (136, 331), this phosphorylation, while demonstrable in vitro, was not detectable in vivo in several laboratories (92, 331, 335). Interpretation of the in vivo results is largely based on the failure of PKG to inhibit responses of cells transfected with RhoA in which Ser-188 was mutated to (nonphosphorylatable) Ala (86, 331, 335), although both NO-induced Ca\(^{2+}\) desensitization of microvessels (34) and transcriptional effects of such mutated RhoA are inhibited by PKG (136). Antagonism of RhoA/ROK may also be affected through phosphorylation of Go\(_{13}\) by PKA that inhibits, in CHO cells, Q\(_{413}\)-mediated activation of RhoA (242). We have also been unable to detect RhoA Ser-188 phosphorylation in mature smooth muscle and found that whereas PKG phosphorylates uncomplexed RhoA in solution, it does not phosphorylate the RhoA · GDI complex (L. Walker, A. V. Somlyo, and A. P. Somlyo, unpublished observations), probably due to the sterically unfavorable position of Ser-188 that is extremely close to the hydrophobic pocket of GDI involved in strong RhoA binding (231). Ser-188 phosphorylation of RhoA in cultured vascular smooth muscle cells, at rest or stimulated with insulin, another activator of PKG (22), was also detected in GTP · RhoA coprecipitated with the RBD domain of Rhotekin, rather than in a GDP · RhoA · GDI complex. PKG may inhibit RhoA/ROK by phosphorylating active GTP · RhoA after it dissociated from GDI, by preventing RhoA binding to ROK and other effectors (86). This interpretation is also supported by findings that Ser-188 is phosphorylated following muscarinic stimulation of intestinal smooth muscle cells, but not in resting cells containing RhoA complexed with GDI (264). Inhibition by PKG of transcriptional regulation by RhoA is also independent of Ser-188 phosphorylation and thought to involve effects of PKG downstream of RhoA (136).

Insulin-induced inhibition of geranyl-geranyl transferase has also been suggested to mediate inhibition of RhoA/ROK by PKG (22), because it inhibits the translocation, required for Ca\(^{2+}\) sensitization, of endogenous RhoA to the cell membrane (130). Whether this mechanism operates to inhibit SIP-mediated translocation in mature (noncultured) cells (34) remains to be determined. Long-term positive regulation of RhoA/ROK by the NO-cGMP-PKG pathway is revealed through inhibition of RhoA expression by PKG inhibitors (333). Inhibition of angiotensin-induced RhoA activation by pretreatment with 8-bromo-cGMP (341) may reflect the effect of stimulants in culture media.

Telokin is another suggested mediator of cGMP-mediated Ca\(^{2+}\) sensitization (428). Phosphorylation of Ser-13 of telokin in situ is significantly increased by 8-bromo-cGMP (428) or forskolin (237, 408). The loss of telokin from permeabilized smooth muscle is accompanied by loss of 8-bromo-cGMP-induced Ca\(^{2+}\) desensitization that.
Ca\textsuperscript{2+} SENSITIVITY OF SMOOTH MUSCLE AND NONMUSCLE MYOSIN II

