Myosin Phosphatase Regulatory Pathways
Different Functions or Redundant Functions?

Thomas M. Lincoln

In many ways, the smooth muscle cell (SMC) has been at the forefront in defining cellular signaling pathways. For example, the initial description of agonist-dependent intracellular calcium ([Ca$^{2+}$]) transients was made on vertebrate vascular SMC using aequorin as the indicator. Delineation of nitric oxide (NO) and cGMP signaling was first accomplished in smooth muscle. And more recently, insights into the general mechanisms of gene transcription that dictate phenotypic traits of cells are being defined by smooth muscle specific gene expression. It should come as no surprise then that the interactions among many signaling pathways in cells should be explained first in the SMC. Perhaps the reason for the central position of the SMC in defining paradigms in cellular biology is that there are well-defined physiologic end points amenable to measurement (ie, contractile force) and even better described and easily quantified biochemical pathways that underlie said physiologic end points (ie, myosin regulatory light chain phosphorylation).

From a historical perspective, the role of protein phosphorylation in smooth muscle contraction as a paradigm for cell signaling follows only that for the control of glycogen metabolism (see 6-8 for reviews). Increases in the levels of cytosolic [Ca$^{2+}$], initiate smooth muscle contraction by binding to the universal intracellular Ca$^{2+}$ receptor protein, calmodulin (CaM), which in turn binds to and activates smooth muscle myosin light chain kinase (MLCK). Activated MLCK catalyzes the phosphorylation serine-19 of the regulatory myosin light chain kinase (MLCK). The question is: how do these kinases, and perhaps others, regulate MLCP activity?

Correspondence to Thomas M. Lincoln, Department of Physiology, College of Medicine, University of South Alabama, Mobile, AL 36688. E-mail tlincoln@usouthal.edu

(Circ Res. 2007;100:10-12.)

© 2007 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org DOI: 10.1161/01.RES.0000255894.25293.82
threonine-853 (numbering according to the mammalian sequence), on MYPT1; phosphorylation of both residues has been correlated with diminished phosphatase activity, but it is T853 phosphorylation that appears to be most specifically associated with specific ROK-induced phosphatase inhibition. Indeed, ROK catalyzes the phosphorylation of T853 more efficiently than T696, suggesting that the actual role of ROK-dependent T696 phosphorylation is more complex. It is now known that several different serine/threonine protein kinases such as ZIP kinase catalyze the T696 phosphorylation of MYPT1. What is not known is whether or not activation of ROK lies upstream of the activation of one or more of these kinases which in turn results in T696 MYPT1 phosphorylation and phosphatase inhibition. Of these the ZIP kinase is a particularly interesting candidate given that, like ROK, it is highly abundant in SMC.

To turn attention to CPI-17, this protein belongs to the larger family (defined by function if not by homology) of low molecular weight protein serine/threonine phosphatase inhibitors that include Inhibitor-1, DARPP-32, and G-substrate. These proteins function as inhibitors in that phosphorylation results in the formation of a “competitive inhibitor” that binds to the phosphatase catalytic subunit denying the binding of phosphoprotein substrates. CPI-17 is of great interest in smooth muscle contraction for several reasons: first, its phosphorylation is catalyzed by PKC, which is activated in response to contractile agonist-dependent activation of PLC by diacylglycerol and increases in [Ca2+]. In fact, it has long been known that PKC activation mediates slow smooth muscle contraction with no increase in [Ca2+], or even diminishing levels of [Ca2+]. Second, CPI-17 is abundant in smooth muscle tissues although it is not universally expressed in all smooth muscle. And finally, ROK and perhaps other kinases such as those mentioned above that catalyze the phosphorylation of MYPT1 in intact SMC also phosphorylate CPI-17. Thus, it appears that the SMC has 2 independent pathways originating at agonist receptor activation that lead to the phosphorylation of 2 proteins, MYPT1 and CPI-17, that inhibit MLCP activity.

The obvious question becomes, why 2 pathways that, in many cases, exist together within the same cell? Dimopoulos et al attempted to answer this question by examining the time courses for CPI-17 and MYPT1 phosphorylation and the correlation of these biochemical events with calcium sensitization. What they found was that CPI-17 phosphorylation proceeded more rapidly following agonist receptor engagement than did MYPT1 phosphorylation. CPI-17 phosphorylation was highly correlated with the rapid release of stored Ca2+ whereas MYPT1 phosphorylation was more dependent on the slower uptake and refilling of Ca2+ stores. Thus, the different pathways leading to MLCP inhibition are essentially controlling the kinetics of tension development. These are interesting and potentially significant findings. Other support for this concept may also be taken from the observations that MYPT1 and CPI-17 demonstrate uneven expression in smooth muscle tissues; generally, MYPT1 is more highly expressed in phasic smooth muscle whereas CPI-17 is more highly expressed in tonic smooth muscle. Rapid phosphorylation of CPI-17 may be important to achieve high levels of MLC phosphorylation needed for sustained tension in the face of declining [Ca2+], observed in tonic smooth muscle, whereas MYPT1 phosphorylation would be important in sustaining MLC phosphorylation with rapidly changing ion channel activity seen in phasic smooth muscle.

