Signal Transduction in Smooth Muscle
Invited Review: cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression

THOMAS M. LINCOLN, NUPUR DEY, AND HASSAN SELLAK
Department of Pathology, Division of Molecular and Cellular Pathology, University of Alabama at Birmingham, Birmingham, Alabama 35294-0019

Lincoln, Thomas M., Nupur Dey, and Hassan Sellak. Invited Review: cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression. J Appl Physiol 91: 1421–1430, 2001.—cGMP is a second messenger that produces its effects by interacting with intracellular receptor proteins. In smooth muscle cells, one of the major receptors for cGMP is the serine/threonine protein kinase, cGMP-dependent protein kinase (PKG). PKG has been shown to catalyze the phosphorylation of a number of physiologically relevant proteins whose function it is to regulate the contractile activity of the smooth muscle cell. These include proteins that regulate free intracellular calcium levels, the cytoskeleton, and the phosphorylation state of the regulatory light chain of smooth muscle myosin. Other studies have shown that vascular smooth muscle cells (VSMCs) that are cultured in vitro may cease to express PKG and will, coincidentally, acquire a noncontractile, synthetic phenotype. The restoration of PKG expression to the synthetic phenotype VSMC results in the cells acquiring a more contractile phenotype. These more recent studies suggest that PKG controls VSMC gene expression that, in turn, regulates phenotypic modulation of the cells. Therefore, the regulation of PKG gene expression appears to be linked to phenotypic modulation of VSMC. Because several vascular disorders are related to the accumulation of synthetic, fibroproliferative VSMC in the vessel wall, it is likely that changes in the activity of the nitric oxide/cGMP/PKG pathway is involved the development of these diseases.

vascular smooth muscle cells; nitric oxide

NITRIC OXIDE (NO) is an important regulator of vascular and nonvascular smooth muscle relaxation. The mechanism by which NO produces relaxation is the subject of much investigation. It is generally conceded that activation of soluble guanylyl cyclase is the principal intracellular event that initiates relaxation, but the events that occur downstream from cGMP formation are still much debated (see Refs. 29, 44, and 63 for reviews). cGMP activates a family of serine/threonine protein kinases, the cGMP-dependent protein kinases (PKG), in smooth muscle cells, and it is likely that there are several different phosphorylation events occurring in response to PKG activation that lead to relaxation. A discussion of the enzymology and functions of the two types of PKG will not be dealt with here because they have been reviewed elsewhere (30, 65). Just how important the NO/cGMP/PKG pathway is in the regulation of smooth muscle tone may be surmised from studies on genetically altered mice lacking components of this pathway. Thus mice lacking endothelial NO synthase or PKG demonstrate moderate hypertension, indicative of increased vascular tone (45, 81).
More recently, another regulatory role for the NO/cGMP/PKG pathway has been proposed in vascular smooth muscle: the control of the vascular smooth muscle cell (VSMC) phenotype. This review will discuss studies that have shown that NO signaling inhibits VSMC proliferation in vitro and in vivo, although the mechanism and physiological significance are still not well understood. Others have shown that NO signaling, via a cGMP mechanism, regulates gene expression in cells. These effects have been extended to VSMCs where cGMP and PKG appear to regulate phenotypic properties of the cells. These later studies may have important implications for our understanding of vascular disorders such as atherosclerosis and restenosis.

MECHANISMS OF SMOOTH MUSCLE RELAXATION

There appear to be three major pathways regulated by NO/cGMP/PKG signaling that induce relaxation in smooth muscle: decreases in intracellular free calcium concentrations, calcium desensitization, and thin filament regulation. It is likely that no single pathway acts exclusively or independently in any one type of smooth muscle cell. However, the relative importance of the various pathways leading to cGMP-induced relaxation is likely to be different in phasic compared with tonic contractile cells and in cells from large arteries compared with those from microvessels.

**Regulation of intracellular calcium.** The first mechanism proposed for cGMP-dependent relaxation of smooth muscle was the reduction of free intracellular cytosolic calcium concentrations (49, 61). Insofar as increases in calcium were required for myosin light-chain (MLC) phosphorylation and contraction, the reduction of calcium by cGMP was seen primarily as a reversal of the contractile mechanism. Numerous studies over the past decade have supported a mechanism that involves the reduction of cytosolic calcium and the subsequent dephosphorylation of MLC. The earliest observations demonstrated that cGMP-dependent relaxation was observed for both agonist-dependent (e.g., α-adrenergic agonists) contraction and depolarization-dependent contraction. Because the former contractile mechanism utilizes a G-protein-coupled pathway to increase intracellular calcium, whereas the latter utilizes the activation of voltage-gated calcium channels to increase calcium, reductions in the rises in calcium to either of these pathways by cGMP-dependent mechanisms made "physiological sense."

