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cGMP-dependent protein kinase I gamma encodes a nuclear localization signal that regulates nuclear compartmentation and function

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Abstract

cGMP-dependent protein kinase I (PKGI) plays an important role in regulating how cGMP specifies vascular smooth muscle cell (SMC) phenotype. Although studies indicate that PKGI nuclear localization controls how cGMP regulates gene expression in SMC, information about the mechanisms that regulate PKGI nuclear compartmentation and its role in directly regulating cell phenotype is limited. Here we characterize a nuclear localization signal sequence (NLS) in PKGI γ , a proteolytically cleaved PKGI kinase fragment that translocates to the nucleus of SMC. Immunolocalization studies using cells expressing native and NLS-mutant PKGI γ , and treated with a small molecule nuclear transport inhibitor, indicated that PKGI γ encodes a constitutively active NLS that requires importin α and β for regulation of its compartmentation. Moreover, studies utilizing a genetically encoded nuclear phospho-CREB biosensor probe and fluorescence lifetime imaging microscopy demonstrated that this NLS controls PKGI γ nuclear function. In addition, although cytosolic PKGI γ -activity was observed to stimulate MAPK/ERK-mediated nuclear CREB signalling in SMC, NLS-mediated PKGI γ nuclear activity alone was determined to increase the expression of differentiation marker proteins in these cells. These results indicate that NLS-mediated nuclear PKGI γ localization plays an important role in how PKGI regulates vascular SMC phenotype.

Keywords

cGMP-dependent protein kinase; cGMP signal transduction; nuclear translocation; smooth muscle cell

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1. Introduction

cGMP-dependent protein kinase I (PKG I) is a cGMP-stimulated phosphokinase that regulates SMC contractility [1–3]. This protein has an NH₂-terminal coiled-coil leucine zipper (LZ)-like autoinhibitory domain, tandem cGMP-binding pockets, and a catalytic region, which contains Mg²⁺/ATP-binding, phosphokinase, and substrate recognition domains. Although PKGI is the product of one gene, alternate splicing of *PKGI* mRNA shuffles the 5' exons and results in the protein expression of two PKGI isoforms, PKGI α and PKGI β [4]. The variant LZ domains direct PKGI isoform homodimerization [5] and differential interactions with cytosolic anchoring proteins [6–8] and cytosolic compartmentation [7, 9–12]. The PKGI isoform LZ domains also encode autoinhibitory (AI) domains that interact with the substrate recognition domain in the catalytic region and inhibit PKGI kinase activity in the absence of cGMP stimulation [13]. The PKGI isoforms exhibit differential cell and tissue expression [14]. Moreover, the PKGI expression is regulated through a number of mechanisms including cytokine-mediated signalling [15–17]. In SMC, cGMP-stimulated PKGI regulates the cytoskeleton by phosphorylating cytosolic protein targets that control intracellular Ca²⁺ levels, Ca²⁺-sensitivity of contractile proteins, and perhaps the thin filament (reviewed in [18]).

PKG I also plays an important role in regulating cell phenotype [1, 2]. PKGI has been detected in the nucleus of several cell types [12, 19, 20] and studies suggest that this compartmentation controls how PKGI influences gene expression. For example, over-expression of PKGI β in PKGI-deficient baby hamster kidney (BHK) cells increases nuclear PKGI immuno-localization, the activation of *fos* promoter, through the activation of the cAMP response element, the AP-1 binding site, and the serum response element, and the induction of *c-fos* mRNA expression [20, 21]. Also in these cells, PKGI transduces gene expression independently of PKA, calmodulin-dependent protein kinase, and MAPK signalling [22]. In SMC, PKGI increases the expression of contractile proteins, such as calponin and smooth muscle myosin heavy chain (MHC) [1, 23–25], and decreases cell proliferation [26]. However, the mechanisms that regulate nuclear PKGI translocation are variable and incompletely understood. For example, in some experiments it is noted that PKGI does not localize to the nucleus or regulate gene expression [9, 27–29]. In addition, the specific function of nuclear PKGI translocation in regulating SMC differentiation is incompletely understood. In some cells, PKGI regulates cytoplasmic signalling systems [30, 31] that can transduce gene expression. These observations make it difficult to know whether PKGI nuclear localization alone controls gene expression. Understanding the mechanisms that regulate nuclear PKGI translocation and function will provide important information about how cGMP signalling regulates SMC phenotype.

Emerging evidence suggests that PKGI post-translational modifications control the nuclear PKGI localization and regulation of gene expression in SMC [32]. In these studies, PKGI cleavage in a hinge region of the molecule, which resides in a β -sheet that projects into the extra-molecular milieu [33], was determined to generate an NH₂-terminal fragment of PKGI that remains in the perinuclear space and a constitutively active COOH-terminal fragment that contains the kinase domain, PKGI γ , that localizes to the nucleus [32]. The PKGI

cleavage and nuclear PKGI γ localization was stimulated by cGMP and mechanisms that increase PKGI expression. Importantly, PKGI proteolysis was determined to be required for nuclear PKGI localization and activity. This is because PKGI cleavage, nuclear immunoreactivity, and transactivation of gene expression were decreased in cells expressing PKGI with mutations in the proteolytic site. Moreover, proprotein convertases (PCs) appear to play a part in regulating PKGI proteolysis and nuclear localization of PKGI γ [34]. This is because PKGI proteolysis was observed to be increased in a variety of cells in which select PCs are overexpressed and decreased in cells in which PC activity is defective or inhibited. Moreover, PC inhibition using membrane-permeable peptide decoys was determined to inhibit proteolysis-dependent PKGI nuclear localization in SMC. However, the mechanisms that govern PKGI γ nuclear localization and function are largely unknown.

Here we examined the mechanisms that regulate PKGI γ nuclear compartmentation and function in cells. Using cells expressing native and mutant PKGI γ , an inhibitor of importin α and β function, and immuno-localization studies, we report that PKGI γ harbors a classical NLS that regulates its nuclear localization. Moreover, employing a nucleus-localizing phospho-CREB biosensor and fluorescence lifetime imaging microscopy (FLIM), we determined that nuclear PKGI γ localization is required for the activation of CREB signalling. Of importance, in studies conducted with a MEK1/2 inhibitor, we observed that nuclear CREB activation by PKGI γ in SMC, but not in BHK cells, is also mediated through MAPK/ERK signalling. Lastly, by assessing the expression levels of SMC contractile proteins in transfected SMC using flow cytometry, we determined that nuclear but not cytoplasmic PKGI γ regulates the expression of cytoskeletal proteins that are indicative of SMC differentiation. Therefore, this work details mechanisms that regulate nuclear PKGI compartmentation and further enhances the view that the nuclear transport of PKGI has an important role in regulating SMC.