X. CALCIUM SENSITIZATION IN PLATELETS AND ENDOTHELIAL CELLS AND CELL MIGRATION

Platelets, endothelial cells, fibroblasts (stress fibers), and other migrating cells are the most extensively investigated nonmuscle cells used for identifying phenotypes of RhoA/ROK-regulated Ca\textsuperscript{2+} sensitivity of myosin II. Platelets, when activated by agonists, such as thrombin, ADP, thromboxane A\textsubscript{2}, serotonin or epinephrine, employ the whole regulatory armamentarium available to smooth muscles: InsP\textsubscript{3}-induced Ca\textsuperscript{2+} release, MLCK, MLCP, RhoA, and ROK. Activation of RhoA results in shape change, phosphorylation of MYPT1 and RLC (Ser-19), and ATP secretion that can be inhibited by the ROK inhibitors HA-1077 and Y-27632 (372). Considering the relationship between the contributions of, respectively, Ca\textsuperscript{2+} release and RhoA/ROK activity to platelet activation, recapitulation (30, 296) of the experimental paradigms of smooth muscle showed that RLC phosphorylation during platelet activation also involves both Ca\textsuperscript{2+} sensitization and increased [Ca\textsuperscript{2+}]. The elegant demonstration that upstream activation of the RhoA/ROK pathway by thromboxane A\textsubscript{2} or thrombin receptors is transmitted through G\textsubscript{Q12} and G\textsubscript{Q13} subunits of trimeric G proteins in G\textsubscript{Q12}-deficient platelets provided one of the most comprehensive characterizations of this pathway, including the rapid activation of at least two Tyr kinases (204). Surprisingly, in platelets a cAMP but not a cGMP analog inhibits RLC phosphorylation and antagonizes shape change induced by thromboxane A\textsubscript{2} or thrombin (204), perhaps also through the ability of PKA to reduce [Ca\textsuperscript{2+}], and thereby MLCK activity. The relatively rapid (within 10 s) Tyr phosphorylation that follows activation of G\textsubscript{Q12} and G\textsubscript{Q13} by thromboxane A\textsubscript{2} receptors and is associated with increased activity of PP-72\textsubscript{NYS} and PP-60-Src suggests that in platelets, as in other cells, Tyr phosphorylation activates GEFs.

Important endothelial functions, cell migration, endothelial permeability, and angiogenesis are powerfully affected through regulation of myosin II by RhoA/ROK (reviewed in Ref. 7). The thrombin-induced increase in endothelial permeability (46) is due to a Ca\textsuperscript{2+}-sensitizing mechanism akin to that of smooth muscle: it is inhibited by C3 and Y-27632 (90), and chelation of Ca\textsuperscript{2+} inhibits the “rapid” phase of the histamine-induced permeability increase, whereas the “tonic” phase induced by thrombin or lysophosphatidic acid is inhibited by Y-27632 (397, 398, 399) and by Tyr kinase inhibitors (387). Blockade of Ca\textsuperscript{2+} entry into endothelial cells also inhibits stress fiber formation that, in the presence of normal Ca\textsuperscript{2+} entry, is inhibited by C3 (243), further indicating that it is the concerted actions of MLCK and RhoA/ROK that increase RLC phosphorylation. Similarly, inhibition of MLCK also blocks thrombin-induced cell rounding and retraction (404). Migration of bovine pulmonary endothelial cells is also stimulated by RhoA/ROK, although the relationship between ROK activity and migration is stimulus dependent: stimulus by S1P is inhibited by Y-27632, whereas vascular endothelial growth factor (VEGF)-stimulated migration is inhibited by MLCK and Tyr kinase inhibitors (227). VEGF-induced endothelial cell migration during capillary lumen formation is also mediated, in some systems, by other Rho family GTPases (Rac, Cdc42; Ref. 21). Thrombin- and histamine-induced increases in endothelial permeability are mediated by several Rho-family proteins (425), whereas Rac1 contributes to cadherin-mediated cell-cell adhesion in some cell types (reviewed in Ref. 179).

Cell migration (reviewed in Ref. 415) is one of the most general biological functions, from the earliest phase of morphogenesis to the latest of cancer. An important
component of endothelial and other cell migration in vitro is actin-polymerization/depolymerization that is controlled by Rac1 and generates movement of the lamellopodial leading edge (355; reviewed in Refs. 138 and 415). This mechanism may be dominant when RhoA activity is low, and consequently, cell attachment to substrates through ROK-induced focal adhesions is weak. Under such conditions, and with PAK conveniently colocalized with actin, phosphorylation of RLC by Rac1-activated PAK2 may provide sufficient retractive force that is otherwise provided by ROK and myosin II (43, 361) for detaching the tail of a migrating cell (440) even without normal ROK activity. This (or difference in cell types) may also explain why inhibition of ROK accelerates cell movement during wound healing (138) but inhibits long-held belief that the tonic response is due solely to influx and/or transiently high \([\text{Ca}^{2+}]_i\) due to influx and/or release, although \([\text{Ca}^{2+}]_i\) does not cause translocation of RhoA to the membrane (indicative of its activation), except at very high \(\text{pCa} 4.5\) levels (127).