Several sites of action have been proposed to account for cGMP-dependent regulation of cytosolic calcium, and these have been reviewed elsewhere (44, 63). These include sequestration and the removal of intracellular calcium through calcium pump mechanisms, inhibition of voltage-gated calcium channels, and inhibition of receptor/G-protein coupling in smooth muscle. However, two newer mechanisms have been reported in the past 2 years that deserve comment. The first of these is an increase in calcium-activated potassium channel gating that is activated by calcium, the so-called BK channels. Increased BK channel activity hyperpolarizes the membrane and reduces intracellular calcium influx through voltage-gated calcium channels. Increased BK channel activity appears to arise from both a direct activation of the channel by PKG and increased activity of spontaneous transient outward currents (STOCs) produced by small bursts of calcium released from the superficial sarcoplasmic reticulum (SR) that activate the channel (75). In the former case, it has been proposed that PKG regulates the phosphorylation of the BK channel either by acting as the direct catalyst for phosphorylation (1, 3, 31) or by activating a protein phosphatase that dephosphorylates the BK channel (111, 117). These results imply that there may be cell- and tissue-specific BK channels that are activated by PKG, one type activated by phosphorylation and the second type activated by dephosphorylation. In the case of increased STOC activity, it has been proposed that PKG increases influx of calcium into the superficial SR or activates a ryanodine receptor or both. The net effect would be a localized release of calcium from the SR into the subplasmalemmal space toward the plasma membrane to activate the BK channel (83). As a result of BK channel activation, a decrease in global intracellular calcium occurs, leading to the dephosphorylation of MLC and relaxation. The molecular mechanisms responsible for the PKG-dependent control of calcium flux into and out of the SR are not clear, although it is generally conceded that phosphorylation of the SR protein, phospholamban, by PKG contributes to SR calcium loading (24, 51, 66, 71, 89). In fact, phospholamban knockout mice have diminished STOC activity and decreased PKG-dependent activation of BK channels (110). A model depicting the PKG-dependent regulation of BK channel activity is shown in Fig. 1.

The other major development in the area of the control of intracellular calcium levels by cGMP/PKG is the regulation of the inositol 1,4,5-trisphosphate (IP₃) receptor. Previous studies from several laboratories demonstrated that the smooth muscle type I IP₃ receptor is phosphorylated by PKG (17, 43, 55–57, 72, 73). This protein was in fact initially termed the G₀ protein substrate by Casnellie and Greengard (16). The result of phosphorylation of the IP₃ receptor has not been clearly defined. The laboratory of Hofmann and Rath and colleagues (91), however, showed that PKG-dependent phosphorylation of the IP₃ receptor decreases calcium release from the SR, and this effect is dependent on the targeting of PKG to the SR. A targeting protein for PKG termed IRAG (IP₃ receptor-associated PKG-I substrate) has been characterized and shown to be necessary for IP₃ receptor phosphorylation.

**Regulation of calcium sensitization.** MLC phosphorylation at serine-19 is necessary for actin activation of myosin ATPase and subsequent cross-bridge cycling. Two key enzymes involved in the control of MLC phosphorylation are myosin light chain kinase (MLCK), a calcium/calmodulin-activated kinase, and MLC phosphatase, a serine/threonine protein phosphatase type I. With respect to regulation of MLCK, there has been
activate BK channels, resulting in the hyperpolarization of the cell. Ryanodine receptor (RyR) activation could result in enhanced release of phospholamban (PLB) and activation of the SR Ca\textsuperscript{2+} by the phosphorylation of the inositol trisphosphate receptor (IP\textsubscript{3}R) or the PLC. Ca\textsuperscript{2+} then activates the BK channel. PKG also catalyzes the phosphorylation of the inositol trisphosphate receptor (IP\textsubscript{3}R) or the ryanodine receptor (RyR), which could result in enhanced release of localized Ca\textsuperscript{2+} into the submembrane space toward the plasma membrane (PL). Ca\textsuperscript{2+} then activates the BK channel. PKG also catalyzes the phosphorylation of the inositol trisphosphate receptor (IP\textsubscript{3}R) or the ryanodine receptor (RyR), which could result in enhanced release of localized Ca\textsuperscript{2+} into the submembrane space toward the plasma membrane (PL). Ca\textsuperscript{2+} then activates the BK channel. PKG also catalyzes the phosphorylation of the inositol trisphosphate receptor (IP\textsubscript{3}R) or the ryanodine receptor (RyR), which could result in enhanced release of localized Ca\textsuperscript{2+} into the submembrane space toward the plasma membrane (PL).

