Proteolytic Processing of cGMP-Dependent Protein Kinase I Mediates Nuclear cGMP Signaling in Vascular Smooth Muscle Cells

Takahiro Sugiura, Hidehiko Nakanishi, Jesse D. Roberts Jr

Abstract—Cyclic GMP modulates gene expression in vascular smooth muscle cells (SMCs) in part by stimulating cGMP-dependent protein kinase I (PKGI) and the phosphorylation of transcription factors. In some cells, cGMP increases nuclear translocation of PKGI and PKGI-dependent phosphorylation of transcription regulators; however, these observations have been variable, and the mechanisms mediating nuclear PKGI translocation are incompletely understood. We tested the hypothesis that proteolytic cleavage of PKGI is required for cGMP-stimulated nuclear compartmentation of PKGI and phosphorylation of transcription factors. We detected an NH₂-terminal PKGI fragment with leucine zipper domain immunoreactivity in the cytosol and endoplasmic reticulum of SMCs, but only a COOH-terminal PKGI fragment containing the catalytic region (now termed PKGIγ) was observed in the Golgi apparatus (GA) and nucleolus. Posttranslational PKGI processing in the GA was critical for nuclear compartmentation of PKGIγ because GA disruption with nocodazole or brefeldin A inhibited PKGIγ nuclear localization. PKGIγ immunoreactivity was particularly abundant in the nucleolus of interphase SMCs where its colocalization with the nucleolar dense fibrillar component protein fibrillarin closely matched the level of nucleolar assembly. Purified nucleolar PKGIγ enzyme activity was insensitive to cGMP stimulation, which is consistent with its lack of the NH₂-terminal autoinhibitory domain. Mutation of a putative proteolytic cleavage region in PKGI inhibited cGMP-mediated phosphorylation of cGMP-activated PKGI and activities of PKGI in SMCs. (Circ Res. 2008;103:53-60.)

Key Words: cGKI ■ guanylate cyclase ■ gene expression regulation ■ signal transduction ■ vascular disease

Cyclic GMP is a key regulator of vascular smooth muscle cell (SMC) cytoskeletal kinetics, proliferation, and differentiation, and abnormalities in cGMP signaling have been associated with pulmonary and peripheral vascular disease. Cyclic GMP is synthesized by guanylyl cyclases, which are activated by nitric oxide, carbon monoxide, and natriuretic peptides, and is metabolized by phosphodiesterases. Through the interaction with cytoplasmic proteins, cGMP influences SMC shape and migration and decreases vascular tone. Recently, cGMP has also been observed to modulate the expression of genes that influence SMC phenotype and proliferation. For example, cGMP has been noted to regulate SMC gene expression by increasing the phosphorylation of transcription factors, such as cAMP response element-binding protein (CREB) and activating transcription factor-1 (ATF-1), altering the expression of transcription regulators such as activator protein-1 (AP-1) and the growth arrest-specific homeobox transcription factor (GAX), and regulating the activity of other nuclear factors, such as serum response factor (SRF).

cGMP-dependent protein kinase I (PKGI) appears to modulate many of the nuclear activities of cGMP. On activation by cGMP, PKGI increases the phosphorylation of several nuclear transcription factors. PKGI is a threonine/serine kinase present in 2 isoforms in SMCs as a result of alternate mRNA splicing. These isoforms, PKGIα and PKGIβ, have distinct NH₂-terminal leucine zipper (LZ) domains. Through these LZ domains, the PKGI isoforms homodimerize and also interact with heterologous proteins, which anchor them in the cytosol and in proximity to their phosphorylation targets. The conserved COOH-terminal portion of PKGI contains 2 cGMP-binding domains and a catalytic region containing Mg²⁺/ATP-binding, kinase, and substrate recognition domains. The importance of PKGI in modulating transcription factor phosphorylation is supported by the observation that in cells lacking PKGI, such as baby hamster kidney (BHK) cells, cGMP does not phosphorylate CREB or ATF-1. In contrast, in cells that express PKGI, such as some low passage SMCs, 3T3 fibroblasts, and BHK cells transfected with PKGI-encoding plasmids, cGMP stimulates phosphorylation of CREB and ATF-1.

Several studies suggest that nuclear translocation of PKGI is important for cGMP-mediated regulation of transcription...
factors in SMCs. PKGI has been detected in nuclei in some SMC lines. Moreover, cGMP induces nuclear localization of recombinant PKGI in BHK cells, which do not express endogenous PKGI, where it mediates cGMP-stimulated phosphorylation of CREB. Importantly, this activity of cGMP in BHK cells is dependent on the activity of a putative monomeric nuclear localization sequence in PKGI that resides within the Mg²⁺/ATP-binding domain. In addition, the nuclear activities of cGMP have been observed to occur independently of the Ca²⁺, PKA, and MAPK signaling pathways. However, there is variability in the PKGI nuclear localization and activities reported in several studies. In some investigations, PKGI was not observed in the nuclei of primary SMCs or SMC lines and did not modulate cGMP-dependent gene expression. One study reported that in cells that lack cGMP-modulated PKGI translocation, cGMP does not modulate CREB phosphorylation or cAMP response element (CRE)-dependent gene expression. This variability in cGMP-mediated PKGI nuclear localization and nuclear function suggests that additional mechanisms might influence PKGI-dependent cGMP nuclear signaling. In this study, we investigated the role of PKGI proteolysis in PKGI function and localization and established its importance in mediating cGMP-induced nuclear events in SMCs.

Materials and Methods
Detailed information is provided in the supplemental materials (available online at http://circres.ahajournals.org).

Antibodies and Reagents
The anti-PKGI regulatory domain (PKGIreg) antibody was kindly provided by D. Bloch (Mass General Hospital, Boston). The other antibodies and reagents were obtained from commercial sources.