2. Materials and methods

2.1 Plasmid constructs and materials

The plasmids encoding NLS-ICAP and NLS-ICAP- S¹³³ were a kind gift of Michael Friedrich [35], and NH₂-terminal FLAG-epitope tagged vasodilator-stimulated phosphoprotein (FLAG-VASP) was from Michael Uhler [28]. pcDNA3-CFP was obtained from Addgene and pAcGFP1-Nuc, which encodes GFP-NLS, was purchased from Clontech. To generate pcDNA3-FLAG-PKGI γ , PCR was used to amplify a cDNA that encodes PKGI γ from pcDNA3-PKGI β [32] with *Bam*HI and *Xba*I restriction site ends. This fragment was then ligated in a 3'-position relative to a DNA fragment that specifies the FLAG epitope tag into pcDNA3. The PKGI γ cDNA fragment encodes a COOH-terminal portion of PKGI β commencing with E¹⁶⁸. This is because amino acid microsequencing of immunopurified A7R5 SMC nuclear PKGI γ suggested that PKGI γ is generated by PKGI cleavage just NH₂-terminal to this amino acid [32]. Using this plasmid and site-directed mutagenesis, additional plasmids were constructed that encode PKGI γ with a mutant NLS (PKGI γ - NLS) and PKGI γ that was predicted to lack kinase activity (PKGI γ -DK). The amino acid changes in the PKGI γ NLS mutation is detailed below; the mutation in PKGI γ -DK corresponds to a T⁵¹⁶->A switch in PKGI β that was determined by Feil and coworkers to abolish PKGI β

phosphokinase activity [36]. The FLAG-PKGI γ and FLAG-PKGI γ - NLS cDNA fragments were also introduced into the first open reading frame (ORF) of pCP2 (Clontech), a bicistronic plasmid with mCherry2 encoded in the second ORF following an internal ribosome entry site. The expression of the native and mutant PKGI γ transgenes was determined in soluble cell lysates as described previously [32]. The authenticity of the plasmid constructs was confirmed using dideoxynucleotide sequencing.

Cell culture and transfection

The fibroblast BHK cells and rat embryonal SMC A10 cells [37] were obtained from the American Tissue Culture Collection (ATCC). Rat aortic SMC (RAoSMC) were obtained using an explant method as previously described [26] and were used in the experiments before the seventh passage. The cells were maintained in DMEM containing 10% v/v FBS, penicillin (100U/ml), and streptomycin (100 μ g/ml) (Invitrogen) in a humidified 37°C incubator with 5% CO₂. Cell transfection occurred using Lipofectamine 2000 (Invitrogen) or Xfect (Clontech) according to the manufacturer's instructions.

Cell imaging

To map PKGI immunoreactivity to the nucleus and cytosol of the cells, laser scanning confocal microscopy was performed using a microscope (Zeiss LSMS Pascal) with a pinhole and slide stage z-axis position set to obtain a ~2 μ m-thick image in the mid-nuclear plane, as determined by the DAPI-image and described previously [32]. To test the effect of ivermectin on nuclear PKGI γ localization, cells were imaged using wide field microscopy (Nikon Eclipse Ti-E) and the mean fluorescence intensity in the nucleus and cytosol was determined using image analysis software (Nikon NIS-Elements). To determine the activation NLS-ICAP in fixed cells, time-correlated single-photon counting FLIM was performed using a Nikon TiE microscope equipped with a dedicated FLIM system (Becker & Hickl GmbH, Berlin Germany). The CFP donor fluorescence of the biosensor was excited with a 448-nm pulsed laser diode. The fluorescence signal from the cells was collected using a photomultiplier tube after the photons passed through a 40x oil immersion objective lens (NA 1.30) and a 460 nm wavelength long-pass filter. The data acquisition time adjusted so that $> 5 \times 10^4$ photons were sampled per pixel. To demonstrate the co-expression of FLAG-PKGI γ transgenes and mCherry2, SMC were transfected with the bicistronic plasmids, described above, fixed, permeabilized, and then exposed to anti-FLAG and Alexa 488-labeled secondary antibodies and then DAPI. The anti-FLAG, mCherry2, and DAPI fluorescence images were merged.

Detection of in vivo PKGI activity

After the cells were transfected with plasmids encoding PKGI γ constructs and FLAG-VASP, a cytosolic PKGI target, soluble proteins were collected in a reducing lysis buffer and resolved using SDS-PAGE. After electro-blotting the proteins onto a charged membrane, their FLAG transgene and phospho-VASP S²³⁹ immunoreactivity were detected as previously described [32].

Flow cytometry

Following transfection with plasmids, the SMC were released from the plates using type IV collagenase, which we observed preserves membrane-bound mCherry2 fluorescence. Subsequently, the cells were fixed for 20 min in 2% paraformaldehyde in phosphate buffered saline, permeabilized with methanol, blocked with 5% bovine serum albumin, and then reacted with fluorescently labeled anti-calponin, MHC, or equivalent amounts of isotype control antibodies. The anti-calponin antibody was purchased from Abcam (ab46794) and the anti-MHC antibody was obtained from Biomedical Technologies (BT-562, Stoughton MA USA). Subsequently, the cells were subjected to flow cytometry (LSRII, BD Biosciences) and at least 1×10^4 events were analyzed.

Data analysis

The lifetime images were analyzed by exponential function fitting and by determining goodness of fit of the photon decay curve using SPC Image software (Becker & Hickl GmbH). The CFP mean fluorescence lifetime (τ_m) was determined using a bi-exponential function model, as detailed in *Results*.

After the flow cytometry data was gated for dead cells and debris, and compensated for fluorescent spill-over, it underwent a linear-log transformation, as described by Klinke and Brundage [38] using custom scripts and a statistical programming language (R, [39]). The data were then gated based on the mCherry fluorescence to permit analysis of SMC contractile protein expression in cells that were transfected with the plasmids.

The experiments were repeated a minimum of three times and typical results are shown. The data are represented as mean $\pm$ S.D. and are compared using ANOVA. When indicated, a Tukey's range test was performed *post hoc*. Where indicated in the figures, $*P < 0.05$.