Direct phosphorylation and PKG-dependent dephosphorylation of BK channels have both been proposed. For increasing the STOC activity, PKG activates the uptake of Ca\textsuperscript{2+} into the superficial sarcoplasmic reticulum (SR) by the phosphorylation of phospholamban (PLB) and activation of the SR Ca\textsuperscript{2+}-ATPase (SERCA). Loading of Ca\textsuperscript{2+} in the SR provides greater Ca\textsuperscript{2+} for release into the submembrane space toward the plasma membrane (PL). Ca\textsuperscript{2+}, then activates the BK channel. PKG also catalyzes the phosphorylation of the inositol trisphosphate receptor (IP\textsubscript{3}R) or the ryanodine receptor (RyR), which could result in enhanced release of localized Ca\textsuperscript{2+} into the submembrane space. Either or both effects activate BK channels, resulting in the hyperpolarization of the cell and a decrease in Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels. The net effect of decreased Ca\textsuperscript{2+} influx would be a decrease in global cell Ca\textsuperscript{2+} and relaxation. PKG may also have direct effects to inhibit Ca\textsuperscript{2+} channel activity.

no firm evidence that PKG-dependent phosphorylation of MLCK inhibits its activity (104). Most of the recent work suggests that PKG activates MLC phosphatase, thereby inhibiting MLC phosphorylation and contraction. The initial studies by Nishimura and van Bremen (76) and subsequently characterized by Lee et al. (60) and others (52) demonstrated that cGMP analogs inhibited agonist-induced calcium sensitization of contraction. Calcium sensitization is the term used to describe the increase in contractile activity produced by G-protein-coupled agonists compared with depolarization. Greater levels of contraction and MLC phosphorylation could be achieved at much lower levels of intracellular calcium using contractile agonists compared with potassium depolarization. An important component of the calcium sensitization mechanism is the Rho-Rho kinase-dependent inhibition of MLC phosphatase following agonist stimulation of contraction (35, 54). PKG apparently opposes Rho kinase-induced inhibition of the phosphatase by phosphorylating the myosin-binding subunit (MBS) of MLC phosphatase, thus activating the catalytic subunit of the phosphatase (100, 103). These studies have also shown that PKG is targeted to the MBS, thus placing it in an ideal location to activate MLC phosphatase. This scenario, similar to the one proposed for targeting of PKG to the IP\textsubscript{3} receptor, relies on the localization of PKG to its substrates for the subsequent induction of relaxation. It is noteworthy that the specific PKG substrate, G\textsubscript{i} protein [also described by Casnelli and Greengard (16)], is likely to be the MBS.

There are further reports that suggest that cGMP/PKG may interfere directly with the Rho-dependent activation of Rho kinase, possibly through the phosphorylation of Rho (90). Several studies have now shown that several of the small GTP binding proteins that converge on the contraction pathway in smooth muscle (e.g., Rho and Rap1) could be physiological substrates for PKG (69, 106). Finally, Wu et al. (114) showed that PKG-mediated desensitization of visceral smooth muscle may involve the phosphorylation of telokin. Clearly, the control of VSMC contraction through the regulation of MLC phosphorylation is an important target in NO/cGMP/PKG signaling.

**Thin filament regulation.** The role of the thin filament in the regulation of smooth muscle cell contraction has been somewhat controversial. Thin filament binding proteins regulate and contribute to the contractile activity of the cell, but there have been very few reports regarding the role of second messenger regulation of thin filament protein function. Two thin filament/actin binding proteins [vasodilatory-stimulated phosphoprotein (VASP) and the 20-kDa heat shock-related protein (HSP20)] have raised interest in possible regulation of smooth muscle cell contraction through thin filament regulation.