Cell Culture and Transfection
Pulmonary artery smooth muscle cells (PASMCs) were isolated from rats; all other cells were obtained from American Type Culture Collection. PASMCs and RFL-6 cells were maintained in RPMI 1640 and the others in DMEM medium. PASMC medium was supplemented with 10% Nuserum, and all other media contained 10% fetal bovine serum. Cells were transfected using a cationic lipid-based reagent. Luciferase activities were measured using a PKGI-specific peptide substrate as previously described.

Quantification of PKGI nuclear localization
Cells transfected with plasmids encoding wild-type and mutant PKGIβ-FLAG and green fluorescent protein with a nuclear localization sequence (GFPnls) were treated with 8-Br-cGMP, exposed to digitonin in PBS containing protease inhibitors, and then fixed as described above. PKGIβ-FLAG, reacted with biotinylated anti-FLAG antibody and Alexa Fluor 610-conjugated streptavidin, and GFPnls were detected using epifluorescence. Identically registered epifluorescent images of 6 randomly-oriented, nonoverlapping 1.0-mm² fields were obtained and analyzed using ImageJ.

Statistical Analysis
Data are represented as mean±SD. Data were analyzed using a randomized complete block design and, because no differences were found between the groups, the results were analyzed using one-way ANOVA. When significant differences were detected, a Scheffé test was used post hoc. Significance was determined at P<0.05.

Results
Nuclear PKGI lacks NH²-terminal LZ domain immunoreactivity
We reasoned that the reported variability in nuclear localization of PKGI could be attributable to a posttranslational modification of PKGI that modifies its immunologic detection. Therefore, several polyclonal antibodies (PKGI epitopes are schematically defined in Figure 1A) were used to localize PKGI functional domains to SMC compartments. Although the nuclei of many pulmonary artery SMCs and cells of SMC and myofibroblast cell lines were observed to harbor PKGIβ immunoreactivity, none exhibited PKGIβ LZ domain reactivity, although it was detected in the cytosol (Figure 1B). However, nuclear PKGIβ staining was not detected in all SMCs. For example, some later SMCs and A7r5 cell passages did not exhibit nuclear PKGIβ immunoreactivity despite the detection of PKGI LZ domain and COOH-terminal immunoreactivity in the cytosol. Moreover, in individual cultures that exhibited nuclear PKGIβ immunoreactivity, expression was nonhomogeneous. For example, in areas where SMCs were confluent, abundant cytosolic and nuclear PKGIβ immunoreactivity was often detected. In contrast, in subconfluent
PKGI is Cleaved in Vascular SMCs

PKGI cleavage might account for the differential compartmentation of PKGI epitopes. We therefore evaluated purified nuclear proteins for evidence of PKGI fragmentation. Plasma membrane disruption and isosmotic density centrifugation permitted the purification of SMC nuclei and effective separation of nuclear and cytosolic proteins (Figure 2A). Immunoblotting using equal amounts of nuclear and cytosolic proteins revealed that SMC nuclear protein fractions did not contain full-length PKGI with NH₂-terminal and COOH-terminal epitope immunoreactivity. However, an ∼18-kDa PKGI fragment with LZ domain immunoreactivity was identified in lysates of BHK cells expressing FLAG-PKGI (Figure 2B, arrowhead), and a 60-kDa fragment (PKGIβ) with PKGILZ and PKGICR immunoreactivity were detected in nuclear proteins. Because we previously found no nuclear immunoreactivity for the PKGIβ LZ domain in intact SMCs (Figure 1B), we performed immunofluorescence studies using purified nuclei to clarify the PKGI LZ domain localization. PKGILZ immunoreactivity mapped to asymmetrical perinuclear structures in purified SMC nuclei (Figure 2C), colocalizing with molecules restricted to the Golgi apparatus (supplemental Figure I). These data suggested that PKGI fragmentation occurs outside the nuclear envelope and that only the COOH-terminal fragment of PKGI enters the nucleus.

Although PKGI expressed from transfected plasmids has been detected in nuclei of some cells, it is not known whether their functional domains are differentially localized. We examined whether the COOH-terminal portion of PKGI overexpressed in RFL6 cells localizes to the nucleus as the endogenous protein does. To improve detection of nuclear PKGI, after RFL6 cells were transiently transfected with plasmids encoding PKGI-FLAG, digitonin was used to permeabilize the plasma membrane and permit the egress of soluble cytosolic PKGI to diffuse from the cell. PKGIβ-FLAG was detected in the nucleus with an anti-FLAG antibody (αFLAG) and colocalized with the GFPNL5 epifluorescence.

Figure 1. PKGI domain nuclear immunoreactivity in SMCs. A, PKGI has a functional leucine zipper (LZ) domain, cGMP-binding regulatory domain (REG), and catalytic region (CR), which were examined using the indicated antibodies. B, In the nucleus of rat pulmonary artery smooth muscle cells (PASMC) and indicated SMC lines, PKGICR immunoreactivity was observed although PKGILZ reactivity was not detected. C, Recombinant PKGI was also localized in cell nuclei. RFL6 cells expressing PKGIβ-FLAG and GFP with a nuclear localization sequence (GFPNL5) were treated with digitonin, which permits soluble cytosolic PKGI to enter the nucleus. PASM Cells expressing PKGIβ-FLAG and FLAG-PKGIβ-FLAG colocalized with DAPI in the nucleus (Figure 1C) but no nuclear PKGIα cr domain immunoreactivity (data not shown).