3. Results

PKGI γ harbors an NLS that regulates PKGI nuclear compartmentation

A putative NLS was previously characterized in PKGI β that appears to regulate its cGMP-dependent nuclear localization and gene expression transactivation [20]. This NLS consists of a single cluster of basic amino acids that resides within the catalytic region of PKGI β (Fig. 1A). Some but not all mutations of the NLS were observed to inhibit cGMP-stimulated PKGI nuclear localization of PKGI. However, others determined that this NLS does not to fully regulate nuclear PKGI β localization. Inspection of the amino acid sequence of PKGI γ reveals that this putative PKGI β NLS is conserved within that kinase fragment (Fig. 1A). To test whether this NLS directs PKGI γ nuclear compartmentation, we used site-directed mutagenesis to make a single amino acid substitution of the P1 of the putative NLS of PKGI γ , as indicated in the figure. A similar approach was employed by Gudi and coworkers to characterize the function of the PKGI β NLS [20]. As shown in Fig. 1B, transient transfection of BHK cells, which are cells that do not express appreciable levels of PKGI [20], with these constructs led to the expression of the PKGI γ transgenes of the expected size. To test the role of the putative NLS in regulating nuclear PKGI γ compartmentation, rat aortic SMC, A10 cells, and BHK cells were transiently transfected with these plasmids and

one that we previously observed expressed PKGI β with a COOH-terminal FLAG tag [32]. Subsequently, the PKGI γ transgenes were localized using indirect immunofluorescence and laser scanning confocal microscopy. As shown in Fig. 1C, whereas nuclear PKGI immunoreactivity was detected in the cells expressing PKGI γ and PKGI β , it was not observed in those that were transfected with PKGI γ - NLS. Moreover, it was noted that the nuclear PKGI γ compartmentation did not require cGMP stimulation, as was described for the full-length PKGI isoforms in other studies [32]. Although, we and others observed that PKGI β overexpression increases nuclear PKGI immunoreactivity [8, 11, 32]. The specificity of the antibodies used to detect the FLAG epitope and the catalytic domain of PKGI is shown in Fig. 1D and elsewhere [32]. These data support the role of the NLS in regulating PKGI γ nuclear compartmentation.

PKGI γ nuclear localization is regulated through a classical NLS

Classical NLSs direct the nuclear importation of cytoplasmic cargo proteins through binding to the adapter protein importin α . Thereafter, NLS-bound importin α recruits importin β to the protein assembly, and importin β facilitates the binding of the trimeric complex to the nuclear pore complex and its transport into the nucleus [40, 41]. Recently, ivermectin was observed to block importin α and β interaction and to inhibit the nuclear transport facilitated by prototypical NLS-mediated mechanism [42]. In order to test whether importin α and β play a role in regulating PKGI γ localization, BHK cells were transfected with plasmids that encode FLAG-PKGI γ and treated with and without ivermectin. Subsequently, the cells were fixed and permeabilized and the PKGI γ transgene was immunolocalized in the cells using an anti-FLAG antibody. To analyze the PKGI γ nuclear compartmentation the mean nuclear and cytoplasmic fluorescence ratios were quantified using a method described by others [42]. In control studies, the cells were transfected plasmids that encode FLAG-PKGI γ - NLS or GFP-NLS, which is GFP that was engineered to have COOH-terminal tandem repeats of the SV40 large T-antigen NLS. As shown in Fig. 2, ivermectin exposure inhibited nuclear PKGI γ localization in the cells to levels detected for PKGI γ with the mutant NLS. Moreover, the disruption of NLS-mediated nuclear localization by ivermectin in these studies was confirmed by observing that it reduced the nuclear compartmentation of the GFP harboring a classical NLS. These studies indicate that the PKGI γ NLS requires importin α and β interaction to facilitate its nuclear localization in cells.

PKGI γ nuclear compartmentation regulates CREB activation in BHK cells

Although the studies detailed above characterized an NLS that regulates nuclear PKGI γ compartmentation, they do not demonstrate how it might control nuclear PKGI function. We and others have shown that nuclear PKGI localization is associated with transactivation of gene expression. However, even when detected in the nucleus, PKGI γ , the PKGI isoforms, and many of their targeted transcription factors also reside in the cytosol. Therefore, it would be important to understand whether the NLS-regulated PKGI γ nuclear compartmentation plays a direct role in controlling nuclear PKGI function. Because cGMP stimulates CREB-mediated signalling [43], and PKGI γ transactivates CREB signalling [32], we examined whether the PKGI γ NLS regulates nuclear CREB activation. Although CREB resides within the nucleus, it is detected and phosphorylated in the cytoplasm as well [44, 45]. To improve the specificity of detecting CREB activation by PKGI γ residing in the

nucleus, a phospho-CREB biosensor that is localized in the nucleus was employed for these studies. Previously, Friedrich and coworkers showed that a fusion protein consisting of the CREB kinase-inducible domain (KID), a linker, and the CREB-binding protein KID-interaction domain (KIX) sandwiched between an NLS and cyan fluorescent protein (CFP) on the NH₂-terminal end and yellow fluorescence protein (YFP) on the COOH-terminal (referred to as *NLS-ICAP*) exhibited reduced fluorescence resonance energy transfer (FRET) when PKA or calmodulin kinase induced CREB KID phosphorylation and KID-KIX interaction decreased CFP and YFP photonic interaction [35]). Because of the high variation in NLS-ICAP expression observed in the transfected cells during pilot studies, we first analyzed whether the activation of this phospho-CREB biosensor could be detected using fluorescence lifetime imaging microscopy (FLIM) instead of FRET. This is because in contrast with FRET, FLIM is insensitive to the expression level or concentration of the donor fluorescent protein.

Using fixed BHK cells expressing NLS-ICAP we defined the criteria to be used to model the photon decay curves of this probe and then tested whether FLIM could detect differences in the kinetics of the decay curves imparted by treatment with agents known to phosphorylate the CREB-based biosensor. As shown in Fig. 3A, the photon decay data of the CFP donor in NLS-ICAP can be modeled using the two-exponent equation as defined by others [46]. The goodness of fit of the modeling equation, particularly within the nuclear region, is shown in the Chi² image. Following sequential application of functions containing increasing numbers of exponential factors, the goodness of fit of the model is first optimal when a two exponent equation is applied. Moreover, inspection of the photon decay curve residual data shows that there is no systematic departure from the model following the application of a bi-exponential equation. These data are in agreement with those of others showing that CFP photon decay is efficiently modeled using a two-exponent equation [47]. Because cell fixation can affect the efficiency of protein fluorescence, we tested whether NLS-ICAP activation could be detected in formaldehyde-fixed cells using FLIM. In these studies, NLS-ICAP was modulated using forskolin because this had been shown to phosphorylate and modulate FRET of this CREB KID-based biosensor [35]. As shown in Fig. 3B, we observed that treating fixed BHK cells with forskolin decreased the CFP τ_m of NLS-ICAP. These studies indicate that FLIM can be used to detect NLS-ICAP activation in fixed BHK cells.