VASP was first identified and characterized by Walter and colleagues (13, 85) from platelets. VASP binds to actin filaments and stress fibers in practically all cells studied. It is particularly abundant in focal adhesions of cultured cells. Both cAMP-dependent protein kinase (PKA) and PKG catalyze the phosphorylation of VASP, resulting in a reduction in the number of focal adhesions (28, 74, 96). It has been reported that VASP phosphorylation on the PKG-specific site decreases the binding of VASP to actin filaments, possibly through its dissociation from the actin binding protein profilin (84). Although it is clear that VASP plays a critical role in cell spreading and movement, its role in smooth muscle contraction is less well defined.

More recently, HSP20 has gained some attention as a potential target for PKG action. Both HSP27 and HSP20 have been implicated in the regulation of smooth muscle contraction (6, 115). HSP20 was reported by Brophy and colleagues (5, 12) to be a specific target for both PKA- and PKG-dependent phosphorylation. Phosphorylation of serine-16 by either kinase is an important target in NO/cGMP/PKG signaling. Thin filament acts as a potential target for PKG action. Both HSP27 and HSP20 have been implicated in the regulation of smooth muscle contraction (6, 115). HSP20 was reported by Brophy and colleagues (5, 12) to be a specific target for both PKA- and PKG-dependent phosphorylation. Phosphorylation of serine-16 by either kinase is an important target in NO/cGMP/PKG signaling.
REGULATION OF VSMC PHENOTYPE

Whereas there is widespread acceptance of the important role for PKG in regulating smooth muscle cell contractility and vascular tone, a newer role for PKG in the regulation of VSMC phenotype is emerging that might prove to be just as important for vascular function. It is important to mention at the outset that it is not our intention here to review the huge volume of literature regarding VSMC phenotypic modulation and growth and the theories and controversies about the development of vascular lesions. Rather, it is our intention to focus on the emerging role of the NO/cGMP/PKG system in VSMC gene expression and how this may relate to vascular diseases.

With these caveats in mind, it has long been established that VSMCs acquire altered phenotypes in response to vessel growth, vascular injury, or even in vitro culturing of VSMCs (15, 18, 19, 36, 70, 78). In contrast to the development of smooth muscle cells from relatively dedifferentiated precursor cells to mature, differentiated contractile cells, the modulation of mature VSMCs between contractile phenotypes and synthetic or secretory phenotypes is a response to injury of the vessel (67, 88). The modulation of VSMC phenotype is a complex process that involves not only cells present in the medial layer of the vessel wall but also myofibroblasts derived from the adventitial layers (112) and even circulating cells derived from the bone marrow (42). Each of these different cell types is capable of migrating into the intimal areas in response to injury. There, the cells proliferate and secrete new matrix proteins in the wound-healing response. To complicate matters further, the medial smooth muscle cells are themselves heterogeneous, some derived from different germ layers, with some existing in a more synthetic state and others maintaining a more contractile phenotype (21, 99).

Regardless of the origin of the intimal cells during the response to injury, it is well established that VSMCs, representing the major cell type present in the vessel wall, acquire the capacity to proliferate and synthesize extracellular matrix (ECM) proteins, hence the terms synthetic or secretory to describe this phenotype. The synthetic phenotype of the VSMC is also acquired during the in vitro culturing of the cells; therefore, cultured VSMCs have become an accepted model for examining mechanisms of phenotypic modulation. Because cells that modulate to the synthetic phenotype cease synthesizing contractile proteins and pharmacomechanical signaling molecules (ion channels for instance) to produce ECM proteins, there are predictably dramatic changes in gene expression in response to injury. Many of the changes in gene expression and pathways leading to such changes have been reviewed elsewhere (18, 78). The role of the NO/cGMP/PKG pathway in regulating smooth muscle gene expression, however, is a relatively newer concept in vascular biology.