nuclear proteins for evidence of PKGI fragmentation. Plasma membrane disruption and isosmotic density centrifugation permitted the purification of SMC nuclei and effective separation of nuclear and cytosolic proteins (Figure 2A). Immunoblotting using equal amounts of nuclear and cytosolic proteins revealed that SMC nuclear protein fractions did not contain full-length PKGI with NH₂-terminal and COOH-terminal epitope immunoreactivity. However, an ∼18-kDa PKGI fragment with LZ domain immunoreactivity was identified in lysates of BHK cells expressing FLAG-PKGI and FLAG-PKGIβ. The apparent masses of the NH₂-terminal and full-length PKGI isoforms (*) were identified in lysates of BHK cells expressing FLAG-PKGIα and FLAG-PKGIβ. The apparent masses of the NH₂-terminal and full-length PKGI isoforms are consistent with the different sizes of their LZ domains. Mass spectroscopy identified peptide portions (red letters) of the LZ domain of PKGIα and the precipitating SBP2 epitope (box).
cGMP Increases PKGIγ Nuclear Localization

Previous studies revealed that cGMP increased PKGI immunoreactivity in the nucleus of BHK and some SMC lines.10 Because only PKGIγ was identified in SMC nuclei, we examined the effect of cGMP on nuclear PKGIγ levels. cGMP was found to increase nuclear PKGIγ abundance in BHK and RFL-6 cells expressing PKGIβ-FLAG (Figure 3A). Moreover, because PKGI expression is increased in postconfluent SMCs,19 the influence of SMC density on PKGIγ levels in purified nuclei was examined. cGMP increased nuclear PKGIγ levels in sparsely plated A7r5 cells, but as SMC density increased, so did the level of nuclear PKGIγ immunoreactivity in the absence of cGMP stimulation (Figure 3B). This suggests that as SMCs become more confluent and PKGI expression increases, PKGI cleavage and nuclear PKGIγ translocation increases independent of cGMP exposure.

PKG1γ fragments were detected in the Golgi apparatus (GA; Figure 2C and supplemental Figure I) and cGMP appeared to increase PKGIγ fractionation, thus we investigated whether cGMP increases localization of PKGIγ to the GA. Exposure of RFL-6 cells to cGMP increased PKGIγ immunoreactivity in the GA and endoplasmic reticulum (ER; Figure 3C and 3D). PKG1CR domain immunoreactivity colocalized with the GA whereas PKGI Laz immunoreactivity also colocalized with the ER. These results suggest that cGMP increases PKGI mobilization to the ER and GA, where cleavage might increase PKGIγ accumulation in the GA and its transfer to the nucleoplasm.

Intact Golgi Apparatus Is Required for Nuclear Trafficking of PKGIγ

The GA contains endopeptidases that process some signaling proteins and thereby regulate their nuclear trafficking.20 We examined the role of the GA in regulating PKGIγ nuclear compartmentation. The effects of both Nz and BFA on PKGI nuclear localization were tested because they dissociate the GA through different mechanisms: whereas Nz causes microtubular depolymerization and relocation of GA fragments into cytосolic islands,21,22 BFA inhibits protein transport from the ER to the GA by inhibiting GTP-exchange factors23 and disassembles the cis/middle- and trans-Golgi complexes, causing them to fuse with the ER.24 PKGIγ nuclear localization was inhibited in cGMP-treated RFL-6 cells exposed to either Nz or BFA. After cGMP exposure, PKG1CR immunoreactivity continued to be associated with the Nz- and BFA-disrupted GA, however PKGIγ nuclear transport decreased (Figure 4A). Objective quantitative analysis revealed that GA disruption with Nz completely blocked cGMP-stimulated PKGIγ nuclear localization while BFA partially inhibited it (Figure 4B). GA disruption did not inhibit PKGIγ fractionation: immunoblotting revealed abundant PKGIγ in cGMP-exposed RFL-6 cells treated with these compounds (data not shown), indicating that the effect of GA disruption appears to be on the nuclear translocation of PKGIγ, and not on proteolysis of PKGI.

PKGIγ Is Associated With the Nucleoli of Interphase SMCs

We further explored the apparent association between PKGIγ and SMC nucleoli (Figures 1B and 3C). PKG1CR immunoreactivity colocalized with SMC nucleoli in situ and with SMC nucleoli purified by ultracentrifugation (Figure 5A and 5B). Moreover, isolated SMC nucleoli exhibited cGMP-independent PKGI enzyme activity at a level similar to that detected in cGMP-treated A7r5 whole cell lysates (Figure 5C). This suggests that removal of the NH2-terminal autoinhibitory pseudosubstrate site of PKGI through proteolytic cleavage releases inhibition of kinase activity, revealing high constitutive PKGIγ activity. This observation is consistent with in vitro studies where the NH2-terminal region of PKGI was removed with purified proteases.25,26

Transcription and processing of precursor rRNA and packaging of rRNA into ribosomal particles occur within struc-
Nuclear colocalization of PKGIabolished by Nz exposure and inhibited by BFA treatment. Cell nucleoli into beaded strands that retained PKGI
decreased PKGI
localization. A, Nz and BFA caused GA disassociation and
disassembly and reassembly, PKGI
noreactivity (Figure 5D). During DRB-mediated nucleolar
production and permits evaluation of nucleolar assembly and
activity.28,29 DRB exposure causes the nucleoli to reversely
unravel into intranuclear structures resembling a string of
beads, which comprised RNA polymerase I and are thought
to be single units of rRNA transcription, and a strand of
nontranscribed DNA spacer regions.28–30 To investigate
whether PKGI
actively associates with the nucleolar sub-
components, SMCs were treated with DRB and the nucleolar
association of PKGI
was assessed. DRB disassembled A7r5
cell nucleoli into beaded strands that retained PKGI
immunoactivity (Figure 5D). During DRB-mediated nucleolar
disassembly and reassembly, PKGI
localized in the nucle-
olus in a similar manner as fibrillarin (Figure 5E), a DFC
protein that processes precursor rRNA,31 indicating that
PKGI
has a dynamic relationship with SMC nucleoli.

