Detailed Materials and Methods

Antibodies and reagents- Anti-PKGIβ_{LZ} (Stressgen; KAP-PK002), anti-PKGI$_{REG}$ (a kind gift of Dr. Janssens[1]), which was generated in rabbits using a purified protein fragment corresponding to amino acids 41-322 of human PKGIβ, and anti-PKGI$_{CR}$ (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 S^{133} (Upstate, 06-519) and anti-phospho-VASP S^{239} (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 disassociate 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 methods[3] 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 $MgCl_2$, 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 SYSPRO 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 $MgCl_2$ 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 $MgCl_2$, 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 NH_2-terminal streptavidin-binding protein (SBP2, as described by others[5,6]), and pcDNA3•FLAG-PKGIα and pcDNA3•FLAG-PKGIβ, which encode NH_2-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.[8,9] 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 NH_2-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 (LC-MS/MS; Proteomics Research Services, Inc.).

The putative sessile amino acids of PKGI were identified by sequencing the NH_2-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 polyvinylidine 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 epifluorescent 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 (Perkin Elmer Life Sciences). To detect PKGI isoform abundance and CREB and VASP phosphorylation, protein fractions were resolved using SDS-PAGE, and the 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.[2,10] Cos7 cells and A7r5 cells and purified nucleoli were homogenized in 0.01 M NaH_2SO_4, 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 PKGI 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 $MgCl_2$, 100 µM 3-isobutyl-1-methylxanthine, 1 µM (R_p)-cAMP-S (Biolog), and 30,000 cpm/µl [γ-^{32}P]-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 PKGIβ 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 PKGIβ-FLAG and GFP were detected using epifluorescence. To quantify PKGI nuclear localization, identically registered epifluorescent images of six randomly oriented, non-overlapping 1.0 mm^2-fields that contained at least 25 cells with nuclear GFP-epifluorescence were obtained. Employing ImageJ[11] and a custom-written macro, the number of cell nuclei with PKGIβ-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 PKGIβ-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, Victor^3). 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

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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-$PKGI_{LZ}$ antibody to detect the NH_2-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 ($Ad.PKGI\beta^2$). (*A*) Equal amounts of total protein obtained from disrupted whole cells or isolated cell nuclei were immunoblotted with an anti-$PKGI_{CR}$ 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 NH_2-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.

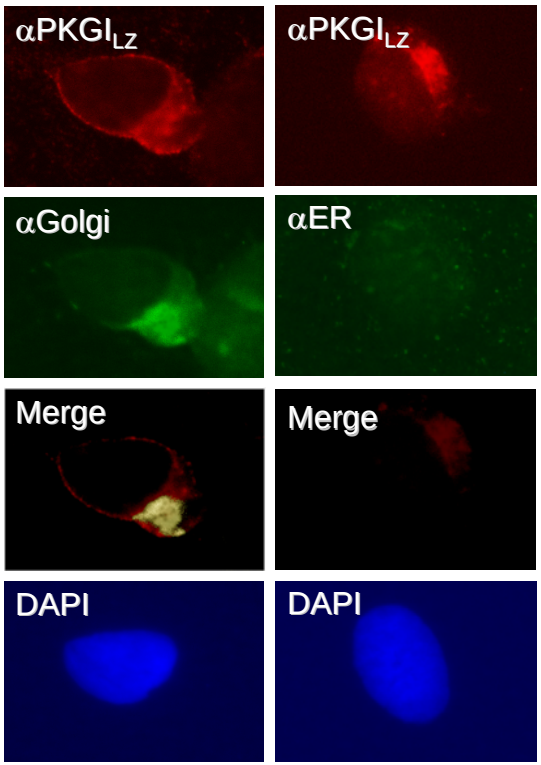
Online Table I

Primers utilized to generate mutant murine PKGIβ:

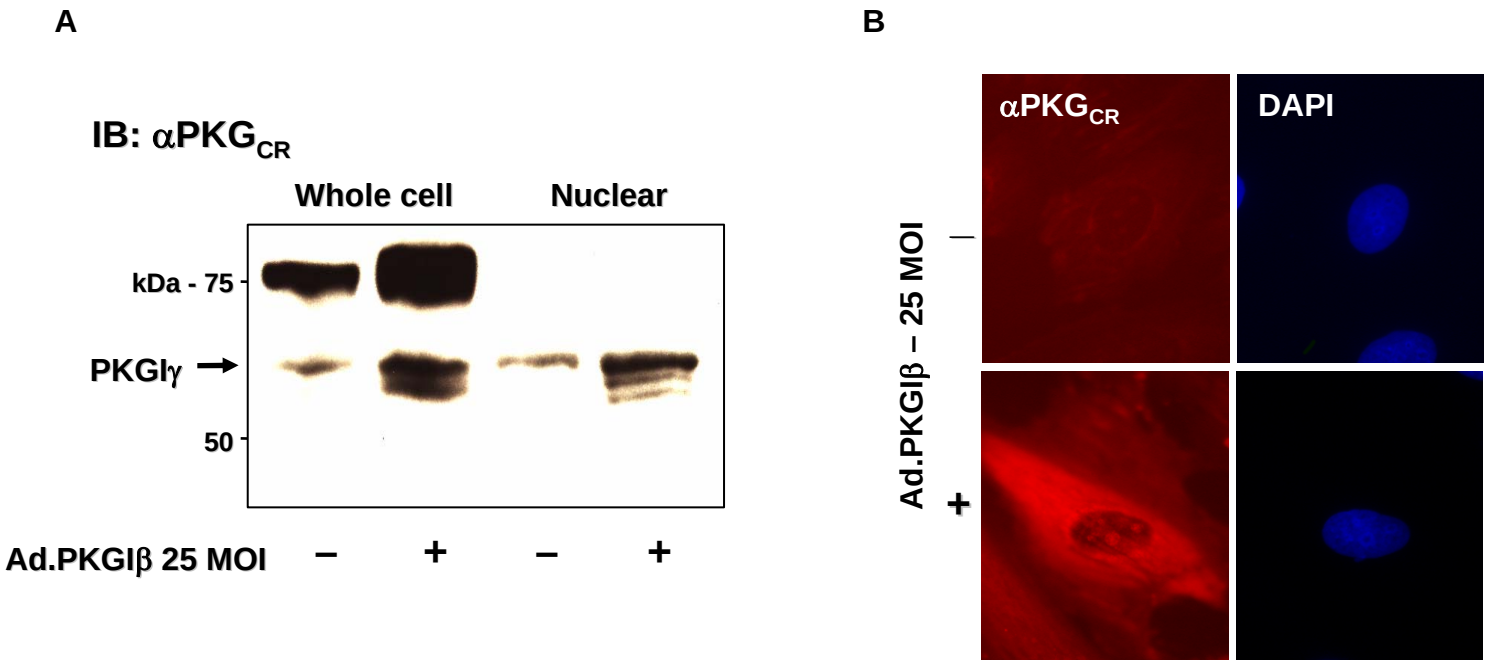
Mutant	Mutated AA*	Sense and antisense primers (5' to 3')
Mut1	141 - 147	gca gca gca gct gca gcg gcc gaa ggc gat gtg ggg tca ctg gtg tac ggc cgc tgc agc tgc tgc tgc gcc gta ttc cac ggg gta cat aca gtc
Mut2	149 - 154	gcc gca gct gcg gca gct tac gtc atg gaa gat ggg agg g agc tgc cgc agc tgc ggc gcc ttc ctt gat gat gca act
Mut3	156 – 161	gcc gcc gcg gcc gct gcg aag gtt gaa gtc aca aaa gaa ggc gtg cgc agc ggc cgc ggc ggc cac cag tga ccc cac atc gcc ttc c
Mut4	162 – 167	gcg gcc gca gcc gca gca gaa ggc gtg aag ctc tgc acc atg gg c tgc tgc ggc tgc ggc cgc ccc atc ttc cat gac gta cac cag tg
Mut5	168 - 173	gca gcc gcg gcg gcc gcc acc atg ggt cct gga aaa gtg ttc ggg ggc ggc cgc cgc ggc tgc ttt tgt gac ttc aac ctt ccc atc ttc

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

Online Figure I

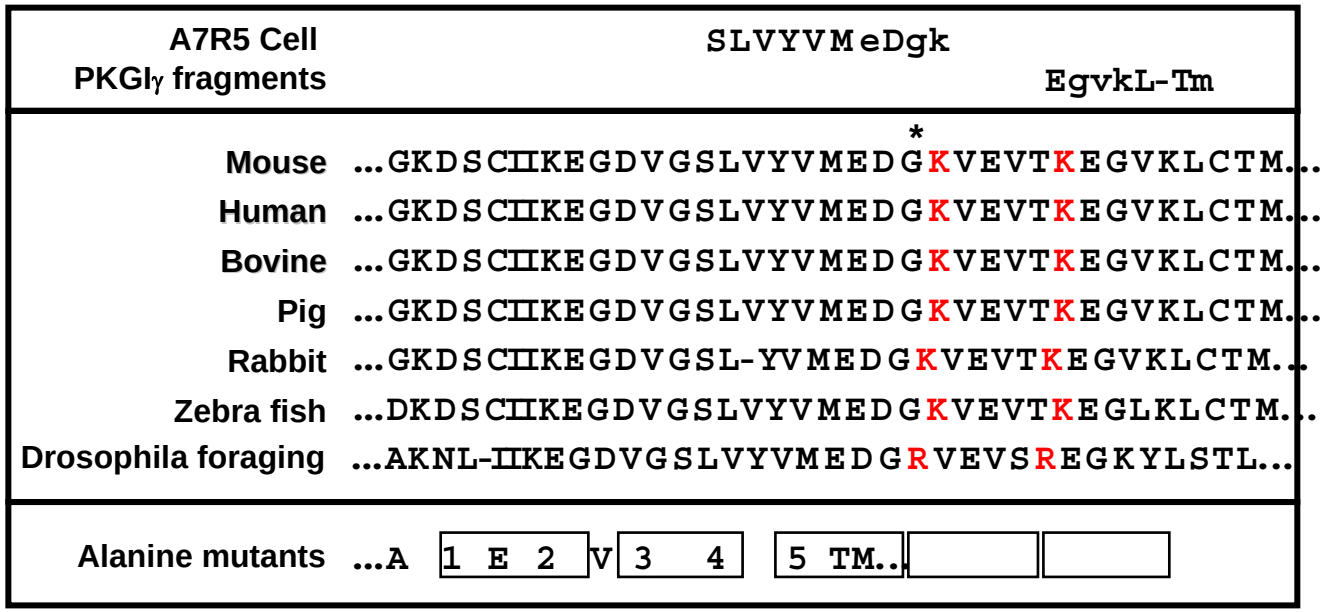


Online Figure II



Online Figure III

Putative PKGI cleavage site:



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