We next tested whether the PKGI γ NLS regulates nuclear PKGI phosphokinase activity through examining the activation of NLS-ICAP using FLIM. The anticipated model of the effect of PKGI-mediated NLS-ICAP phosphorylation on the CFP-YFP interaction is shown in Fig. 4A. The interaction between the photon donor CFP and acceptor YFP is expected to shorten the photon relaxation time in non-phosphorylated NLS-ICAP. In contrast, phosphorylation of the CREB KID is expected to increase phospho-KID and KIX interaction, decrease the CFP-YFP interaction, and prolong the photon relaxation time. For these studies, BHK cells were co-transfected with plasmids that encode NLS-ICAP and PKGI γ or PKGI γ - NLS, fixed, and then the mean CFP photon relaxation time (τ_m) was determined following FLIM. The cellular compartmentation of the NLS-ICAP sensor is shown in Fig. 4B in typical BHK cells in the CFP fluorescence intensity image. The CFP intensity images confirmed the localization of NLS-ICAP within the nucleus of the cells.

Moreover, the CFP τ_m heat map images demonstrate that this CREB KID-based biosensor is activated by PKGI γ primarily in the nucleus. Moreover, very little activation of NLS-ICAP is observed in the cytosol of the cells by PKGI γ and PKGI γ - NLS. This supports the nuclear partitioning of this probe. The composite photon relaxation curve plot obtained from the cell nuclei and shown in Fig. 4C, confirms a shift in the CFP photon release kinetics in the PKGI γ - but not the PKGI γ - NLS-expressing BHK cells.

Next were defined the mechanisms through which nuclear PKGI γ regulates the phospho-CREB biosensor. In these studies, BHK cells were transfected with the indicated plasmids and either: 1) fixed and subjected to FLIM and the CFP τ_m was measured in the nuclear region, or 2) lysed and the indicated protein levels were detected using antibodies and protein blot hybridization. As shown in Fig. 5A, whereas PKGI γ increased the CFP τ_m of NLS-ICAP, PKGI γ - NLS did not modulate the mean relaxation time of this phospho-CREB sensor. The data in Fig. 5B indicate that the lack of CFP τ_m modulation by PKGI γ - NLS was not due to a deficiency in PKGI γ - NLS kinase activity; PKGI γ - NLS was observed to phosphorylate over-expressed VASP, a PKGI phosphorylation target and member of the Ena/VASP family of proteins that resides in the cytosol, and to cause a known shift in its apparent molecular weight when over-expressed in the BHK cells. Because the NLS-ICAP CREB KID harbors several amino acid residues that are targeted by kinases that phosphorylate CREB [35, 48], we tested whether PKGI γ modulates the NLS-ICAP CFP τ_m through the consensus cyclic nucleotide-dependent phosphorylation site (-RRFS-, [49]). As shown in Fig. 5A, in contrast with the increase in CFP τ_m observed in the nuclei of BHK cells expressing PKGI γ and NLS-ICAP, those expressing PKGI γ and NLS-ICAP with a mutation in the KID residue mapping to CREB S¹³³ did not exhibit a change in the photon relaxation time. Previous studies show that the control plasmid used in this experiment expresses NLS-ICAP S¹³³ and that the mutant NLS-ICAP is not phosphorylated by PKA [35].

To determine whether the activation of NLS-ICAP was due to the interaction of PKGI γ with another kinase, we tested whether NLS-ICAP CFP τ_m increased in cells that expressed an inactive PKGI γ kinase (PKGI γ -DK). Previous studies by Feil and colleagues demonstrated that mutation in an auto-phosphorylation site of PKGI α inhibited PKGI kinase activity [36]. Although it was unknown whether a corresponding mutation in PKGI γ would inhibit its activity, as shown in Fig. 6A, over-expressed PKGI γ -DK was observed to not phosphorylate FLAG-VASP in BHK cell lysates as determined by immunoblotting and exposure to phospho-VASP antibodies. In additional studies using anti-FLAG antibodies, we observed that BHK cells transfected with pcDNA3-FLAG-PKGI γ -DK, expressed a protein of the expected size by immunoblotting and that the protein was observed within the nucleus by immunofluorescence microscopy (data not shown). Importantly, we found that BHK cells co-transfected with the plasmid that encodes PKGI γ -DK and NLS-ICAP did not exhibit the increase in CFP τ_m observed in the cells co-expressing PKGI γ (Fig. 6B).

Moreover, we tested whether the nuclear activation of NLS-ICAP by PKGI γ was mediated through PKA signalling. These studies were performed because under certain conditions, PKGI can cross-activate PKA signalling and because this biosensor was optimized to identify nuclear PKA activity. As shown in Fig. 7, the activation of NLS-ICAP by PKGI γ

persisted during treatment of the BHK cells with myr-PKI, a membrane-permeable PKA inhibitor, even though this compound inhibited the forskolin-mediated increase in CFP t_m . Together these studies indicate that the PKGI γ NLS regulates nuclear PKGI phosphokinase activity in BHK cells.

Nuclear and cytoplasmic PKGI γ regulate CREB activation in vascular SMC

Although the studies detailed in BHK cells above indicated that PKGI γ nuclear localization alone activates CREB, others observed in vascular SMC that PKGI can stimulate cytosolic MAPK signalling [30], which could indirectly regulate nuclear CREB signalling. To examine this possibility, we tested whether NLS-mediated PKGI γ nuclear localization alone regulates CREB activation in vascular SMC. For these studies, early passage rat aortic SMC were co-transfected with plasmids that encode NLS-ICAP and PKGI γ , with or without the NLS mutation detailed above, and the NLS-ICAP activation was assessed using FLIM after the cells were fixed. Pilot studies for this investigation confirmed that NLS-ICAP localizes to the nuclei of the SMC and that a bi-exponential model reliably details the photon relaxation curve of the CFP donor (data not shown).

Similar to the results in BHK cells, we observed that PKGI γ prolonged CFP t_m in the aortic SMC (Fig. 8A). However, in contrast, PKGI γ - NLS expression increased the CFP t_m to a level that was intermediate between that determined in PKGI γ expressing and pcDNA3 transfected control SMC. To determine whether this change in NLS-ICAP activation was mediated through the cytoplasmic stimulation of MAPK signalling, aortic SMC were co-transfected with plasmids that encode NLS-ICAP and PKGI γ or PKGI γ - NLS, and then treated with U0126, a MEK1/2 inhibitor, or DMSO, the diluent. As shown in Fig. 8B, U0126 treatment fully inhibited the PKGI γ - NLS modulation of CREB activation. However, U0126 treatment only partially inhibited the change in CFP t_m associated with PKGI γ expression. These data suggest that in SMC PKGI γ modulates CREB activation through direct nuclear mechanisms as well as cytosolic ones that require MAPK.