Regulation of Gene Expression by cGMP Requires Nuclear PKGI
Previous studies indicate that nuclear PKGI modulates gene
expression in part by phosphorylating CREB.2–4 Because
cGMP induces nuclear localization that appears dependent on
PKGI cleavage, we examined whether mutation of a putative
cleavage site inhibits cGMP-dependent CREB phosphoryla-
tion and activity and PKGI
nuclear localization. To identify
the PKGI cleavage site, the NH2-terminal end of immunopu-
rified PKGI
was detailed using Edman-based amino acid
sequencing. Accounting for a protein fragment contributed by
bovine serum albumin, peptides commencing with serine
and glutamic acid of PKGI
were identified (detailed in
supplemental Figure III). We generated plasmids with alanine
substitutions in this region (mapped in Figure 6A and
supplemental Figure III) and assessed their ability to express
catalytically active PKGI
and support cGMP-dependent
CREB phosphorylation and CRE-dependent gene expression
in BHK cells. Transfection conditions in these experiments
were optimized so that wild-type and mutant PKGI had
equivalent immunoreactivity levels. The wild-type and mu-
tant PKGI forms exhibited similar cGMP-stimulated cytos-
olic kinase activity, as shown by the ability of cGMP to
stimulate PKGI-dependent VASP serine phosphorylation
(Figure 6B). However, Mut3 and Mut4 exhibited decreased
cGMP-dependent CREB phosphorylation. Of interest, these 2
mutants had amino acid substitutions associated with the
amino acid sequence KVEVTK, which has similarity to a
putative proprotein convertase motif.32 Moreover, the amino
acids in this area have a high level of species homology in
PKGI (supplemental Figure III). Previous studies found that
nuclear localization of PKGI critical for cGMP-dependent
stimulation of CRE-mediated gene expression.10 We exam-
ined the ability of PKGI Mut3 to confer cGMP-dependent
CRE-promoter activity. cGMP increased CRE-dependent
promoter activity in BHK cells expressing wild-type PKGI
but not in cells expressing PKGI Mut3 (Figure 6C). Addi-
tional studies revealed that this PKGI mutant exhibited less
cGMP-stimulated PKGI
nuclear localization (Figure 6D).
These studies suggest that proteolytic cleavage of PKGI
within the cGMP-binding domain is critical for the nuclear
activities of cGMP.

Discussion
The principal objective of this investigation was to examine
mechanisms that regulate the nuclear localization of PKGI
and modulation of gene expression by cGMP. In SMCs,
cGMP exposure facilitated the cleavage of the NH2-terminal
LZ domain from PKGI and the nuclear localization of
PKGI, a constitutively active kinase fragment of PKGI. The
Golgi apparatus appeared to have an important role in
modulating the nuclear translocation of PKGI; Golgi apparatu-
sus disruption with either nocodazole or brefeldin A inhibited
the cGMP-stimulated nuclear localization of PKGI. Addi-
tionally, PKGI nuclear localization appeared to be required
for the regulation of gene expression by cGMP. Mutation of
PKGI in the putative proteolysis site inhibited cGMP-
mediated CREB-phosphorylation, CRE-dependent gene
expression, and the nuclear localization of PKGI. cGMP
regulates the expression of genes that modulate SMC prolif-
eration and differentiation, therefore these results support a
central role for PKGI in mediating the influence of cGMP on
cell phenotype. Moreover, they provide additional evidence
that proteolytic pathways regulate some canonical signaling
systems. Protein cleavage is also a critical step in the nuclear

Figure 4. An intact GA was important for PKGI
nuclear localization. A, Nz and BFA caused GA disassociation and
decreased PKGI
nuclear localization in 8-Br-cGMP-treated
RFL-6 cells. B, cGMP-induced nuclear PKGI
localization was abolished by Nz exposure and inhibited by BFA treatment.
Nuclear colocalization of PKGI
-FLAG and GFP
, shown in the inset, was objectively quantified in digitonin-treated RFL-6 cells
detailed in the text. Results are expressed as means±SD, n=6 per group, and typical of 3 independent experiments. *and
indicated treatment vs the other treatment groups.
translocation and activities of sterol regulatory element binding protein (SREBP) and Notch. In these pathways, proteolytic removal of a membrane-binding domain releases a protein fragment that can enter the nucleus and modulate gene expression. Similarly, NH2-terminal cleavage of PKGI removes an LZ domain that tethers PKGI to cytosolic proteins. The resulting active kinase PKGI fragment enters the SMC nucleus and phosphorylates proteins that regulate gene expression.

Although the functional domains of PKGI and PKA are arranged similarly, the requirement for proteolytic processing to enable cyclic nucleotide-dependent nuclear signaling distinguishes PKGI from PKA. The PKA heterodimer consists of separate regulatory and catalytic subunits. On cAMP binding, the catalytic subunit is released from the regulatory unit which anchors it in the cytosol and enters the nucleus where it modulates gene expression.33 In PKGI, the anchoring (LZ) and regulatory and catalytic (PKGI\textsubscript{H9253}) domains are on a single protein molecule that appear to require cleavage to allow PKGI\textsubscript{H9253} nuclear translocation. Another difference between PKA and PKGI is the requirement for a nuclear localization sequence (NLS) for PKGI nuclear localization. Unlike the PKA catalytic subunit, which is small enough to passively diffuse into the nucleus, PKGI\textsubscript{H9253} is too large to enter the nucleus without an NLS to facilitate docking to nuclear pore complexes. Gudi and coworkers identified a monomeric NLS in PKGI that facilitated nuclear PKGI\textsubscript{H9253} localization in BHK cells on cGMP treatment.3,10 This NLS is in the Mg\textsuperscript{2+}/ATP binding domain of PKGI and is present in PKGI\textsubscript{H9253}.