Cells utilize both Ca/CaM-activated MLCK and \([\text{Ca}^{2+}]_i\) also contributes to cell migration, as RLC phosphorylation and the resulting actomyosin ATPase activity provide force for detaching the tails of migrating cells from the substrate. Y-27632 inhibits tail detachment of migrating human cancer cells (361), and transendothelial migration of monocytes (427) and neutrophils (5). Consistent with mechanisms coordinated between ROK and MLCK, C3 and Y-27632 inhibit neutrophil migration, stress fiber formation, and RLC phosphorylation (324). Similar complementarity of action between MLCK and RhoA/ROK is required for endothelial cells to contract and open the gaps that permit transendothelial migration: endothelial cell retraction is accompanied by \([\text{Ca}^{2+}]_i\)-dependent RLC phosphorylation (430) that is enhanced through inhibition of MLCP by RhoA/ROK (91).

ROK activity in whole animals received, until recently, little or no attention, perhaps because an important (first) study of a selective ROK inhibitor, Y-27632, found it to have little systemic effect at doses that reduced blood pressure of several hypertensive animal models, but not of normotensives (393). More recent studies showed that Y-27632 reduces the amplitude of gastric contractions in conscious rats (387), but the delivery routes were different (oral vs. intravenous), precluding direct comparison of these results. Inhibition of ROK with Y-27632 also reduces the amplitude of the spontaneous and oxytocin-induced tonic component of contractions of (isolated) human myometrium and even the force induced by high K\(^+\), without affecting \([\text{Ca}^{2+}]_i\) (220). Physiological tonic activity of RhoA/ROK downregulates erectile function and maintains the flaccid (contracted) state, whereas inhibition of ROK with Y-27632 and adenosine gene transfer of dominant negative RhoA enhances erectile function (63) and relaxes corpus cavernosum smooth muscle causing vasodilation and erection (66, 251, 307, 412). All the known components of the RhoA/ROK pathway, including MYPT1 and CPI-17, are present in corpus smooth muscle in which, moreover, RhoA content is 17-fold higher than in ileum smooth muscle and RhoA activation is demonstrable (412). Naturally, the therapeutic use of ROK inhibitors for erectile dysfunction is being considered (180). ADP-ribosylation of endogenous RhoA with transfected C3 reduces the sensitivity of hamster resistance vessels to extracellular \([\text{Ca}^{2+}]_i\) (34), and Y-27632 dilates the basilar artery of anesthetized normotensive rats, suggesting that in these blood vessels, like in corpus cavernosum, RhoA/ROK is constitutively active, and it is likely to be a tonically active regulator in several tissues of the whole organism.

The physiological importance of the RhoA/ROK pathway is also indicated by its early phylogenetic appearance in important cellular functions, cytokinesis and embryonic morphogenesis, in Caenorhabditis elegans (302, 423, 424) and Drosophila (170).
B. Diseases of Smooth Muscle

Recent comprehensive reviews show increasing recognition of the roles of RhoA/ROK in disease and as a therapeutic target (31, 74, 421). It is not surprising, given the early history of G protein-coupled Ca\(^{2+}\) sensitization of smooth muscle by RhoA/ROK (reviewed in Ref. 358), that abnormalities of smooth muscle function, hypertension, cerebral and coronary vasospasm, bronchial asthma, preterm labor, and erectile dysfunction lead the list of diseases in which RhoA/ROK signaling is upregulated (reviewed in Ref. 421). Hyperactivity of RhoA/ROK plays a role in the three experimental models of hypertension in which Narumiya’s group originally showed that inhibition of ROK reduced elevated blood pressure (393): vascular ROK mRNA levels are increased in the SHR model (259), and levels of active GTP \( \gamma \) RhoA are increased in aortic smooth muscle of angiotensin II-induced hypertensive rats, spontaneously hypertensive rats, and \( N^2 \)-nitro-L-arginine methyl ester (L-NAME)-treated rats (340, 341). Similar upregulation of ROK mRNA, produced by exposure of coronary artery adventitia to interleukin-1, accompanies the resulting MYPT1 (Ser-854) phosphorylation and hyperresponsiveness to 5-hydroxytryptamine (serotonin) that are inhibited by Y-27632 and by hydroxyfasudil (182, 347). In this context, the responsiveness of human vascular smooth muscle to ROK inhibition (20, 412) also points to the clinical potential of ROK inhibitors.

pronounced Ca\(^{2+}\) sensitization of coronary arterial smooth muscle by serotonin is found in genetically hypertensive rats (330), and oxidized low-density lipoprotein (LDL), strongly implicated in atherosclerosis, also increases the Ca\(^{2+}\) sensitivity of depolarized hamster resistance arteries (33) and endothelial cell contraction and permeability (91).

