The maintenance of vascular tone is central to the regulation of blood pressure and tissue perfusion and plays a role in the pathogenesis of hypertension and atherosclerosis. Vascular tone is determined by the balance of vasodilator and vasoconstrictor stimuli. After several decades of research, the NO/cGMP/cGMP-dependent protein kinase (cGK) pathway is now recognized as an important mediator of vasodilation. However, the mechanisms by which cGK causes smooth muscle relaxation continue to be an important question.

Smooth muscle contraction and relaxation are tightly coupled to the phosphorylation and dephosphorylation, respectively, of the regulatory myosin light chain. Myosin light chain phosphorylation state is determined by the relative activities of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). MLCK phosphorylates MLC leading to contraction, and MLCP dephosphorylates MLC, leading to relaxation (Figure). Both MLCK and MLCP activities are highly regulated. MLCK activity is activated by the binding of calcium/calmodulin and thus is the primary mechanism linking intracellular calcium concentration to smooth muscle contractility. MLCP activity is regulated by both vasodilator and vasoconstrictor stimuli, and is therefore responsible for much of the calcium-independent regulation of contractility (reviewed in 5). cGK mediates vasorelaxation by both calcium-dependent and calcium-independent pathways. Initial experiments found that cGMP inhibited calcium elevations in freshly isolated aortic smooth muscle cells. However, in passaged aortic smooth muscle cells in which cGK expression is downregulated, cGMP failed to inhibit calcium elevations, supporting the hypothesis that smooth muscle relaxation, in part, results from the inhibition of intracellular calcium mobilization. In permeabilized smooth muscle strips in which transmembrane calcium concentrations can be clamped without loss of intracellular proteins, cGMP induces smooth muscle relaxation without a change in intracellular calcium, suggesting that cGK could mediate calcium-independent relaxation via activation of MLCP. Perhaps the most definitive evidence to date comes from the complete cGKI knockout mouse model. These mice have multiple cardiovascular, gastrointestinal, hematopoietic, and neurological abnormalities, resulting in early postnatal mortality and making them difficult to study. Nevertheless, the aortas from these mice failed to relax after stimulation of the NO/cGMP/cGK pathway, supporting a critical role for cGKI in vascular relaxation.

The cGK family includes two distinct genes, prkG1 and prkG2, that encode cGKI and cGKII, respectively. cGKI is the isoform expressed in smooth muscle tissues. The amino terminus of cGKI is encoded by two alternatively spliced exons, resulting in the two isoforms cGKIα and cGKIβ, that differ only in the amino-terminal leucine zipper domains. Several studies have shown that both cGKI isoforms are expressed in smooth muscle, raising the question whether one or both isoforms are responsible for smooth muscle relaxation, which is addressed by Weber et al in this issue of Circulation Research.

In recent years, molecular studies have contributed to the hypothesis that both cGKI isoforms contribute to smooth muscle relaxation and are targeted to specific substrates via their amino-terminal leucine zipper domains. In a screen for cGKIα interacting proteins, our laboratory found that cGKIα interacts directly with the regulatory myosin binding subunit (MYPT1) of MLCP. This interaction targets cGKIα to MLCP and is critical for cGKI-mediated activation of MLCP. Although MYPT1 was originally thought to target MLCP to contractile fibers, we found that targeting of MLCP is more complex and involves a molecular scaffold, myosin phosphate-rho interacting protein, that anchors MLCP to actin and colocalizes RhoA to regulate MLCP. Tang et al demonstrated that cGKIα inhibits thrombin receptor-mediated calcium release through a cGKIα-regulator of G protein signaling (RGS)2 interaction. This leucine zipper-mediated interaction targets cGKIα to phospholipase and activate RGS2, which in turn increases the GTPase activity of Goq, terminating thrombin receptor signaling. cGKIβ was found to regulate inositol 1,4,5-triphosphate (IP3)-mediated calcium release by binding to and phosphorylating the inositol 1,4,5-triphosphate receptor-associated cGMP kinase substrate (IRAG). The cGKIβ-IRAG interaction leads to phosphorylation of IRAG and inhibition of calcium release via the inositol triphosphate type I receptor. These isoform-specific leucine zipper-mediated interactions have formed the basis of a targeting hypothesis in which both cGKI isoforms have distinct functional roles in smooth muscle relaxation by virtue of their specific targets (Figure).

Previous approaches have used cell culture models to test the targeting hypothesis. In one study, GFP-tagged cGKIα or cGKIβ were transfected into cGKI-null smooth muscle cells and...
Figure. MLC phosphorylation determines smooth muscle contractility. Contractile agonists lead to inositol 1,4,5 triphosphate (IP3) production or activation of RhoA (RhoA-GTP). IP3 binding to its receptor in the sarcoplasmic reticulum leads to release of Ca2+. Ca2+/calmodulin binds to and activates MLCK, which in turn phosphorylates MLC (calcium-dependent contraction). Activated RhoA binds to and activates ROCK, leading to phosphorylation and inhibition of MLCP, inhibiting MLC dephosphorylation (calcium-independent contraction). cGKI mediates relaxation by inhibiting both calcium-dependent and -independent contraction. cGKIα activates MLCP by a direct interaction and by inhibition of RhoA, and activates RGS2 to inhibit Gα signaling; cGKIβ activates IRAG, which then inhibits Ca2+ release by the IP3 receptor.

tested for inhibition of calcium release. Only the cGKIα-containing cells exhibited calcium regulation by cGMP. A potential limitation in this study is the possibility that the GFP moiety interfered with cGKI targeting. In a recent study by Christensen and Mendelsohn, the role of PKGI isoforms in thrombin receptor-mediated calcium mobilization was studied in both CHO cells stably expressing either cGKI isoform and human aortic smooth muscle cells expressing primarily cGKIα or cGKIβ. In CHO cells, cGKIα had a significantly greater calcium-lowering effect, whereas in cultured human aorta smooth muscle cells, only cGKIα lowered calcium in response to cGMP. Thus neither of these cell culture-based studies that manipulated the expression of cGKI isoforms confirmed the expected isoform-specific functions based on cGKI targeting.