Nuclear PKGI γ regulates SMC differentiation

Previous studies indicate that cGMP regulates SMC differentiation through stimulating PKGI [1, 43]. However, it is unknown whether PKGI can regulate SMC differentiation through nuclear mechanisms alone. Because the NLS detailed above was determined to regulate nuclear PKGI γ localization and activity, we next tested whether or not the PKGI γ compartmentation influences SMC differentiation. Primary vascular SMC are difficult to transfect. To facilitate our studies, we employed plasmids and analytic techniques that would allow us to study PKGI γ -mediated changes of differentiation primarily in transfected SMC. We constructed bicistronic plasmids in which cDNA encoding PKGI γ or PKGI γ - NLS was inserted into the first ORF while sequence encoding mCherry2, a red fluorescent protein that localizes to the plasma membrane [50], resided in the second ORF. Because of the relative efficiency of gene transcription, we observed that each SMC transfected with this construct that exhibited mCherry2 fluorescence also expressed the PKGI γ transgene encoded by the first ORF (as shown in typical cells in Fig. 9A). Therefore, to examine the effect of PKGI γ nuclear compartmentation on SMC differentiation, we gated the flow cytometry fluorescence data to select for SMC that exhibited mCherry2 fluorescence.

Rat aortic SMC transfected with plasmids that encode PKGI γ exhibited increased SMC contractile protein expression in comparison with those exposed to the PKGI γ - NLS-encoding or control vectors. As shown in Figs. 9B and C, SMC expressing PKGI γ but not PKGI γ - NLS had increased numbers of cells exhibiting a higher mean fluorescence signal associated with calponin and MHC expression. Together these data indicate that PKGI γ regulates SMC differentiation through direct nuclear mechanisms.

4. Discussion

PKGI has a pivotal role in regulating how cGMP controls vascular SMC phenotype. Previous investigations demonstrated that PKGI increases the differentiation of SMC [1, 23, 25, 26, 51]. These studies led to the investigation of whether nuclear PKGI localization has a role in regulating the expression of SMC genes. Investigations identified a cryptic, cGMP-regulated NLS residing in the catalytic domain of PKGI β that is required for PKGI nuclear compartmentation. Gudi and coworkers detected a single cluster of basic amino acids in PKGI β that regulated the appearance of nuclear PKGI immunofluorescence and function, as detected by *fos* promoter activation [20]. This PKGI β NLS amino acid sequence was thought to have a similar structure as one distinguished in interleukin-1 α [20, 52]. Although the general structure of NLSs is variable, we note that the putative PKGI β NLS follows the consensus sequence of (K/R)X(K/R), which is thought to be typical of monopartite NLSs [53]. Moreover, this PKGI β NLS resides within the Mg²⁺/ATP binding region of the small lobe of the catalytic domain, which is likely to have access to the extramolecular environment and the adaptor proteins that are required to guide nuclear pore-dependent protein transport. Gudi and coworkers showed that the nuclear PKGI translocation was ATP-dependent, supporting the role of energy-dependent, nuclear pore-regulated PKGI nucleoplasm transport. The PKGI β NLS also appeared to be cryptic because cGMP stimulation appeared to be important in activating the nuclear PKGI localization and activity. While characterizing the NLS activity, they observed that switching the P1 lysine to a negatively charged amino acid in the putative NLS generated a mutant PKGI β that was catalytically active although it no longer exhibited cGMP-stimulated nuclear localization or transactivation of a *c-fos* promoter. In addition, fusing the putative NLS with β -galactosidase was found to be sufficient to cause the nuclear localization of that enzyme. However, in studies by other investigators, PKGI has been observed not to localize to the nucleus of cells and the putative PKGI β NLS was shown to incompletely regulate cGMP-dependent PKGI nuclear compartmentation. For example, Collins and Euler observed that mutations of P1 and P3 amino acids in the putative PKGI β NLS did not completely block the cGMP-stimulated PKGI nuclear activity in HEK293 cells [28]. Therefore, the mechanisms regulating nuclear PKGI translocation and activity are incompletely understood.

The work presented here provides important refinements to the knowledge about the mechanisms that regulate nuclear cGMP signalling. Previously we showed that PKGI proteolysis plays a critical role in regulating nuclear PKGI localization [32]. In these studies, cGMP-treatment or PKGI overexpression in SMC or cell lines was observed to cause PKGI proteolysis and the generation of a COOH-terminal kinase fragment with constitutive activity. Moreover, we determined that although the NH₂-terminal LZ portion of cleaved PKGI is retained in the cytosol, the kinase fragment localizes to the nucleus. Using

antibodies that detect the NH₂-terminal portion of the PKGI isoforms, we have not been able to detect full-length, uncleaved PKGI in the nucleoplasm. Although we observed that PKGI proteolysis was critical for nuclear PKGI γ localization and the regulation of gene expression by PKGI γ , the mechanisms controlling nuclear PKGI γ compartmentation were unknown.

PKGI γ is ~60 kDa and too large to passively diffuse into the nucleus. This is supported by our observation that PKGI γ with a mutation in the NLS was not immunolocalized to the nucleus and did not activate NLS-ICAP, unless mediated through cytosolic MAPK signalling. Moreover, the role of the NLS in PKGI γ nuclear transport is supported by studies of the compartmentation of synthetic PKGI catalytic domain fragments. For example, although Gudi and colleagues showed that whereas the putative NLS was not required for regulation of the nuclear compartmentation of a ~37 kDa fragment PKGI β [20], it did control the nuclear localization and transactivation of gene expression by a 65 kDa fragment that contained more NH₂-terminal amino acids. Browning and colleagues showed that a ~63 kDa PKGI kinase fragment that was fused with GFP and contained the putative NLS, localized to the nucleus of cells [29]. Moreover, other investigators showed that COOH-terminal fragments of PKGI had important activities in regulating vascular SMC phenotype in cultured cells [23, 54, 55] and in injured systemic vessels [54]. However, in these studies, the role of nuclear localization in mediating these effects of the PKGI fragments in SMC was not investigated.

To further detail how the NLS characterized in this report influences PKGI nuclear localization, we examined whether importin α plays a part in regulating PKGI γ nuclear compartmentation. For these studies, we utilized a recently identified specific inhibitor of importin α and β -dependent protein localization. During the use of an AlphaScreen protein-protein binding assay designed to detect small molecules that disrupt viral protein nuclear import, ivermectin was identified as a potential inhibitor of importin α and β interaction [56]. Subsequently, this compound was determined to be a specific inhibitor of importin α and β -dependent nuclear localization using a variety of reference proteins [42]. Of interest, ivermectin did not inhibit the nuclear translocation of proteins that alone require importin β 1 (e.g. parathyroid hormone-related protein [57]) or importin 13 (such as the SUMO-conjugating enzyme, UBC9 [58]) [42]. Our observation that ivermectin inhibits nuclear PKGI γ localization to similar levels observed for PKGI γ -NLS indicate that importin α and β regulate PKGI γ nuclear compartmentation. The inhibition of GFP-NLS nuclear compartmentation by ivermectin was expected because the SV40 large T-antigen NLS encoded in this transgene has been shown to require importin α and β for nuclear localization regulation [59]. Moreover, Wagstaff showed that ivermectin inhibited the nuclear compartmentation of another fluorescent protein-SV40 large T-antigen fusion protein [42]. Together these results determine for the first time mechanisms that regulate PKGI γ nuclear localization in cells.