Studies performed more than 10 years ago established a potentially important role for NO in the inhibition of VSMC proliferation in vitro and in vivo (33, 68). Recent studies suggest a role for NO in the regulation of the expression of cell cycle control proteins such as p21 (Waf1/Cip1) and p53 (37, 38, 53, 102). In many of these studies, the mediation of the effects of NO was linked to cGMP and PKG activation. In other studies, however, the effects of NO were found to be either independent of cGMP or at least independent of PKG activation. Although these studies suggest an important role for NO/cGMP signaling in regulation of VSMC cell cycle events, it is important to distinguish between the proliferation of VSMC on the one hand from the changes in the pattern of gene expression resulting in different proteins being made in the cells on the other. Both contractile cells and synthetic cells are capable of proliferation in vitro and in vivo (99). When injury and the liberation of growth factors during injury occur, there is an immediate proliferative response of contractile and synthetic cells that lasts for 7–14 days, depending on the species and type of injury. The proliferation of cells in the intima is accompanied by the migration of new smooth muscle cells into the intima. The new population of cells in the intima constitutes only a minor portion of the mass of tissue that results from injury. The major contributing factor to the new mass of intimal tissue is the synthesis and secretion of ECM by intimal cells, a process that continues even after the proliferative response has subsided (99). The changes in gene expression and phenotype of VSMCs that permit the cells to acquire the capacity to synthesize new ECM and shut down production of contractile protein synthesis are processes that may be unrelated to the proliferative activity of the cell. Thus the process by which NO inhibits VSMC proliferation may be distinct from the processes by which NO regulates gene expression and phenotype of VSMC. Because most of the investigations on the role of NO in arterial response to injury have focused only on proliferation, the mechanisms by which NO signaling may change VSMC gene expression and phenotype are largely unknown.

Much of the early work on the mechanisms by which PKG regulates gene expression in cells were performed in cells other than VSMCs. Pilz, Boss, and colleagues (39, 40, 47, 82) have shown that PKG is a key regulator of protooncogene expression in cells such as BK cells transfected with the cDNA encoding the kinase. Studies in these and other cell types by other investigators have shown a similar potential for cGMP/PKG signaling in regulating specific mRNA transcription (8, 23, 32, 34, 41, 48, 58, 63, 80, 93, 107). The molecular mechanisms involving PKG-regulated transcription are not well understood, although it has been reported that activation of the cAMP response element, serum response element, and activator protein-1 elements by PKG is involved in the mechanism (34, 39, 40, 48, 93). In VSMCs, it is well established that specific promoter
elements control the expression of contractile protein genes in development, and many of these promoter elements may be involved in gene expression in phenotypic modulation. A more thorough discussion of the transcriptional control for smooth muscle cell gene expression is beyond the scope of this review, and the reader is referred to a recent summary relating to smooth muscle cell-specific gene expression (79). Nevertheless, there are several common elements found in smooth muscle-specific promoters for contractile proteins, ECM proteins, and other smooth muscle-specific genes. At this time, however, there are no studies relating to the regulation of these promoters or the specific regulatory elements contained within these promoters by NO or PKG signaling. Findings that directly implicated a specific role for cGMP and PKG in the regulation of VSMC phenotype and gene expression were first reported by Boerth et al. (9, 10, 27, 62). In these studies, adult rat aortic VSMCs that were highly passaged and deficient in PKG expression were found to demonstrate increased production of smooth muscle myosin heavy chain, α-actin, and other markers of the contractile phenotype in response to the restoration of PKG expression. Likewise, inhibition of ECM protein production was also found to occur in response to restoration of PKG expression. In some cases, increased expression was found to be dependent on the increased steady-state production of the mRNA for these proteins, whereas, in others, the processing of mRNA may be regulated by cGMP-dependent protein phosphorylation (107). In the case of the decrease in matrix protein production, some level of posttranscriptional control appears to be present (27). In a preliminary report (20), PKG was found to regulate the expression of ~123 genes in cultured human VSMCs. Collectively, these studies indicate that the NO/cGMP/PKG signaling pathway has the capacity to control the expression of proteins in cultured VSMCs and that the expression of these proteins may regulate the morphology and the phenotype of the VSMC.

There are likely to be major regulatory differences in the development of VSMCs from mesenchymal stem cells on the one hand and the modulation of adult VSMCs between contractile and synthetic phenotypes on the other. As mentioned earlier, elegant studies by several laboratories have established important elements in the genes of smooth muscle-specific proteins that must be turned on during development. However, there is no evidence at present that the NO/cGMP/PKG pathway controls the program for smooth muscle cell development. In fact, PKG-deficient mice appear to have relatively normal vessel function, at least as juvenile animals; thus it is unlikely that PKG has a role in gene expression during development (81). On the
VSMC to the synthetic phenotype. bFGF, bovine fibroblast growth factor (PKA). cGMP activates PKA through cross-activation. Prostanoid molecules are shown to activate cAMP-dependent protein kinase (COX)-2 in the vessel wall following injury. The resultant increase in cyclic nucleotide levels from nitric oxide (NO) and cytokine inflammatory mediators. In the latter case, interleukin (IL)-1β and tumor necrosis factor (TNF)-α induce the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 in the vessel wall following injury. The resultant increases in cyclic nucleotide levels from nitric oxide (NO) and prostanoid molecules are shown to activate cAMP-dependent protein kinase (PKA). cGMP activates PKA through cross-activation. Through mechanisms not known at this point, it is proposed that PKA inhibits PKG mRNA production, resulting in modulation of the VSMCs to the synthetic phenotype. bFGF, bovine fibroblast growth factor; PDGF, platelet-derived growth factor; PG, prostaglandin.