However, there is another aspect to the role of these 2 pathways regulating MLCP, and that is the process of “calcium desensitization.” It has long been known that relaxation of agonist-contracted smooth muscle by NO-containing vasodilators is more efficient than relaxation of depolarized smooth muscle tissue. NO mediates SMC relaxation by increasing the levels of cyclic GMP that, in turn, activate PKG. In SMC, PKG is known to lower [Ca2+]i by activating calcium-dependent potassium channels, inhibiting agonist-dependent activation of PLC, stimulating Ca2+ uptake into the sarcoplasmic reticulum (SR) and inhibiting inositol trisphosphate (IP3)-mediated Ca2+ release from the SR (see for a review). As illustrated in Figure 1, cGMP, via PKG-Iα activation, also activates MLCP. Surks et al demonstrated that the PKG-Iα isoform binds to what turns out to be the spliced out variant of MYPT1 that contains the C-terminal leucine zipper domain (LZ+) thereby targeting the kinase in an appropriate position to catalyze MYPT1 phosphorylation. The importance of this finding was demonstrated by

![Diagram of Calcium Sensitization and Desensitization](image)

**Calcium sensitization and calcium desensitization pathways converge on MLCP in smooth muscle. MLCP exists in an inactive (MLCP) or an active (MLCPA) state depending on the phosphorylation of either CPI-17 or MYPT1. Calcium sensitization promotes CPI-17 and MYPT1 phosphorylation (shown here by phosphorylation of threonine-868, T868) by activation of protein kinases such as PKC and ROK. CPI-17 phosphorylation proceeds on a faster time course associated with the rapid generation of cross-bridge cycling and force development; MYPT1 phosphorylation, on the other hand, develops more slowly and is associated with the maintenance of force development. In both cases, MLCP activity is diminished even as [Ca2+]i is declining to maintain MLCP phosphorylation. Calcium desensitization, on the other hand, is dependent on activation of PKG, and possibly cAMP-dependent protein kinase (PKA). PKG activation results in PP2A-dependent dephosphorylation of CPI-17 and activation of MLCP, and the phosphorylation of S695 of the LZ+ isoform of MYPT1. This prevents ROK-dependent phosphorylation of T696 resulting in the activation of MLCP. Because the expression of CPI-17 and the LZ+ isoform of MYPT1 varies from tissue to tissue, the sensitization of Ca2+ dependent contraction will also vary from tissue to tissue, thereby creating the opportunity for fine-tune control of smooth muscle tone in different tissues. Abbreviations: MLCP, myosin light chain phosphatase; CPI-17, MLCP inhibitor; MYPT1, MLCP targeting subunit; LZ+; spliced-out isoform of MYPT1 containing the C-terminal leucine zipper domain; PKC, protein kinase C; ROK, RhoA-activated kinase; PKG, cGMP-dependent protein kinase; PP2A, protein phosphatase 2A.
Wooldridge et al24 where PKG-I catalyzes the phosphorylation of serine-695 of MYPT1. Recall that calcium-sensitizing kinases (ROK, ZIPK) inhibit MLCP in part at least through phosphorylation of T696. According to the model published by these latter authors, S695 phosphorylation and T696 phosphorylation cannot occur together because of steric exclusion. Thus, S695 phosphorylation prevents phosphorylation of T696 thus blocking MLCP inhibition. Fisher, Brozovich and coworkers20,21 demonstrated that NO/cGMP-dependent relaxation of smooth muscle was highly correlated with the expression of the spliced-out LZ+ isoform of MYPT1, which binds PKG-I. Thus, one possible outcome for having two separate pathways for the regulation of MLCP is to have at least one pathway available for calcium desensitization.

On the other hand, what is known about calcium desensitization and CPI-17? At least 2 laboratories have demonstrated that PKG activation leads to the dephosphorylation of CPI-17.25,26 Bonnevier and Arner20 found that the mere activation of PKC under conditions where Ca2+ levels were not increased in skinned smooth muscle preparations led to contractions that were attenuated by cGMP and PKG activation. Relaxation was entirely dependent on PKG-dependent dephosphorylation of CPI-17, presumably through the activation of a protein phosphatase 2A (PP2A). Thus, both MYPT1-T696 phosphorylation and CPI-17 phosphorylation are attenuated in calcium desensitization.

The regulation of MLC dephosphorylation through distinct signaling pathways is but one more example of redundancy for the regulation of a highly complex and important physiological process. As shown by Dimopoulos et al.,15 one outcome of this redundancy may be to fine-tune the contractile process in different tissues. This could be true for both calcium sensitization and calcium desensitization. Yet, it is also possible that these pathways really do represent “back-up” systems so that changes in expression of one or more of these components during development, physiological stress, or even disease do not compromise the control of smooth muscle tone which is so important for vascular function, breathing, digestion and reproduction.

**Sources of Funding**
Supported by grants from the NIH (HL053426, HL066164).

**Disclosures**
None.

**References**

**Key Words:** phosphorylation | calcium | cyclic GMP | smooth muscle
Myosin Phosphatase Regulatory Pathways: Different Functions or Redundant Functions?

Thomas M. Lincoln

*Circ Res.* 2007;100:10-12
doi: 10.1161/01.RES.0000255894.25293.82

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/100/1/10