Studies suggest that cGMP-induced conformational changes in PKGI may expose a cryptic proteolytic site to endopeptidases, regulating PKGI cleavage. For example, in vitro studies indicate that cGMP-binding unfolds PKGI and increases the sensitivity of PKGI to endopeptidase-induced fractionation.36,37 In these experiments, PKGI was not cleaved in the cGMP-binding region, as we report here. This suggests that SMCs contain an endopeptidase that cleaves amino acid residues in the cGMP-binding region and is distinct from those investigated in the in vitro studies. It
Figure 6. Mutation of the putative proteolytic cleavage area in PKGI inhibited cGMP-mediated gene expression and nuclear localization of PKGI. A, PCR generated plasmids encoding mutant PKGIβ-FLAG (Mut1–5) with alamines (box) in a putative proteolysis area suggested by NH2-terminal amino acid sequence analysis of a fragment of immuno-purified A7r5 cell PKGIβ. B, Mutation of the putative PKGI cleavage site inhibits cGMP-mediated CREB phosphorylation. Immunoblotting with antiphospho-CREB antibodies revealed a decrease in phospho-CREB in lysates of cGMP-treated BHK cells expressing mutant PKGIβ-FLAG compared to those expressing wild-type PKGI-FLAG. The cells had equivalent cGMP-stimulated cytosolic PKGI activity levels as reflected by phospho-VASP immunoreactivity. C, PKGI cleavage site mutation inhibits cGMP-dependent CRE-activated gene transactivation. BHK cells transfected with a CRE-luciferase reporter plasmid and expressing Mut3 PKGI-FLAG did not have increased luciferase activity when exposed to 8-Br-cGMP and examined for FLAG immunoreactivity. 8-Br-cGMP did not increase nuclear PKGIγ immunoreactivity in cells expressing Mut3. Results are expressed as means ± SD, n = 6 each group, and typical of 3 independent experiments. *P < 0.05, indicated treatment vs the other treatment groups. D, PKGI cleavage site mutation inhibited PKGIγ nuclear localization. BHK cells expressing Mut3 or wild-type PKGI-FLAG were treated with 8-Br-cGMP and examined for FLAG immunoreactivity. 8-Br-cGMP and examined for FLAG immunoreactivity. 8-Br-cGMP treatment vs the other treatment groups. D, PKGI cleavage site mutation inhibited PKGIγ nuclear localization. BHK cells expressing Mut3 or wild-type PKGI-FLAG were treated with 8-Br-cGMP and examined for FLAG immunoreactivity.

might also indicate that additional PKGI posttranslational modifications or protein interactions are required to permit PKGI proteolysis within the cGMP-binding region.

The association of PKGIγ with the nucleolus in SMCs is a novel finding. The PKGIγ nuclear compartmentation we report, particularly during nucleolar disassociation and reassembly, and the presence of PKGI enzyme activity within purified nucleoli support the notion that PKGI is actively integrated within the nucleolus of SMCs. These data suggest that cGMP signaling might influence nucleolar function. Recent proteomic screens indicate that the nucleolus contains not only proteins required for ribosomal biogenesis, but also kinases such as protein kinase C and PKA, which likely transduce cytoplasmic signals.

The identification of PKGI proteolysis in SMCs has important implications for vascular disease mechanisms and the development of novel therapies. Indirect evidence suggests that abnormalities in PKGI processing contributes to neointima formation in injured vessels. For example, Monks and coworkers observed that carotid artery injury in rats increases PKGI expression in neointimal SMCs. However, because the increased PKGI was detected in the perinuclear region of SMCs and not within the nucleus, PKGI proteolysis likely was diminished in the injured vessels. Decreased nuclear PKGI activity might contribute to SMC proliferation in injured carotid arteries. In early passaged SMCs, in which we detected PKGI proteolysis and nuclear PKGIγ, PKGI decreases cell proliferation. In contrast, in murine SMCs, in which PKGI does not localize in the nucleus, PKGI increases cell proliferation. Such an attenuation of PKGI proteolysis might inhibit the cytostatic effect of PKGI-based therapies. For example, Sinnaeve and coworkers found that although overexpression of PKGI does not decrease neointimal formation in the injured porcine aorta, transduction of an LZ domain-deficient mutant PKGI, which could passively diffuse into SMC nuclei, is protective. Thus, although PKGI expression is upregulated in some injured vessels, deficient proteolysis could prevent PKGIγ generation and nuclear translocation, blocking cGMP-induced protection against neointimal cell proliferation. Our data indicate that PKGIγ- like molecules may be able to protect against vascular diseases in which PKGI proteolytic mechanisms are diminished.

In summary, we identified a novel mechanism that regulates cGMP nuclear signaling. The observation that PKGI proteolytic cleavage critically regulates this signaling pathway has important implications for understanding how cyclic nucleotides regulate gene expression in health and disease.

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Disclosures

None.