In the current study, Weber et al took the interesting approach of creating mouse lines that express either cGKIα or cGKIβ on a cGKI null background. Expression of the cGKI genes was driven by the endogenous SM22α promoter, resulting in smooth muscle–specific expression. The cGKI isoforms were expressed at levels and activity 1.5- to 2.0-fold greater than control mice. The transgenic rescue mice had a life expectancy greater than the cGKI null mice, but less than the wild-type control mice. Interestingly, all of the tested smooth muscle functions in the cGKI transgenics were equivalent to those in the wild-type mice. These included intestinal transit of barium, jejunal, and aortic smooth muscle relaxation and inhibition of norepinephrine-induced calcium transients by cGMP. Although surprising, these data could still be explained by the known interactions between cGKIα and cGKIβ with RGS2 and IRAG, respectively.

Weber et al performed a thorough examination of blood pressure in the null, wild-type, and the cGKI α and cGKI β rescue mice. The basal blood pressure was not different between wild-type and rescue mice, but was elevated as expected in the cGKI-null mice. Moreover, the hypertensive effect of nitravasodilators, carbachol, and the ROCK inhibitor Y27632 were all preserved in the cGKI rescue mice. The latter manipulation is particularly interesting because cGKIα opposes the inhibitory effect of RhoA/ROCK on MLCP activity and inhibits RhoA directly by phosphorylation at Ser188. One would therefore expect that the cGKIα rescue mice would have less RhoA/ROCK activity and therefore less hypotension from Y27632.

The elegant approach by Weber et al avoids many of the pitfalls of the earlier studies of cGKI isoform-specific functions in the vasculature, including reliance on cell culture models and use of epitope-tagged cGKI isoforms. How then can we explain the apparently equivalent physiologic effect of cGKIα and cGKIβ rescue? The authors provide two hypotheses. First, it is possible that the specificity for each isoform for their respective targets is less pronounced in vivo. This is possible because most of the experiments that characterized the cGKI isoform specific targets were performed in cell culture systems and with purified proteins. If there were more overlap between cGKI isoforms and their target interactions, then expression of individual cGKI isoforms might exhibit subtle, if any, differences from wild-type mice. Second, each cGKI isoform alone is sufficient to maintain circulatory physiology based on its known interactions. For example, cGKIα rescue can mediate cGMP-mediated vasodilation because it can lower calcium via its interaction with RGS2, and activate MLCP via its interaction with MYPT1. It is more difficult to reconcile how cGKIβ rescue can fully reconstitute cGMP-mediated vasodilation without regulating MLCP.

There are several additional possibilities that may explain the apparent functional equivalence of cGKIα and cGKIβ to rescue vascular function. The cGKI targets discussed here have well-described isoform-specific interactions, yet there are additional cGKI targets that do not bind in an isoform specific manner, whose role in vascular physiology is less clear, and there are likely more targets that are undiscovered. These cGKI targets may allow functional overlap between the cGKI isoforms. Furthermore, although Weber et al observed similar expression of the cGKI isoform targets, IRAG, MYPT1, and RhoA, other critical proteins within these signaling pathways may be upregulated. Moreover, posttranslational modification (eg, phosphorylation) of these isoform-specific targets or splice variation may also contribute to altered activity of these signaling pathways without apparent differences in their protein expression levels. A third possibility represents a limitation to any transgenic approach when used to explore pathways regulated by differential targeting. Even modestly increased levels of protein overexpression may be adequate to obscure the specificity of protein targeting, particularly if the protein in question is present in excess of the targeting protein.
Future experiments with this model may provide important insights into cGKI isoform specificity. Biochemical experiments comparing wild-type, null, and rescue mouse vascular tissue for isoform-specific binding to respective targets and regulation of these pathways would be revealing. For example, is there cGMP-mediated activation of MLCP and phosphorylation/inhibition of RhoA in both cGKI isoform rescue mice? Can cGMP induce cGKI interaction with and phosphorylation of IRAG in both rescue mice?

Ultimately, additional genetic models in mice may be the best approach. Among these, inducible, tissue-specific deletion of each cGKI isoform will provide the opportunity to dissect their roles in vascular function while avoiding any concerns about loss of specificity in cGKI overexpression mice. Finally, specific knock-in mutations to disrupt cGKI isoform targeting will also be informative. Our laboratory has created a mouse in which the leucines and isoleucines in the cGKIα leucine zipper domain have been mutated to alanine, disrupting its interaction with MLCP without affecting kinase activity. Preliminary studies show that these mice are hypertensive and have abnormalities of vascular relaxation, supporting the isoform specific targeting hypothesis and the importance of cGKIα in the regulation of vascular tone.24 cGKI is critical for the maintenance of vascular tone by mediating vasodilation in response to nitrovasodilators. The two cGKI isoforms interact with specific targets in smooth muscle via their amino-terminal leucine zipper domains, and there is debate about their relative roles in smooth muscle relaxation. This carefully performed, elegant study by Weber et al adds an interesting wrinkle to the targeting hypothesis by showing that both cGKIα and cGKIβ can rescue vascular relaxation and intestinal motility in cGKI null mice. Future biochemical studies with this model, as well as additional genetic targeting approaches to delete or mutate specific cGKI isoforms, will provide a wealth of information about these pathways in the near future.

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References


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