Pleiotropic Regulation of Vascular Smooth Muscle Tone by Cyclic GMP–Dependent Protein Kinase

Thomas M. Lincoln, Padmini Komalavilas, Trudy L. Cornwell

Abstract  Cyclic GMP (cGMP) mediates vascular smooth muscle relaxation in response to nitric oxide and atrial natriuretic peptides. One mechanism by which cGMP decreases vascular tone is by lowering cytosolic Ca\(^{2+}\) levels in smooth muscle cells. Although mechanisms by which cGMP regulates cytosolic Ca\(^{2+}\) are unclear, an important role for the cGMP-dependent protein kinase in regulating Ca\(^{2+}\) has been proposed. Cyclic GMP–dependent protein kinase has been shown to regulate several pathways that control cytosolic Ca\(^{2+}\) levels: inositol 1,4,5-trisphosphate production and action, Ca\(^{2+}\)-ATPase activation, and activation of Ca\(^{2+}\)-activated K\(^{+}\) channels. The pleiotropic action of cGMP-dependent protein kinase is proposed to occur through the phosphorylation of important proteins that control several signaling pathways in smooth muscle cells. One potential target for cGMP-dependent protein kinase is the class of okadaic acid-sensitive protein phosphatases that appears to regulate K\(^{+}\) channels among other potentially important events to reduce cytosolic Ca\(^{2+}\) and tone. In addition, cytoskeletal proteins are targets for cGMP-dependent protein phosphorylation, and it is now appreciated that the cytoskeleton may play a key role in signal transduction. (Hypertension. 1994;23[part 2]:1141-1147.)

Key Words  • calcium • phosphorylation • ion channels • phosphatases • cytoskeleton • nitric oxide • atrial natriuretic factor

In the 1970s vasodilators such as nitroglycerin and nitroprusside were found to relax blood vessels through the generation of cyclic GMP (cGMP) in smooth muscle cells.\(^{1,2}\) Studies from the laboratories of Ignarro (Gruetter et al\(^{4}\)) and Murad (Arnold et al\(^{5}\)) identified the free radical nitric oxide (NO) as the substance derived from these drugs that elevated cGMP by activating soluble guanylate cyclase. In 1980, Furchgott and colleagues (Reference 6 and Cherry et al\(^{7}\)) discovered an endothelium-derived substance that relaxed contracted arterial strips. This substance, termed endothelium-derived relaxing factor (EDRF), elevated cGMP in vascular smooth muscle.\(^{8-10}\) The pharmacologic similarities between NO and EDRF led Furchgott,\(^{11}\) Ignarro et al,\(^{12}\) and Palmer et al\(^{13}\) to propose that EDRF was in fact NO. Thus began one of the most fascinating areas of not only vascular biology but intercellular communication in general.

The role of cGMP as a mediator of NO signaling has recently focused attention on another area of investigation established in the mid-1970s, ie, the role of cGMP-dependent protein kinase and other potential cGMP receptor proteins in cell function. Vascular smooth muscle cells are a rich source of cGMP-dependent protein kinase,\(^{14}\) a serine-threonine protein kinase selectively activated by cGMP, and a member of the large protein kinase family (see Reference 15 for a recent review). Cyclic GMP–dependent protein kinase is closely related in structure and function to the cyclic AMP (cAMP)–dependent protein kinase, but unlike the cAMP-dependent protein kinase, little is known of the function of the cGMP-dependent protein kinase. Specific substrate proteins are not well characterized in many instances, and the precise mechanism of cGMP-dependent protein kinase in the relaxation of contracted vascular smooth muscle is still unclear. This is partly because of the complex mechanisms by which vascular smooth muscle cells regulate contractile activity as well as the apparently less ubiquitous role of cGMP-dependent protein kinase in the regulation of cellular activity.

The purpose of this article is to review the role of cGMP and cGMP-dependent protein kinase in the regulation of vascular tone. In addition, newly emerging roles for this enzyme in the regulation of other vascular activity such as growth and differentiation will be addressed. The various model systems used to define the role of cGMP-dependent protein kinase will be discussed because our current information is derived from a collection of studies performed in a variety of systems.