Previous studies have shown that PKGI activates gene expression. For example, PKGI proteolysis and nuclear PKGI compartmentation were observed to correlate with transactivation of phospho-CREB-dependent gene expression [20, 32]. However, it is possible that this CREB-dependent gene regulation was modulated by activation of CREB in the cytosol and the nuclear translocation of phospho-CREB. For example, phospho-CREB

has been detected in the cytosol of cells and other kinases have been observed to stimulate the nuclear localization of cytosolic phospho-CREB [44, 45]. Therefore, to determine the role of the NLS characterized above in partitioning nuclear PKGI γ function, we employed a nuclear phospho-CREB based biosensor and mapped its activation by PKGI γ in the nuclear compartment using FLIM. Friedrich and coworkers used phospho-specific antibodies and immunoblotting to show that PKA and CAM kinase phosphorylate this sensor and FRET methods to demonstrate that the biosensor is modulated within the nucleus of cells. Moreover, in the FLIM studies detailed here we observed that this sensor detects selective nuclear CREB activation, as we did not observe appreciable cytosolic NLS-ICAP CFPtm prolongations in cells expressing PKGI γ or PKGI γ -NLS despite showing that the phosphorylation of a cytoplasmic PKGI target, FLAG-VASP. Moreover, using mutant NLS-ICAP and PKGI γ and PKA inhibitors, we confirmed that PKGI γ phosphorylated a typical PKA/PKGI motif, and did not modulate NLS-ICAP through cross-activation of PKA signalling. These data indicate that PKGI γ exhibits direct nuclear function, which is regulated through its nuclear compartmentation.

We found it interesting to note that, in contrast with the results observed in BHK cells, cytosolic PKGI γ activity regulated CREB activation in aortic SMC. These observations suggested that PKGI γ stimulates a cytosolic CREB-regulating signalling pathway that is not present in BHK cells. Previous investigators observed that PKGI activates MAPK signalling in aortic SMC [30]. Because this pathway stimulates CREB-signalling, we tested whether PKGI γ might regulate the phospho-CREB biosensor through cytoplasmic as well as nuclear mechanisms. Our observation of NLS-ICAP activation by PKGI γ -NLS as well as PKGI γ in rat aortic SMC suggested this possibility. It is interesting that the lack of phospho-CREB sensor activation by PKGI γ -NLS in BHK cells is consistent with the observations by others that PKGI β does not stimulate MAPK signalling in these cells. This suggests that either the effector molecules might not be expressed or that PKGI might not share a similar cytosolic compartment with MAPK signalling components in these cells. Moreover, the inhibition of the NLS-ICAP activation by PKGI γ -NLS by U0126 supports the potential role of MEK signalling in cytosolic PKGI γ activity. Nevertheless, the persistence of NLS-ICAP modulation in PKGI γ expressing U0126-treated cells supports a direct stimulation of CREB by nuclear PKGI γ in these cells.

Several studies indicate that PKGI regulates SMC differentiation. For example, PKGI expression reduction via cell passaging or RNAi, PKGI inhibition using membrane-permeable inhibitory peptides, and PKGI rescue by over-expression has been observed to modulate the mRNA and protein levels of contractile proteins in vascular SMC [1, 25, 51, 60]. Moreover, over-expression of a COOH-terminal PKGI fragment that was engineered to have constitutive phosphokinase activity was observed to increase SMC contractile protein expression [23] and to decrease glucose- and serum-stimulated SMC proliferation in culture [54, 55]. However, although PKGI has been determined to regulate nuclear signalling systems [20, 22], it was unknown whether PKGI regulates SMC differentiation through nuclear or cytosolic mechanisms. We observed that PKGI γ but not PKGI γ -NLS increased the protein expression of calponin and MHC in the SMC. These studies suggest that although PKGI γ can be observed to regulate cytosolic signalling systems, such as MAPK, the nuclear localization has an important role in controlling how it regulates SMC

differentiation. These results lead us to speculate that mechanisms that inhibit the nuclear localization and activity of PKGI γ may play an important role in the pathogenesis of vascular diseases associated with SMC dedifferentiation.

5. Conclusion

We determined for the first time that an NLS residing in PKGI γ , an important constitutively active kinase fragment of PKGI in SMC, defines PKGI's role in regulating nuclear signalling and SMC phenotype determination. These findings are significant because they demonstrate that although can PKGI regulate nuclear signalling through cytoplasmic as well as nuclear pathways in SMC, it is the nuclear compartmentation of PKGI γ itself plays a role in regulating SMC phenotype. Moreover, investigations detailed here promote the utility of using a nucleus-localizing CREB KID-based biosensor and FLIM in detailing nuclear PKGI function and the use of bicistronic plasmids with a fluorescent protein marker to examine the effects of gene expression on cell function in transfected SMC.

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The abbreviations used are

PKGI	cGMP-dependent protein kinase I
NLS	nuclear localization signal
CREB	cAMP response element-binding protein

References

1. Lincoln TM, Wu X, Sellak H, Dey N, Choi CS. *Front Biosci.* 2006; 11:356–367. [PubMed: 16146737]
2. Francis SH, Busch JL, Corbin JD, Sibley D. *Pharmacological reviews.* 2010; 62:525–563. [PubMed: 20716671]
3. Hofmann F, Wegener JW. *Methods in molecular biology.* 2013; 1020:17–50. [PubMed: 23709024]
4. Ruth P, Pfeifer A, Kamm S, Klatt P, Dostmann WR, Hofmann F. *The Journal of biological chemistry.* 1997; 272:10522–10528. [PubMed: 9099696]
5. Richie-Jannetta R, Francis SH, Corbin JD. *The Journal of biological chemistry.* 2003; 278:50070–50079. [PubMed: 12933804]
6. MacMillan-Crow LA, Murphy-Ullrich JE, Lincoln TM. *Biochemical and biophysical research communications.* 1994; 201:531–537. [PubMed: 8002983]
7. Wang Y, El-Zaru MR, Surks HK, Mendelsohn ME. *The Journal of biological chemistry.* 2004; 279:24420–24426. [PubMed: 15051728]
8. Casteel DE, Zhang T, Zhuang S, Pilz RB. *Cellular signalling.* 2008; 20:1392–1399. [PubMed: 18450420]
9. Feil R, Gappa N, Rutz M, Schlossmann J, Rose CR, Konnerth A, Brummer S, Kuhbandner S, Hofmann F. *Circulation research.* 2002; 90:1080–1086. [PubMed: 12039797]

