Cyclic GMP Regulation of Myosin Phosphatase: A New Piece for the Puzzle?

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The contractile state of smooth muscle (SM) reflects the ratio of activities of myosin light-chain kinase (MLCK) and myosin light chain phosphatase (MLCP), which determines the extent of regulatory light chain (RLC) phosphorylation and actin-activated myosin II activity and can be changed by modulating the activities of the calcium-calmodulin–dependent MLCK or of MLCP. Agonists through G protein–coupled receptors, which activate the small G protein RhoA and its effector, Rho-kinase (ROK), results in inhibitory phosphorylation of the regulatory subunit (MYPT1) of MLCP leading to an increase in RLC phosphorylation and force independent of a change in [Ca\(^{2+}\)]. A process termed Ca\(^{2+}\) sensitization (reviewed in1). The importance of regulation of MLCP activity has focused attention on potential phosphorylation sites on MYPT1 especially Thr-696, Thr-853, Ser-692, Ser-695, and Ser-852 (numbering for the human MW133 isoform) (Figure) but the physiologically relevant sites remain to be fully understood. CPI-17, found in some SMs is another potential mediator of Ca\(^{2+}\) sensitization, which on phosphorylation by a variety of kinases inhibits the MLCP catalytic subunit, PP1c\(^{\alpha}\) resulting in Ca\(^{2+}\)-sensitized force independent of phosphorylation of MYPT1. Conversely, cyclic nucleotides can relax Ca\(^{2+}\)-sensitized force and reduce RLC phosphorylation through activation or dis-inhibition of MLCP activity leading to Ca\(^{2+}\) desensitization. Early studies showed that cyclic GMP decreased Ca\(^{2+}\) sensitivity and reversed agonist-induced Ca\(^{2+}\)-sensitized force at constant Ca\(^{2+}\). Urocorin-induced Ca\(^{2+}\) desensitization through PKA activation leads to a decrease in both Thr-696 and Thr-853. Direct phosphorylation of MYPT1 has been shown for both PKA on Ser-695 and by PKG on a C-terminal Ser residue. However, phosphorylation of these sites did not activate phosphatase activity raising the question as to the underlying mechanism. In an elegant study by Haystead and colleagues, all the major sites phosphorylated by PKA and PKG, which included Ser-692, Ser-695, and Ser-852, were identified in radioactive peptides of MYPT1 using Edman sequencing (Figure). Importantly, they demonstrated that phosphorylation of Ser-695, which is immediately adjacent to the inactivating Thr-696 prevented phosphorylation of Thr-696 by MYPT1 kinase. Additionally, 8-bromo-cGMP inhibited Thr-696 phosphorylation and Ca\(^{2+}\)-sensitization of ileum SM. Thus, phosphorylation of Ser-695 prevented phosphorylation of Thr-696 and its inhibition of MLCP. The role of PKA/PKG induced phosphorylation of Ser692 and Ser852 remains to be determined. In the current issue of Circulation Research, Nakamura et al further examined the antagonism between MYPT1 Ser-695 and Thr-696 phosphorylation through the generation of a diphospho antibody, which only recognizes MYPT1 diphosphorylated at Ser-695 and Thr-696, and a phospho Ser-695 antibody, which specifically recognizes phospho Ser-695/ unphosphorylated Thr-695 MYPT1 and not the diphospho form. An additional phospho Thr-696 antibody recognizes both the diphospho and the monophosphorylated protein. Using these tools, Nakamura et al found that cGMP treatment of phenylephrine-stimulated α-toxin–permeabilized, femoral arteries lead to a decrease in Thr-696 phosphorylation and a significant increase in Ser-695 phosphorylation, as expected from Haystead et al. They also detected 0.27 mol/mol of diphospho-Ser695/Thr696 MYPT1, which was not significantly different under the 3 conditions examined: pCa 6.5, phenylephrine stimulation, or phenylephrine plus 8-bromo-cGMP stimulation. This diphospho form represented approximately 20% of the total pool of MYPT1 in the femoral artery. This is surprising if phosphorylation of Ser-695 prevents phosphorylation at the adjacent Thr-696 site as demonstrated by Wooldridge et al and this discrepancy remains to be resolved. Ultimately, direct site analysis showing the presence of phosphate at both sites is important. Both groups found that kinases that phosphorylate Thr-696 are much more effective in phosphorylating this site when Ser-695 is unphosphorylated, possibly reflecting decreased accessibility attributable to the bulky phosphate group. Curiously, no increase in phosphorylation of Thr-696 or the diphospho Ser-695/Thr-696 sites in the α-toxin–permeabilized artery sensitized to Ca\(^{2+}\) with phenylephrine was observed, in agreement with several previous reports using only the commercially available phospho-Thr-696 antibodies. It is unclear whether ROK directly phosphorylates MYPT1 at Thr-696, in contrast to phosphorylation at Thr-853, which is reduced by treatment with ROK inhibitors (reviewed in1). The Thr-853 site was not explored in the present study. They report that Thr-696 phosphorylation decreased by only 50% with 8-bromo-cGMP stimulation, whereas the tension and RLC phosphorylation fell to baseline presumably indicating that Ser-695 phosphorylation is not the sole explanation of PKG-induced Ca\(^{2+}\) desensitization. It is important to emphasize that the increase in Ser-695 phosphorylation does not directly increase phosphatase activity as direct measurements...
of phosphatase activity by these authors as well as others have demonstrated, but rather leads to a decrease in the inhibited state of the phosphatase. In another approach, the authors used recombinant MYPT1 phosphorylated by Rho-kinase, PKG, or both as a substrate and followed the ability of a homogenate of femoral artery with or without 8-bromo-cGMP treatment to differentially dephosphorylate this substrate in the absence of ATP. This approach lead to the novel observation that the phosphatase in the tissue homogenate that induced dephosphorylation of phospho Thr-696 substrate, is activated by PKG. To date, the identity of the phosphatase responsible for dephosphorylating MYPT1 is unknown, and the present finding suggests that this MYPT-phosphatase-phosphatase is also regulated.