RhoA/ROK activity is also prominent in cerebral vasospasm. Experimental cerebral vasospasm induced by subarachnoid hemorrhage is accompanied by elevated ROK activity and MYPT1 phosphorylation (albeit at Ser-854; Ref. 329), and cerebral vasodilation by Y-27632 is significantly greater in spontaneously hypertensive rats than Wistar-Kyoto controls (67). Relaxation of cerebral vasospasm by the ROK inhibitor hydroxyfasudil is accompanied by a decrease in RLC phosphorylation, consistent with ROK inhibition (268), and ROK inhibitors are now clinically used in Japan for treating cerebral vasospasm (328). Long-term effects of vascular injury, such as arteriosclerotic coronary lesions, are associated with MYPT1 phosphorylation and regress when treated with a ROK inhibitor (346). Inhibition of Rho with C3 or ROK with Y-27632 inhibits angiotensin II-induced protein synthesis (432), perhaps indicating usefulness of ROK inhibition for reducing not only blood pressure, but also hypertensive DNA synthesis (340) and vascular remodeling (292). Stimulation of vascular smooth muscle cell migration by thrombin is mediated by RhoA/ROK and is likely to have major pathological significance through its contribution to neointimal thickening by migrated smooth muscle cells (334, 339). Inclusion of ROK inhibitors(s) into drug-eluting stents may be useful for prevention of postangioplasty restenosis.

Y-27632 and C3 also reverse antigen-induced Ca\(^{2+}\) sensitization and increased RhoA expression in airway smooth muscle (59, 160, 161), suggesting that ROK inhibitors may also be useful for the treatment of asthma. Ca\(^{2+}\) sensitization by tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \); Ref. 294) implicated in asthma seems to utilize RhoA/ROK as its effector, as indicated by the response of cultured airway smooth muscle cells (157). The long sought after mechanism of hypoxic pulmonary vasoconstriction also involves ROK; it is reversed in perfused lungs by Y-27632, and hypoxia-induced RLC phosphorylation in pulmonary resistance vessels is inhibited by both C3 and Y-27632 (414).

C. Cancer

The importance of RhoA for Ras-mediated oncogenic transformation in cancer is well known (323; reviewed in Refs. 31, 303, 322, 445), and its role in cancer cell migration and invasiveness is increasingly recognized (169, 361, 362). More recent is the demonstration of the role of RhoA/ROK in angiogenesis, including cancer neoangiogenesis (361, 362).

The contribution of RhoA to Ras-mediated oncogenic transformation and transcriptional regulation is not a manifestly direct effect of ROK, although it requires ROK activity (136, 238, 322). Modulation of transcription of smooth muscle-specific genes by ROK activity is related to G/F actin (228, 238) and nuclear/cytoplasmic serum response factor (SRF) ratios (228) and, perhaps, nuclear lipoma preferred partner protein (LPP) (198). However, it is important to recall in this context that ROK is not the only effector of RhoA and, therefore, the consequences of upstream inhibition of RhoA with C3 or dominant negative RhoA expression are not necessarily identical to those of downstream inhibition of ROK with Y-27632. For example, upstream inhibition of RhoA by C3 or a statin inhibitor inhibits cell proliferation (82), whereas ROK inhibitors do not block proliferation in vitro (361). Cancer cell motility, migration (169), and metastasis are the mechanisms in which the direct role of ROK in cancer has been most closely identified. Human prostate cancer cell migration is inhibited by Y-27632 both in vitro and in vivo (361, 362), indicating the importance of actomyosin activity regulated by RhoA/ROK. Passage of cancer cells into and out of the circulation, through the endothelial barrier, is also expected to be facilitated by Ca\(^{2+}\) sensitization of endothelial RhoA/ROK and actomyosin-mediated endothelial contraction and formation of gaps.
Tumor growth beyond a certain size also requires neoangiogenesis (103) that is supported by ROK: both endothelial vacuole formation, the earliest phase of angiogenesis, and the later phase of endothelial sprouting and endothelial cell migration are inhibited by ROK inhibitors, Y-27632 and WI-536 (361, 362). Some cancer cells (e.g., LnCAP prostate cancer) also possess Ser proteinase-activated receptors that can activate RhoA (135), and the role of some of these proteinases as invasion regulators (82) may be responsible for multiple contributions of RhoA to cancer progression, and explain the beneficial effects of combined ROK and urokinase-type plasminogen activator inhibition (273). Other human cancers also migrate by mechanisms facilitated by ROK: migration of human pancreatic cancer cell lines is inhibited by antisense oligonucleotide against ROK (183), whereas HMG CoA reductase inhibitors (statins and geranyl-geranyl transferase inhibitors) inhibit the invasiveness of aggressive breast cancer cells (82), by inhibiting geranyl-geranylation of RhoA that is required for its association with the cell membrane and activation.