References


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Detailed Materials and Methods

Antibodies and reagents- Anti-PKGIβLZ (Stressgen; KAP-PK002), anti-PKGIβREG (a kind gift of Dr. Janssens1), which was generated in rabbits using a purified protein fragment corresponding to amino acids 41-322 of human PKGIβ, and anti-PKGIβCR (Stressgen; KAP-PK005) were used to map the leucine zipper domain of PKGIβ and the regulatory and catalytic domains of PKGI to intracellular compartments, respectively. The FLAG-epitope was detected using biotinylated anti-FLAG (Sigma; F9291). Anti-CREB antibody (Upstate, 06-863) and human serum with autoantibodies against pyruvate dehydrogenase (kindly proved by Dr. Donald Bloch) were used to detect nuclear and cytoplasmic proteins. Isolectin IB4-conjugated with Alexa Fluor 488 (Molecular probes, I-21411) and anti-disulfide isomerase antibody (Stressgen, SPA-891) were used to detect the GA and ER, respectively. Anti-fibrillarin antibody (Cytoskeleton, AFB01) was used to detail intranucleolar structures. Anti-phospho-CREB S133 (Upstate, 06-519) and anti-phospho-VASP S239 (Cell Signaling, 3114) were used to assess nuclear and cytosolic PKGI activity. To stimulate PKGI, membrane-permeant cGMP analogues, 8-Br-cGMP (Sigma, B1381) and 8-(p-chlorophenylthio)-cGMP (Sigma, C5438; 8-CPT-cGMP) were used. Nz (Sigma, M1404) and BFA (Fluka, WA13082) were used to disrupt the GA and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; Sigma, 35290) was used to dissociate the nucleolus.

Cell culture and transfection- Rat PASMC were obtained from pulmonary arteries, as previously described.2 A7r5, A10, RFL6, and BHK cells were obtained from American Type Culture Collection (Manassas VA). Rat PASMC and RFL-6 cells were maintained in RPMI 1640 (Invitrogen) and A7r5, BHK, and A10 cells were maintained DMEM medium (Invitrogen). The medium for the rat PASMC was supplemented with 10% Nuserum (BD Bioscience), the medium for the other cells contained 10% heat-inactivated fetal bovine serum (Hyclone); the medium for all cells was supplemented with penicillin and streptomycin. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen), as described by the manufacturer. Generally, 1.0 x 10^5 cells were exposed to 0.5 – 2 µg of plasmid and 1 – 4 µl of Lipofectamine 2000 reagent for 6 hours in Opti-MEM medium (Invitrogen) before the medium was changed and the cells were incubated for an additional 18 – 24 hours before being studied. The amount of plasmid used in the transfection reactions was carefully titrated during pilot studies to cause the expression of equivalent levels of PKGI expression as determined by immunoblotting in the different treatment groups.

Subcellular fractionation- SMC nuclei were purified according to previously described methods3 at 4 °C and in the presence of protease inhibitors (Sigma). The crude nuclear suspension, generated using a small glass homogenizer (Pyrex no. 7726) to disrupt A7r5 cells swelled in a hypotonic buffer (5 mM MgCl₂, 10 mM NaCl, 10 mM Tris HCl (pH 7.5) containing 1% IGEPAL CA-630 (Sigma), was purified using an iodixanol step density gradient (Sigma, Optiprep) and centrifuged at 9 850 x g (9 000 rpm using a SW 55 rotor) for 40 min at 4 °C with no braking (Beckman L8-80M). Purified nuclei were collected at the 30 — 35% (w/v) iodixanol interphase, washed again with the hypotonic buffer, and re-suspended in a buffer suitable for the studies. The purity of the nuclei was confirmed by inspection using phase contrast microscopy, by SDS-PAGE analysis of the nuclear and cytosolic fractions using SYPRO red protein stain (Molecular Probes) and epifluorescent scanning densitometry, and by immunoblotting to detect differential expression of the markers CREB (nuclear) and pyruvate dehydrogenase E2 subunit (cytosolic).

Nucleoli were obtained from purified SMC nuclei using the method described by Muramatsu et al.4 at 4 °C and in the presence of protease inhibitors (Sigma). After washing, purified A7r5 cell
nuclei were suspended in ~10 volumes of 0.34 M sucrose containing 0.25 mM MgCl₂ and disrupted using sonication. The resulting nuclear materials were centrifuged at 2,000 x g for 20 min through a 0.88 M sucrose cushion containing 0.25 mM MgCl₂, and the purified nucleoli were collected and resuspended in a buffer that was suitable for further studies. The purity of the isolated nucleoli was confirmed by their characteristic appearance in phase contrast microscopy and by staining with Azure C and fluorescent RNA-binding dyes (Molecular Probes, SYTO RNASelect).

Plasmid construction and mutagenesis - pcDNA3•PKGIβ-FLAG, an expression plasmid that encodes COOH-terminal FLAG-tagged murine PKGIβ, pcDNA3•SBP2-PKGIα, which encodes an NH₂-terminal streptavidin-binding protein (SBP2, as described by others⁵,⁶), and pcDNA3•FLAG-PKGIα and pcDNA3•FLAG-PKGIβ, which encode NH₂-terminal FLAG-tagged murine PKGIα and PKGIβ, respectively, were constructed using standard techniques.⁷ Plasmids encoding mutant murine PKGIβ, in which alanine had been introduced into a putative proteolytic region, were generated using PCR and the oligonucleotides detailed in the Online Table I according to the methods described by others.⁸ To exclude mutations inadvertently introduced by the DNA polymerase, the mutated cDNA fragment was excised with Pst I and Xho I and ligated into a parental plasmid. The expression and kinase activity levels of the mutant PKGIβ were evaluated in lysates from 8-Br-cGMP-treated BHK cells transfected with the constructs by immunoblotting with anti-FLAG and anti-phospho-VASP antibodies. Automated DNA sequencing was used to verify the authenticity of the constructs.

Purification and analysis of PKGI fragments - The identity of a putative NH₂-terminal fragment of proteolytically cleaved PKGIα was determined using LC-MS/MS. BHK cells were transiently transfected with pcDNA3•SBP2-PKGIα, treated with 1 mM 8-Br-cGMP, and then lysed in RIPA buffer containing protease inhibitors (Sigma). Subsequently, the cell lysates were pre-cleared and the SBP2-tagged protein fragments were collected using streptavidin immobilized on magnetic beads. After extensive washing, the SBP2-tagged proteins were released from the beads in heated SDS-loading buffer, split into two fractions and then resolved using SDS-PAGE. The proteins from one sample portion were transferred to a PVDF membrane and identified using peroxidase-conjugated streptavidin and enhanced chemiluminescence; the proteins from the other aliquot were fixed in the gel using methanol and stained with a fluorescent total protein stain. A ~20-kDa protein fragment identified to have the same relative migration as a fragment with peroxidase-conjugated streptavidin reactivity, was digested with trypsin and fragments were analyzed by liquid chromatography / tandem mass spectrometry (Proteomics Research Services, Inc.).