Regulation of Intracellular Calcium Levels by Cyclic GMP

Early studies on the effects of cGMP on vascular smooth muscle relaxation were performed on isolated arterial strips. Several of these studies indicated that a reduction in intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) produced by cGMP was an important component of the mechanism of cGMP-dependent relaxation because enzymes whose activity depended on elevated [Ca\(^{2+}\)], (phosphorylase b kinase and myosin light chain kinase) were inhibited after elevation of cGMP.\(^{16,17}\) In the early 1980s Morgan and Morgan\(^{18}\) provided direct evidence using aequorin-loaded smooth muscle strips in which several vasodilators reduced [Ca\(^{2+}\)]. Subsequently, our laboratory (see
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number of steps in the \([\text{Ca}^{2+}]\) flux pathways have been

Inhibition of IP_3 Formation

It is well known that cGMP-elevating agents induce relaxation in vascular preparations contracted with agonists more effectively than those contracted by depolarization. This has led several investigators to examine the effect of cGMP to inhibit agonist-evoked IP_3 formation. Rapoport et al first demonstrated that nitrogen oxide-containing vasodilators inhibited inositol phosphate accumulation in contracted aortic strips. A similar proposal for the effects of cGMP on phoshoinositide turnover in platelets had previously been made. The mechanism of this action has not been elucidated. The inhibition of phospholipase C by cGMP-dependent protein kinase has been proposed, although no direct effect of cGMP-dependent protein kinase on the phosphorylation of purified phospholipase C forms has been reported. Hirata et al and Light et al suggested that cGMP-dependent protein kinase inhibits phospholipase C activation through phosphorylation of G proteins. Ruth et al demonstrated that transfected cGMP-dependent protein kinase inhibited IP_3 formation and thrombin-evoked \([\text{Ca}^{2+}]\) mobilization in CHO cells and suggested that cGMP-dependent protein kinase could inhibit G protein function in CHO cells. Unfortunately, no direct effect of cGMP-dependent protein kinase on G protein phosphorylation has been observed despite repeated attempts. It is likely that an indirect inhibition of phospholipase C occurs, presumably through the phosphorylation of a regulatory protein for phospholipase C. One potential candidate for the cGMP-dependent protein kinase–mediated inhibition of IP_3 formation is the actin-binding protein VASP (vasodilator-stimulated phosphoprotein). This 46/50-kD protein is found in platelets and smooth muscle cells, and its phosphorylation is correlated with the time- and concentration-dependent inhibition of phospholipase C activation in platelets. The mechanism by which VASP phosphorylation regulates platelet phospholipase C activity is unknown, but because of its cytoskeletal localization, it could serve to shuttle phospholipase C from the plasma membrane to the soluble fraction of cells. This would in effect remove the enzyme from its substrate (phosphatidylinositol 4,5-bisphosphate) in the cell.

Inhibition of Ca^{2+}-Activated K^+ Channels

Activation of Ca^{2+}-activated K^+ channels leads to hyperpolarization in excitable cells and inhibits Ca^{2+} entry through voltage-activated Ca^{2+} channels. Because depolarizing concentrations of K^+ would inhibit the gating action of the Ca^{2+}-activated K^+ channel, agents that activate this channel would be less effective in relaxing depolarized tissues compared with agonist-activated tissues. Early observations indicated that NO and 8-bromo-cGMP hyperpolarized smooth muscle, suggesting that these mediators stimulated the Ca^{2+}-activated K^+ channel. Subsequently, cGMP-dependent protein kinase was shown to activate Ca^{2+}-activated K^+ channels in both GH_3C_1 cells and vascular smooth muscle cells. In the case of the GH_3 cell...
channel, the most dramatic effect of cGMP-dependent protein kinase was to increase the opening probability of the channel, and cAMP decreased the probability of channel opening (Fig 2). Interestingly, the effect of cGMP-dependent protein kinase to stimulate the channel was blocked with the protein phosphatase inhibitor okadaic acid. This suggests that the channel is inactive in the phosphorylated state, whereas cGMP-dependent dephosphorylation increases the opening probability.