10. John TA, Ibe BO, Raj JU. Clin Exp Pharmacol Physiol. 2008; 35:148–158. [PubMed: 17892503]
11. Desch M, Sigl K, Hieke B, Salb K, Kees F, Bernhard D, Jochim A, Spiessberger B, Hocheil K, Feil R, Feil S, Lukowski R, Wegener JW, Hofmann F, Schlossmann J. Cardiovascular research. 2010
12. Cornwell TL, Pryzwansky KB, Wyatt TA, Lincoln TM. Mol Pharmacol. 1991; 40:923–931. [PubMed: 1836834]
13. Heil WG, Landgraf W, Hofmann F. Eur J Biochem. 1987; 168:117–121. [PubMed: 2822399]
14. Orstavik S, Natarajan V, Tasken K, Jahnsen T, Sandberg M. Genomics. 1997; 42:311–318. [PubMed: 9192852]
15. Browner NC, Sellak H, Lincoln TM. American journal of physiology Cell physiology. 2004; 287:C88–96. [PubMed: 14985234]
16. Bachiller PR, Nakanishi H, Roberts JD Jr. American journal of physiology Lung cellular and molecular physiology. 2010; 298:L324–334. [PubMed: 20023176]
17. Sellak H, Choi CS, Dey NB, Lincoln TM. Cardiovascular research. 2013; 97:200–207. [PubMed: 23139241]
18. Lincoln TM, Dey N, Sellak H. Journal of applied physiology: respiratory, environmental and exercise physiology. 2001; 91:1421–1430.
19. Pryzwansky KB, Kidao S, Wyatt TA, Reed W, Lincoln TM. J Leukoc Biol. 1995; 57:670–678. [PubMed: 7722424]
20. Gudi T, Lohmann SM, Pilz RB. Molecular and cellular biology. 1997; 17:5244–5254. [PubMed: 9271402]
21. Gudi T, Huvar I, Meinecke M, Lohmann SM, Boss GR, Pilz RB. The Journal of biological chemistry. 1996; 271:4597–4600. [PubMed: 8617718]
22. Gudi T, Casteel DE, Vinson C, Boss GR, Pilz RB. Oncogene. 2000; 19:6324–6333. [PubMed: 11175347]
23. Boerth NJ, Dey NB, Cornwell TL, Lincoln TM. Journal of vascular research. 1997; 34:245–259. [PubMed: 9256084]
24. Zhang T, Zhuang S, Casteel DE, Looney DJ, Boss GR, Pilz RB. The Journal of biological chemistry. 2007; 282:33367–33380. [PubMed: 17878170]
25. Zhou W, Dasgupta C, Negash S, Raj JU. American journal of physiology Lung cellular and molecular physiology. 2007; 292:L1459–1466. [PubMed: 17322285]
26. Chiche JD, Schlutsmeyer SM, Bloch DB, de la Monte SM, Roberts JD Jr, Filippov G, Janssens SP, Rosenzweig A, Bloch KD. The Journal of biological chemistry. 1998; 273:34263–34271. [PubMed: 9852090]
27. Joyce NC, DeCamilli P, Boyles J. Microvascular research. 1984; 28:206–219. [PubMed: 6094989]
28. Collins SP, Uhler MD. The Journal of biological chemistry. 1999; 274:8391–8404. [PubMed: 10085070]
29. Browning DD, Mc Shane M, Marty C, Ye RD. The Journal of biological chemistry. 2001; 276:13039–13048. [PubMed: 11278473]
30. Komalavilas P, Shah PK, Jo H, Lincoln TM. The Journal of biological chemistry. 1999; 274:34301–34309. [PubMed: 10567406]
31. Schwappacher R, Weiske J, Heining E, Ezerski V, Marom B, Henis YI, Huber O, Knaus P. The EMBO journal. 2009; 28:1537–1550. [PubMed: 19424179]
32. Sugiura T, Nakanishi H, Roberts JD Jr. Circulation research. 2008; 103:53–60. [PubMed: 18535260]
33. Kim JJ, Casteel DE, Huang G, Kwon TH, Ren RK, Zwart P, Headd JJ, Brown NG, Chow DC, Palzkill T, Kim C. PloS one. 2011; 6:e18413. [PubMed: 21526164]
34. Kato S, Zhang R, Roberts JD Jr. American journal of physiology Lung cellular and molecular physiology. 2013; 305:L130–140. [PubMed: 23686857]
35. Friedrich MW, Aramuni G, Mank M, Mackinnon JA, Griesbeck O. The Journal of biological chemistry. 2010; 285:23285–23295. [PubMed: 20484048]
36. Feil R, Kellermann J, Hofmann F. Biochemistry. 1995; 34:13152–13158. [PubMed: 7548077]

37. Kimes BW, Brandt BL. *Experimental cell research*. 1976; 98:367–381. [PubMed: 943302]
38. Klink DJ 2nd, Brundage KM. *Cytometry Part A: the journal of the International Society for Analytical Cytology*. 2009; 75:699–706. [PubMed: 19582872]
39. R_Core_Team. *R Foundation for Statistical Computing*. Vienna, Austria: 2012.
40. Goldfarb DS, Corbett AH, Mason DA, Harreman MT, Adam SA. *Trends in cell biology*. 2004; 14:505–514. [PubMed: 15350979]
41. Harel A, Forbes DJ. *Molecular cell*. 2004; 16:319–330. [PubMed: 15525506]
42. Wagstaff KM, Sivakumaran H, Heaton SM, Harrich D, Jans DA. *The Biochemical journal*. 2012; 443:851–856. [PubMed: 22417684]
43. Pilz RB, Broderick KE. *Front Biosci*. 2005; 10:1239–1268. [PubMed: 15769622]
44. Sue YM, Chung CP, Lin H, Chou Y, Jen CY, Li HF, Chang CC, Juan SH. *American journal of physiology Cell physiology*. 2009; 297:C321–329. [PubMed: 19587222]
45. Chen WJ, Chen YH, Lin KH, Ting CH, Yeh YH. *Arteriosclerosis, thrombosis, and vascular biology*. 2011; 31:2106–2113.
46. Becker W. *Journal of microscopy*. 2012
47. Ai HW, Henderson JN, Remington SJ, Campbell RE. *The Biochemical journal*. 2006; 400:531–540. [PubMed: 16859491]
48. Johannessen M, Moens U. *Front Biosci*. 2007; 12:1814–1832. [PubMed: 17127423]
49. Gonzalez GA, Menzel P, Leonard J, Fischer WH, Montminy MR. *Molecular and cellular biology*. 1991; 11:1306–1312. [PubMed: 1671708]
50. Winnard PT Jr, Kluth JB, Kato Y, Artemov D, Raman V. *Cancer Biol Ther*. 2007; 6:1889–1899. [PubMed: 18059163]
51. Dey NB, Foley KF, Lincoln TM, Dostmann WR. *J Cardiovasc Pharmacol*. 2005; 45:404–413. [PubMed: 15821435]
52. Maier JA, Statuto M, Ragnotti G. *Molecular and cellular biology*. 1994; 14:1845–1851. [PubMed: 8114717]
53. Lange A, Mills RE, Lange CJ, Stewart M, Devine SE, Corbett AH. *The Journal of biological chemistry*. 2007; 282:5101–5105. [PubMed: 17170104]
54. Sinnaeve P, Chiche JD, Gillijns H, Van Pelt N, Wirthlin D, Van De Werf F, Collen D, Bloch KD, Janssens S. *Circulation*. 2002; 105:2911–2916. [PubMed: 12070122]
55. Wang S, Li Y. *American journal of physiology Heart and circulatory physiology*. 2009; 297:H2075–2083. [PubMed: 19717728]
56. Wagstaff KM, Jans DA. *Analytical biochemistry*. 2006; 348:49–56. [PubMed: 16300722]
57. Lam MH, Hu W, Xiao CY, Gillespie MT, Jans DA. *Biochemical and biophysical research communications*. 2001; 282:629–634. [PubMed: 11401507]
58. Mingot JM, Kostka S, Kraft R, Hartmann E, Gorlich D. *The EMBO journal*. 2001; 20:3685–3694. [PubMed: 11447110]
59. Xiao CY, Hubner S, Jans DA. *The Journal of biological chemistry*. 1997; 272:22191–22198. [PubMed: 9268364]
60. Li P, Wang D, Lucas J, Oparil S, Xing D, Cao X, Novak L, Renfrow MB, Chen YF. *Circulation research*. 2008; 102:185–192. [PubMed: 17991884]