Fig. 3. Model for the role of PKG in phenotypic modulation of vascular smooth muscle cells (VSMCs) in disease. Injury and inflammation lead to decreased PKG expression that, at least in part, regulates the phenotype of the VSMCs. Decreased PKG expression occurs as a result of direct suppression of the PKG gene by growth factors and cytokine inflammatory mediators. In the latter case, interleukin (IL)-1β and tumor necrosis factor (TNF)-α induce the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 in the vessel wall following injury. The resultant increase in cyclic nucleotide levels from nitric oxide (NO) and prostanoid molecules are shown to activate cAMP-dependent protein kinase (PKA). cGMP activates PKA through cross-activation. Through mechanisms not known at this point, it is proposed that PKA inhibits PKG mRNA production, resulting in modulation of the VSMCs to the synthetic phenotype. bFGF, bovine fibroblast growth factor; PDGF, platelet-derived growth factor; PG, prostaglandin.

Other hand, the cGMP/PKG pathway may regulate phenotypic modulation of adult VSMCs between phenotypes. This may have some physiological relevance in that endothelial dysfunction or disruption following injury would decrease NO production and inhibit activation of the PKG pathway in the smooth muscle cells. It would be as a result of this type of injury that wound-healing activity of the cells would be important for vessel repair and function. The studies using cultured VSMCs suggest that PKG is at least one key factor regulating the establishment of a contractile-like phenotype.

If there are very few studies that implicate an involvement of the NO/cGMP/PKG pathway in the control of VSMC gene expression in vitro, there are fewer yet in vivo studies (2, 109). Anderson et al. (2) showed that, subsequent to balloon coronary artery injury in the swine, PKG expression is decreased within days after angioplasty and coincident with the loss of calponin expression, a marker protein for the contractile phenotype. As shown in Fig. 2, there was also a corresponding increase in time with the expression of osteopontin, a marker for the synthetic phenotype. It was interesting to note that only in the VSMCs that were undergoing proliferation and were presumably actively involved in wound-healing activity did PKG levels drop in the cells. These results suggest that the decreases in PKG expression may be causally related to the injury response of the VSMC. Therefore, these in vivo studies, coupled with the in vitro studies demonstrating dramatic effects of PKG on VSMC morphology and protein and gene expression, suggest that PKG occupies a central switch in the modulation of VSMC phenotype in response to injury.

Regulation of PKG gene expression in VSMCs. It has been known for decades that PKG expression in mammalian cells is nonuniform, unlike the expression of PKG’s closest relative, PKA. PKG expression is robust in all contractile smooth muscle cells and mesangial cells, platelets, and cerebellum; it is measurable in polymorphonuclear cells, endocrine secretory cells, heart, and some endothelial cells. It appears to be absent in skeletal muscle fibers, many neurons in the central nervous system, and erythrocytes. Other cell types may or may not express PKG, since the levels are so low that they probably reflect the levels in the vasculature. As mentioned earlier, PKG-null mice develop relatively normally and only acquire a lethal phenotype presumably from gastrointestinal disorders. If PKG, as the data suggest, is anything but a “housekeeping” gene, then there are important control mechanisms that enable its expression to be closely modulated.