The mechanism of action of cGMP-dependent protein kinase to activate protein phosphatase is not well understood. Direct phosphorylation of protein phosphatase 1 or 2A (the phosphatases sensitive to okadaic acid inhibition) has not been demonstrated. Thus, as in the case for cGMP-dependent protein kinase inhibition of phospholipase C, the effect of kinase appears to be the phosphorylation of a protein that leads to activation of the protein phosphatase. There appears to be a wide tissue variability with the effects of cGMP on K+ channel activity. In rat aorta, for instance, charybdoxin, tetraethylammonium, and other K+ channel inhibitors have no effect on cGMP-induced relaxation or on cGMP-dependent inhibition of [Ca2+]i flux.34 On the other hand, swine carotid artery relaxation, like that of bronchial smooth muscle, appears to depend at least in part on K+ channel activation.33 Thus, it appears that the effects of cGMP on inhibition of K+ channels vary from tissue to tissue and perhaps from species to species.

**Activation of Ca2+-ATPase Activity**

Cyclic GMP has been demonstrated to decrease [Ca2+], levels in K+-treated arterial smooth muscle cells. Because [Ca2+]i elevations in this instance are unrelated to generation of IP3, it has been proposed that cGMP leads to activation of Ca2+-ATPase activity.19 Direct demonstration of such activation has been documented in several instances,19,36,38 but the mechanism underlying Ca2+-ATPase activation is in some cases unclear. One potential site of action of cGMP-dependent protein kinase is the sarcoplasmic reticulum Ca2+-ATPase regulatory protein phospholamban. cGMP-dependent protein kinase catalyzes the phosphorylation of this protein in vitro39 and in intact early-passaged cultured arterial smooth muscle cells.40,41 In the latter instance, phosphorylation was correlated with Ca2+-ATPase activation (Fig 3) and a reduction in [Ca2+]. Because phospholamban is a substrate for both cAMP- and cGMP-dependent protein kinases, it has been proposed that colocalization of cGMP-dependent protein kinase with the sarcoplasmic reticulum is an important factor for cGMP-dependent phosphorylation of this protein.40
Indeed, other smooth muscle sarcoplasmic reticulum proteins such as the IP$_3$ receptor are also phosphorylated in vitro and in intact aortic smooth muscle cells by cGMP. The significance of this effect has yet to be determined. Further support for a role for cGMP in the regulation of sarcoplasmic reticulum Ca$^{2+}$-ATPase was obtained by Luo et al., who demonstrated that inhibitors of the ATPase (puromycin, cyclopiazonic acid, and 2,5-di-[tert-butyl]-1,4-benzohydroquinone) blocked nitroglycerin and atrial peptide-induced relaxation in the contracted rabbit aorta. Thus, in at least some smooth muscle preparations stimulation of Ca$^{2+}$ resequestration by cGMP-dependent phosphorylation plays a role in relaxation.

**Inhibition of IP$_3$ Receptor Activity**

Several investigators have suggested that cGMP elevations inhibit the release of Ca$^{2+}$ from IP$_3$-sensitive storage sites in smooth muscle cells. Murthy et al. found that both cGMP and cAMP inhibited the IP$_3$-dependent increase in agonist-evoked Ca$^{2+}$ transients and suggested that cGMP-dependent protein kinase may inhibit IP$_3$ receptor function. Recently, our laboratory has demonstrated that the IP$_3$ receptor purified from cerebellum is an excellent substrate protein for cGMP-dependent protein kinase. Like the cAMP-dependent protein kinase, the cGMP-dependent enzyme catalyzes the phosphorylation of serine-1755 in the protein. Supattapone et al. suggested that the phosphorylation of this residue by cAMP-dependent protein kinase inhibits the capacity of IP$_3$ to release Ca$^{2+}$ from brain microsomes. A similar situation may exist for cGMP-dependent phosphorylation of the protein because the IP$_3$ receptor from primary cultures of vascular smooth muscle cells is phosphorylated in the intact cell. However, more studies are needed to verify this action of cGMP.