Phenylephrine-induced Ca\textsuperscript{2+} sensitization in α-toxin–permeabilized femoral artery increased the phosphatase inhibitory phospho-CPI-17 at Thr-38 as expected, but Nakamura et al detected no change after stimulation with 8-bromo-cGMP. However, nitric oxide–induced relaxation to histamine contracted intact carotid artery has been shown to cause a rapid increase in cGMP content coincident with a fall in RLC phosphorylation, a transient increase MLCP activity and a reciprocal transient fall in phospho-CPI-17, which peaked at 5 minutes. The present study may have missed the change in phospho-CPI-17 as the measurement was made 20 minutes after the addition of 8-bromo-cGMP at a time when Etter et al observed an ∼60% recovery of phosphorylation of CPI-17. Thus, it would appear relevant to also time resolve changes in MYPT1 phosphorylation coincident with the 8-bromo-cGMP-induced fall in force.

PKG-induced Ca\textsuperscript{2+} desensitization operates in parallel with mechanisms that reduce [Ca\textsuperscript{2+}], and also lead to an inhibitory phosphorylation of phospholipase C-β3 and phosphorylate and inhibit the InsP\textsubscript{3} receptor resulting in a decrease in [Ca\textsuperscript{2+}], NO induces relaxation of rat aorta through inhibition of Rho-kinase signaling, and PKG can inhibit RhoA/ROK activity by phosphorylating Ser-188 of RhoA. The leucine zipper region at the C terminus of some MYPT1 isoforms and its interaction with the leucine zipper domain of PKG has been shown to play a role in PKG-mediated Ca\textsuperscript{2+} desensitization. Leucine zipper negative isoforms are resistant to cGMP and are unable to dephosphorylate myosin and induce relaxation in response to 8-bromo-cGMP after Ca\textsuperscript{2+} activation. Although the MYPT1 leucine zipper may serve to target PKG, it is not known whether this is required for phosphorylation of Ser-695 or structurally whether this is feasible.

Mechanisms of Ca\textsuperscript{2+} sensitization and Ca\textsuperscript{2+} desensitization warrant further investigation as RhoA/ROK signaling and changes in MYPT1 phosphorylation are implicated in hypertension, cerebral and coronary vasospasm, erectile dysfunction, and bronchial asthma.

In conclusion, the report by Nakamura et al adds to accumulating information on the mechanisms underlying cGMP regulation of MLCP. Based on their newly generated diphospho Ser-695/Thr-696 antibody, they have examined in greater detail the interplay between phosphorylation of Ser-695 and Thr-696 and report that 20% of MYPT1 exists as a diphospho Ser-695/Thr-696 species, which does not change with phenylephrine or cGMP stimulation, whereas phosphoSer-695 is increased and phosphoThr-696 decreased by cGMP. Interestingly, they demonstrate that the unknown phosphatase responsible for dephosphorylation of the MYPT1 sites is activated either directly or indirectly by cGMP.
Clearly, many questions remain to be explored. Knowledge of the kinetics, time course, and contributions of each of the upstream and downstream regulatory mechanisms as well as their spatial distribution and structural studies to reveal how phosphorylation of MYPT1 regulates its activity are necessary before a cohesive picture emerges to explain cyclic nucleotide regulation of myosin phosphatase and Ca\(^{2+}\) desensitization.

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**References**

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