The putative sessile amino acids of PKGI were identified by sequencing the NH₂-terminal end of immunopurified PKGIγ. The nuclei of nearly confluent 8-Br-cGMP-treated A7r5 cells were collected and purified as described above, and homogenized in a buffer containing 150 mM NaCl and 0.1 M sodium phosphate. After centrifugation, PKGIβ and PKGIγ in the supernatant were collected using the anti-PKG CD antibody immobilized on a solid substrate (Pierce; MicroLink Protein Coupling kit). Subsequently, the collected proteins were released into heated SDS-loading buffer, resolved using SDS-PAGE, and electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane (ProBlott; Applied Biosystems). The protein corresponding to PKGIγ based on its relative MW was identified using Ponceau S staining and subjected to automated protein microsequencing using Edman degradation and high performance liquid chromatography (HPLC) (Applied Biosystems; Model 491A Protein Sequencer).
Immunodetection of proteins- The intracellular localization of PKGI was determined using indirect immunofluorescence. Cells and purified nuclei and nucleoli were fixed with 4% paraformaldehyde in PBS, permeabilized with 100% methanol or, in the case of fibrillarin detection, fixed in 100% methanol and permeabilized with 1% Triton X-100, and then blocked with 1% serum in PBS. After incubation with a primary antibody or pre-immune serum, the cells and organelles were exposed to biotin-conjugated secondary antibodies and a fluoroprobe-linked strepavidin or a fluorescently labeled secondary antibody and the antigen-antibody complexes were detected using epifluorescence microscopy. In the case of FLAG-epitope detection, the biotinylated primary antibody was detected using strepavidin conjugated with a fluoroprobe. Protein blot hybridization was used to document the separation of the cytosolic and nuclear proteins using the methods described above. Nuclear and cytoplasmic protein fractions were resolved using SDS-PAGE and then transferred to a PDVF membrane. After blocking with 1% serum in PBS, the protein blot was exposed to antibodies recognizing CREB and E2, and the antigen-antibody complexes were detected using enzyme-conjugated secondary antibodies and chemiluminescence. To detect PKGI isoform abundance and CREB and VASP phosphorylation, protein fractions were resolved using SDS-PAGE, and transferred to a PDVF membrane. After the protein blot was blocked with 5% nonfat milk in TBS containing 0.1% Tween 20, it was incubated with the primary antibody and exposed to a horseradish peroxidase-conjugated secondary antibody before having the antigen-antibody complexes detected using chemiluminescence. In the cases in which the biotinylated anti-FLAG antibody was used to detect the FLAG epitope, the blots were exposed to ABC-peroxidase (Vector Labs) instead of the secondary antibody.

PKG enzyme activity- Nucleolar PKG enzyme activity was measured using methods described previously. Cos7 cells and A7r5 cells and purified nucleoli were homogenized in 0.01 M NaH₂SO₄, 2 mM EDTA (pH 6.8). After centrifugation, the protein concentration of the supernatant was determined using a bicinchoninic acid-based protein assay method (Pierce), and the PKG enzyme activity was determined using a reaction mixture containing 20 µM Tris (pH 7.4), 200 µM ATP, 136 µg/ml of the PKGI-phosphorylation substrate RKISASEF (Genosys Biotechnologies), 20 mM MgCl₂, 100 µM 3-isobutyl-1-methylxanthine, 1 µM (Rp)-cAMP-S (Biolog), and 30,000 cpm/µl [γ-32P]-ATP. Assays were conducted in the absence and presence of 10 µM cGMP and were terminated by absorption of the peptide onto a phosphocellulose membrane (Whatman; P-81 paper). After washing the membranes with 75 mM phosphoric acid, radiolabeled peptide was quantitated by liquid scintillation spectrometry. The PKG enzyme activity is expressed as picomoles of peptide phosphorylated / min · mg of protein.

Quantification of PKGIγ nuclear localization- RFL-6 and BHK cells were transfected with plasmids encoding native and mutant PKGIγ with a COOH-terminal FLAG-tag and GFP with a nuclear localizing sequence (Clontech, pAcGFP1-Nuc). Subsequently, the cells were exposed to medium with and without 1 mM 8-Br-cGMP, briefly washed with PBS, and treated with 40 µg/ml digitonin dissolved in PBS containing protease inhibitors for 10 min. After washing the cells with PBS, they were exposed to a biotinylated anti-FLAG antibody and Alexa Fluor 610 conjugated streptavidin (Molecular Probes), and PKGIγ-FLAG and GFP were detected using epifluorescence. To quantify PKGI nuclear localization, identically registered epifluorescent images of six randomly oriented, non-overlapping 1.0 mm²-fields that contained at least 25 cells with nuclear GFP-epifluorescence were obtained. Employing ImageJ and a custom-written macro, the number of cell nuclei with PKGIγ-FLAG immunofluorescence and GFP fluorescence was determined in the following manner: first the image with GFP-epifluorescence was thresholded and the number of nuclei from the transfected cells with transgene expression was then determined using a particle analysis algorithm. Subsequently, the image with the PKGIγ-FLAG detected using Alexa Fluor 610 epifluorescence was thresholded and then underwent a
bit-wise multiplication operation using a mask obtained from the GFP-fluorescent image. The resulting number of nuclei with both fluorescent signals was then enumerated as above. The percentage of cells with PKGIγ was determined by dividing the number of cells with both anti-FLAG immunoreactivity and GFP epifluorescence by the total number of cells with GFP epifluorescence. During the obtainment and analysis of the fluorescent image data, the investigator was not aware of the plasmids employed in the transfection experiment or used to treat the cells.