**Decreased Sensitivity of Contractile Proteins**

A number of studies have suggested that cGMP inhibits the sensitivity of contractile proteins to the effects of Ca$^{2+}$. Investigators have predicted that cGMP would oppose proliferation and phenotypic modulation of vascular smooth muscle cells derived from some vessels may rely more heavily on one type of control system than another. In the rat aortic smooth muscle cell, for example, cGMP-dependent protein kinase is primarily localized to the sarcoplasmic reticulum where [Ca$^{2+}$]$_i$ fluxes appear to be regulated via the phosphorylation of proteins such as the IP$_3$ receptor and phospholamban. In other vessels such as the carotid artery the regulation of Ca$^{2+}$-activated K$^+$ channels by cGMP-dependent protein kinase may be more important in controlling [Ca$^{2+}$]$_i$. Still, there may be mechanisms as yet not described that are regulated by cGMP-dependent protein kinase that may be involved in regulating contractile activity in all smooth muscle cells. One possibility, as illustrated in Fig 4, is the regulation of protein phosphatase activity, which may underlie the regulation not only of K$^+$ channels but of phospholipase C activity, contractile proteins, and ATPases. Such a scheme may involve the regulation of phosphorylation of cytoskeletal proteins that could be involved in trafficking of proteins between the plasma membrane and cytosolic compartments.

**Regulation of Vascular Smooth Muscle Cell Proliferation**

Medial layer vascular smooth muscle cells exist as differentiated, contractile cells in blood vessels. Under physiological stimuli (e.g., angiogenesis) or pathological situations (e.g., restenosis after angioplasty), vascular smooth muscle cells undergo waves of proliferation, resulting in the expansion of the medial layer, or in the case of restenosis, migration of the cells into the intimal space of blood vessels where the cells proliferate. In addition to proliferation, vascular smooth muscle cells also undergo morphologic and phenotypic changes resulting in cellular dedifferentiation. These cells acquire the capacity to synthesize and secrete large amounts of extracellular matrix proteins, which make up a large part of the neointimal mass in restenotic tissue. The process of proliferation and phenotypic modulation has been studied extensively over the last decade. Growth factors such as platelet-derived growth factor, epidermal growth factor, and basic fibroblast growth factor have all been implicated in the onset of proliferation, migration, and phenotypic differentiation (see Reference 54 for a review). Processes that oppose proliferation and phenotypic modulation are less well understood. In 1989 Garg and Hassid and Abell et al. demonstrated that smooth muscles that elevate cGMP inhibit cultured smooth muscle cell proliferation. The attractiveness of this hypothesis was that endothe-
and NO in regulating vascular smooth muscle cell growth is unclear at present. It should be noted that practically all studies of vascular smooth muscle cell growth have used passaged cells that have already undergone phenotypic modulation or dedifferentiation in culture. The relevance of these models to the in vivo situation is not established.

A more important but less thoroughly investigated role for cGMP may be in the regulation of the smooth muscle cell phenotype. As mentioned above, vascular smooth muscle cells placed in culture rapidly dedifferentiate. Concurrently, the cells cease to express cGMP-dependent protein kinase. Thus, evaluation of the role of cGMP in morphologic and phenotypic events is difficult because the cultured cells do not express the receptor protein for cGMP. Recent studies in our laboratory using cultured vascular smooth muscle cells stably transfected with the cDNA encoding cGMP-dependent protein kinase \( \alpha \) demonstrate that cGMP evokes a morphology similar to that of contractile, nonmodulated cells.\(^6\) Whether these cells are in fact identical to medial layer, contractile cells is not known. Nevertheless, this transfected cell system could represent an important model system for studying cGMP-dependent protein kinase and phenotypic control in vascular cells.

**Summary and Perspective**

The emergence of NO as a general paracrine regulator of cellular activity has resulted in a newly awakened interest in the role of cGMP in cell function. Much of the current investigation in this field is still at the level of NO and cGMP synthesis. However, to fully appreciate and understand the variety of NO-cGMP signaling mechanisms, it is important to understand the "downstream" effects of cGMP in cells. This means an understanding of the biologic role of cGMP-dependent protein kinase, the major receptor protein for cGMP in cells. To date, relatively few laboratories are actively engaged in probing the role of this enzyme in cellular activity despite a general appreciation of its importance. It is becoming clear that cGMP-dependent protein kinase may control a large number of diverse cellular activities, and thus it is rather naive to assume that any one action of the enzyme or any one phosphorylated protein explains its role, even in smooth muscle relaxation. Perhaps many of the targets of cGMP-dependent protein phosphorylation may be events that have not been biochemically elucidated, such as the activation of protein phosphatases. In any event, the frontiers for research in the NO-cGMP signaling pathways in cells will include cGMP-dependent protein kinase.

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