XII. WANTED: RHOAD MAPS THROUGH DOMAINS TORTUOUS, BUT RICH

The major pathway of RhoA/ROK signaling is now well established: RhoA activates ROK to inhibit myosin phosphatase, leading to phosphorylation of the RLC of smooth muscle and nonmuscle myosin II, thus increasing actomyosin ATPase-based motility and force development. Significant evidence indicates that this mechanism operates under physiological conditions and, when altered, as a contributor to a number of disease states. A great number of agonists and other stimuli can initiate signaling through RhoA/ROK, but numerous questions remain unanswered about the target and outcome selectivity of different stimuli: how they can produce a variety of responses in different cells and organs, regulating processes ranging from axonal guidance and vasculogenesis in the embryo through the reproductive, erectile state to cancer metastasis.

Biological, including RhoA-mediated, signal transduction resembles the operation of a huge Lego set in which the various pieces can be assembled differently for appropriate cellular localization and function of the components: receptors, trimeric and monomeric G proteins, RhoGEFs, receptor and nonreceptor Tyr kinases, RhoGAPs and effectors, acting with appropriate kinetics, to yield a cohesive image of function and phenotype (Fig. 4).

Some of the most important recent information about pieces of this mosaic is the identity of RhoGEFs that deliver the upstream signal from an activated receptor to yield GTP · RhoA from the inactive GDP · RhoA · GDI complex. However, not withstanding the great value of the information already derived from studies of a variety of transfected, cultured cells expressing, coexpressing, and overexpressing different receptors, trimeric G proteins, and RhoGEFs, information about their physiological roles at the cellular level and within the whole organism is limited, and the quantitative contributions of the professional actors, namely, the endogenous, not transfected, cellular proteins to the signaling drama are not yet sufficiently revealed. We believe that it will be important to determine how endogenous RhoGEFs operate cell and pathway selectively: motivated not only by curiosity about the role of this fundamental biological mechanism, but also because GEFs may be attractive targets for therapeutic intervention in, for example, tumor angiogenesis.

The “Lego set” of signaling also has the characteristics of a fractal mosaic, breaking through increases in our understanding into ever smaller components. The smallest components now visualized under the microscopes of structural genomics and structural biology are protein domains: amino acid sequences within a protein capable of independently folding into structural entities having distinct functional roles. The domain structures of signaling proteins, whether targeting to the membrane and to trimeric G proteins, like RGS, PDZ or PH domains of RhoGEFs or to other partner proteins or substrates like LIM domains, provide essential directional cues for signaling. In this case, again, determining how the different activated receptors, GPCRs, plexins, or ephrins utilize their cytoplasmic tails or other mechanisms to attract the appropriate RhoGEFs to initiate signaling within a cellular, localization-specific context to yield distinctive responses or phenotype awaits exploration.

The mechanisms that can selectively inhibit RhoA/ROK by, for example, inhibiting Rho prenylation to prevent ROK activation at the cell membrane or by activating cyclic nucleotide-regulated protein kinases upstream through insulin receptors and with nitric oxide, are, similarly, potential therapeutic targets. As the picture of RhoA/ROK signaling emerges after an era dominated by the fruitful studies of electrical signaling in biology, as part of the more complex panorama of chemical signal transduction, we now have to learn, hopefully soon, how Maxwell’s demon, hidden in the details, manipulates and selects the various domains of signaling proteins and modifies their kinetics to produce this large and moving mosaic.

Until, “quindi uscimmo a riveder le stelle” (Dante Alighieri).

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