PKGIγ nuclear signal transduction- PKGI-dependent CREB phosphorylation was determined by immunoblotting of the lysates from 8-Br-cGMP-treated BHK cells transiently transfected in 12-well culture plates with mutant and wild-type pcDNA3•PKGIβ-FLAG. To quantify CRE-dependent transcription, BHK cells were transiently transfected with 0.12 µg pcDNA3•PKGIβ and 1.08 µg pcDNA3, 0.60 µg pcDNA3•PKGIβ-Mut3 and 0.60 µg pcDNA3, or 1.2 µg pcDNA3, and with 0.3 µg pCRE-luc and 0.06 µg pRL-CMV (both from Promega). After 6 hours, the cells were cultured in DMEM supplemented with penicillin, streptomycin and 0.2% FBS for 12 hours. 1 mM 8-Br-cGMP was added to some wells and all were cultured for another 12 hours after which cells were lysed and the Photinus pyralis (firefly) and Renilla reniformis luciferase enzyme activities were measured using a commercially available kit (Promega, E1910) and a luminometer (Perkin Elmer, Victor3). The data were normalized as follows: firefly / Renilla luciferase activity for each sample was expressed relative to the average value observed in the cGMP-treated pcDNA3•PKGIβ transfected group in the same experiment.

Statistical analysis- The data are represented as mean ± SD. The experiments were independently repeated three times. The effect of the treatments and mutagenesis of PKGIβ on nuclear localization and CRE-dependent luciferase activation data were analyzed between individual experiments by using a randomized complete block design. Since this method indicated that there was no significant difference between groups, the data from a typical experiment was analyzed using a one-way ANOVA. When significant differences were detected, a Scheffe test was used post hoc. Significance was determined at P < 0.05.
References


Supplemental figure legends

Online Figure I. The PKGIβ LZ domain-containing fragment in nuclear protein fractions is localized to the Golgi apparatus (GA). Purified A7r5 nuclei were affixed to glass slides and reacted with the anti-PKGIβ LZ antibody to detect the NH₂-terminal portion of PKGIβ, isolectin IB4 to detect the GA (αGolgi), and anti-disulfide isomerase antibody to identify endoplasmic reticulum (αER).

Online Figure II. Over-expression of PKGIβ in SMC using adenoviruses increases PKGIγ generation and nuclear localization. Sub-confluent A7r5 cells were infected with an adenovirus encoding human PKGIβ (Ad.PKGIβ²). (A) Equal amounts of total protein obtained from disrupted whole cells or isolated cell nuclei were immunoblotted with an anti-PKGIγ antibody. PKGIγ was increased with elevated PKGIβ expression. (B) SMC infected with adenovirus encoding human PKGIβ (Ad.PKGIβ) exhibited increased nuclear PKGIγ immunoreactivity.

Online Figure III. The putative proteolysis region of PKGI is highly conserved between species. The NH₂-terminal sequence of PKGIγ immunopurified from the nuclei of cGMP-treated A7r5 cells was determined by Edman degradation-based protein microsequencing. Accounting for a protein fragment of bovine serum albumin, which was co-purified with PKGIγ, two peptide sequences were detected as indicated; large case letters refer to confirmed amino acid residues and small case letters correspond to amino acid residues that are suggested by the HPLC data. The amino acid sequences from the indicated species were determined by data base interrogation (BLAST). Red amino acids are part of a putative proprotein convertase site identified by inspection.
**Online Table I**

**Primers utilized to generate mutant murine PKGIβ:**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Mutated AA*</th>
<th>Sense and antisense primers (5’ to 3’)</th>
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<tbody>
<tr>
<td>Mut1</td>
<td>141 - 147</td>
<td>gca gca gca gct gca ggc gaa ggc gat gtg ggg tca ctg gta ttc cac ggg gta cat aca gtc</td>
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<tr>
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<td></td>
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<td>149 - 154</td>
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</tr>
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<td></td>
<td></td>
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</tr>
<tr>
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<td>156 – 161</td>
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<td>cgc agc ggc cgc ggc ggc cac cac ccc cac act gcc ttc c</td>
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<td></td>
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</tr>
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</table>

* * amino acid sequence number is based on murine PKGIβ (Accession number: NP_035290; GI: 6755156).
Online Figure I

αPKGI\textsubscript{LZ}  αPKGI\textsubscript{LZ}

αGolgi  αER

Merge  Merge

DAPI  DAPI
Online Figure II

A

IB: $\alpha$PKG$_{\text{CR}}$

<table>
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<th>Whole cell</th>
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<tr>
<td>kDa 75</td>
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<td>PKG$_{\text{I} \gamma}$</td>
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</tr>
<tr>
<td>50</td>
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</tbody>
</table>

Ad.PKG$_{\beta}$ 25 MOI

- - + +

B

$\alpha$PKG$_{\text{CR}}$  DAPI

- + - +
Online Figure III

Putative PKGI cleavage site:

<table>
<thead>
<tr>
<th>A7R5 Cell PKGIγ fragments</th>
<th>SLVYVMeDgk</th>
<th>EgvKL-Tm</th>
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<tr>
<td>Mouse</td>
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<td>Drosophila foraging</td>
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Alanine mutants ...A 1 E 2 V 3 4 5 TM...

* Protein convertase recognition site: (K/R)-Xn-(K/R); n = 0, 2, 4, 6 and not C.