Cultured VSMCs from rodent and rabbit vessels cease to express normal levels of PKG during multiple passaging; in many cases, expression drops to unmeasurable levels. The mechanism responsible for the decrease in PKG expression is unknown. High cell density appears to increase, or at least maintain, higher expression in vitro (25). Serum-derived growth factors such as platelet-derived growth factor may reduce PKG expression in vitro, suggesting that withdrawal of growth factors may enhance expression (101). Nevertheless, the molecular pathway leading to increased PKG levels in cultured cells or tissues is unknown at this time. In contrast, there is more information available on the mechanisms that decrease PKG expression. Soff et al. (98) demonstrated that high concentrations of NO donor drugs decrease the mRNA levels for PKG in primary cultures of bovine aortic smooth muscle cells. In the only intact tissue study performed to ask the same question, inducible NO synthase (iNOS) expression was found temporally and spatially correlated with decreased PKG expression (2). These results have led to our hypothesis that injury and inflammatory conditions that promote the production of inflammatory cytokines such as interleukin-1β and tumor necrosis factor-α are involved in the reduction in PKG expression in VSMCs (27, 62). We have postulated that these inflammatory cytokines induce the expression of iNOS and, by virtue of high and persistent NO output, inhibit PKG mRNA production. The drop in PKG expression coupled with the production of growth factors and other mediators of cell proliferation and inflammation then would allow modulation of VSMCs to the wound-healing phenotype. If this hypothesis is correct, then it would be predicted that...
animals deficient in iNOS would demonstrate reduced intimal lesion formation in response to injury. In fact, Chyu et al. (22) demonstrated that there was a dramatically reduced neointimal thickening after arterial wall injury in the iNOS knockout mouse.

The molecular mechanisms by which inflammatory events reduce PKG gene expression in VSMCs are unknown. It has been established that inflammatory cytokines induce not only iNOS expression but also expression of cyclooxygenase-2 (COX-2) in VSMCs and that both gene products have been shown to be abundant in atherosclerotic lesions (7, 4, 14, 26, 46, 50, 97, 99, 113, 116). One net effect of iNOS and COX-2 expression in the vessel wall in response to injury would be a sustained production of cyclic nucleotides (both cAMP and cGMP), resulting from the eicosanoid-dependent activation of adenylyl cyclase and the NO-dependent activation of guanylyl cyclase, respectively. Because cyclic nucleotide analogs have been shown to suppress PKG-I mRNA expression in cultured VSMCs (98), it is conceivable that high-output cyclic nucleotide production in response to inflammation suppresses PKG expression in the vessel wall in response to injury. Furthermore, cAMP analogs were found to be more potent than cGMP analogs in reducing PKG mRNA expression, suggesting that PKA may mediate the effects of both cAMP and cGMP through cross activation of PKA. A hypothetical model describing the role of iNOS, COX-2, and cyclic nucleotides in suppressing PKG expression is shown in Fig. 3. Clearly, more studies using pharmacological and molecular tools are needed to confirm whether these pathways in fact suppress PKG expression in vitro and in vivo.

Interestingly, the promoter and gene structures of human PKG-I also suggest a possible role for PKA in regulating PKG mRNA expression. The published sequence of the human PKG-I gene (77) plus unpublished data from our laboratory suggest that PKG gene expression is driven by an Sp1-dependent mechanism. Sp1 proteins are ubiquitous transcription factors that either increase or decrease expression of genes (59). Previous studies have shown that Sp1 function is regulated by both NO (11, 108) and cAMP-dependent PKA activation (87, 105). These studies immediately suggest a link between iNOS and COX-2 induction and the decrease in PKG promoter activity, since inhibition of Sp1 activity by NO and PKA would decrease the expression of PKG in VSMCs. Alternatively, Sp1 proteins interact with a variety of coactivators and corepressors in many different cells. In VSMCs, Sp1 competes with the immediate early gene, egr-1, to increase the expression of other early-onset genes in response to injury (for example, platelet-derived growth factor-A chains, thrombospodin-1) (59, 94); egr-1 is repressed by a gene product, NAB2 (NGF1-A binding protein), to reduce the proliferate response in VSMCs (95). Therefore, it is possible that PKG-I gene expression is controlled by a factor or factors that interact with Sp1 or egr-1 and that NO and PKA regulate the activity of such proteins.

CONCLUSIONS

The NO signaling pathway has become one of the more intensely studied over the past decade. Although NO signaling in cells is complex as a result of its interactions with reactive oxygen species, heme groups on proteins, sulfhydryl groups, and other cellular targets, the activation of guanylyl cyclase remains the most important pathway in mediating NO function. The role of cGMP and its activation of PKG in smooth muscle relaxation has been intensively investigated for more than two decades. We now have a substantial amount of information regarding the mechanism of cGMP/PKG effects in smooth muscle relaxation. The role of NO/cGMP/PKG in smooth muscle cell proliferation and gene expression is now emerging as a major area of investigation. New knowledge on the mechanisms by which this pathway regulates VSMC growth and phenotype will surely add to our understanding on the development of vascular diseases and disorders from graft restenosis and atherosclerosis to hypertension.

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