Highlights

- PKGI γ encodes a monopartite amino acid sequence that determines its nuclear translocation.
- A classical nuclear localization mechanism regulates PKGI γ compartmentation.
- Nuclear PKGI γ localization controls its activation of CREB signaling.
- Nuclear but not cytoplasmic PKGI γ activity controls PKGI regulation of vascular SMC differentiation.

Fig. 1. PKGI γ encodes a NLS that directs its nuclear compartmentation. (A) The PKGI α/β isoforms have linearly arranged variant leucine zipper (LZ) – autoinhibitory (AI) domains and a conserved cGMP-binding domain and a catalytic region, which is also common with the PKGI γ proteolytic fragment. The PKGI molecules also encode a putative NLS, which contains a cluster of positively charged amino acids (+), that is located as indicated. Plasmids were made that encode NH₂-terminal FLAG-tagged wild type (WT) PKGI γ and the indicated NLS K $\rightarrow$ E mutant (ΔNLS). (B) Lysates from BHK cells transfected with the FLAG-PKGI γ plasmid constructs exhibited expression of the transgenes by immunoblotting. After transfection with the plasmids encoding the indicated genes or pcDNA3, the cell lysates were resolved using SDS-PAGE, electroblotted on a charged membrane, exposed to the indicated antibodies, enzyme labeled secondary antibodies, and detected using chemiluminescence and a CCD camera system. (C) Although cells expressing PKGI γ and PKGI β exhibited nuclear PKGI immunoreactivity, those expressing PKGI γ with a mutant NLS did not. The cells transfected with plasmids that encode the indicated PKGI transgenes

were fixed, permeabilized, and interacted with the indicated antibodies that detect the FLAG epitope or catalytic region of PKGI (α PKGI_{CR}), and Alexa 488-conjugated secondary antibodies. Immunoreactivity was then assessed in a mid-nuclear optical slice obtained using laser scanning confocal microscopy. (D) The specificity of the antibodies used to detect the PKGI transgenes is shown here. The indicated cells were transfected with a plasmid that encodes PKGI β -FLAG or pcDNA3, fixed, permeabilized, and then exposed to the indicated primary antibodies, Alexa 488-labeled secondary antibodies, DAPI, and assessed using laser scanning confocal microscopy.

Fig. 2.

Ivermectin inhibits nuclear PKGI γ localization. Rat aortic SMC were transfected with plasmids encoding the indicated PKGI γ constructs or GFP with three tandem repeats of the simian virus 40 large T-antigen NLS (GFP-NLS). After 1 hr treatment with 25 μ M ivermectin, or DMSO, and the cells were then fixed, permeabilized, and reacted with anti-PKGI γ CR and fluorescent secondary antibodies. Subsequently, wide-field fluorescence microscopy images were obtained and the ratio of nuclear-to-cytoplasmic fluorescence intensity was determined for a sample of cells whose images were obtained by an investigator masked with respect to the treatment group. The results were normalized to the mean value obtained from the DMSO-treated cells. N=10 each group.

Fig. 3. Nuclear phospho-CREB biosensor fluorescence lifetime microscopy (FLIM) modeling. (A) BHK cells were transfected with a plasmid that encodes NLS-ICAP, a uni-molecular fluorescence resonance energy transfer (FRET)-based biosensor that localizes to the nucleus and is targeted by kinases that phosphorylate the CREB kinase-inducible domain (KID) (Friedrich 2010). NLS-ICAP phosphorylation increases CREB KID binding to the CBP KID-interaction domain (KIX) and thereby decreases the FRET of adjacent CFP and YFP molecules. After 40 hours of serum starvation, the cells were fixed and then subjected to time-correlated single-photon counting FLIM. After step-wise application of a photon lifetime model equation with the indicated number of exponent time factors, representative Chi^2 images, model residuals, and Chi^2 data were obtained from a region of interest that included the whole cell. These data indicate that a two exponent model represents effectively the photon decay data as it results in a Chi^2 close to one without systematic variations in the model residuals. $N=5$; * $P < 0.05$. (B) One day after BHK cells were

transfected with plasmids that encode CFP or NLS-ICAP, as indicated, and serum starved as described above, the cells were treated with 40 μ M forskolin or DDW, fixed, and then subjected to FLIM and the mean photon decay times (t_m) were determined using a two exponent photon decay model. Forskolin-treatment increases NLS-ICAP CFP t_m similarly to the change in NLS-ICAP FRET reported previously [35]. N=10 each group.

Fig. 4.

Nuclear but not cytoplasmic PKGI γ regulates phospho-CREB biosensor activation. (A) BHK cells were transfected with plasmids that encode NLS-ICAP with or without those that encode the indicated PKGI γ constructs or pcDNA3. Shown are the anticipated molecular configurations of NLS-ICAP residing in the nucleus in the PKGI γ -expressing cells. Should PKGI γ phosphorylate nuclear NLS-ICAP then the CFP-YFP distance would increase as reflected by a greater τ_m . In contrast, should nuclear NLS-ICAP not be phosphorylated by PKGI γ -NLS, then the τ_m would likely be unchanged and the CFP τ_m would be comparable with the one determined in the control cells. (B) Representative CFP fluorescence intensity images confirm that NLS-ICAP predominantly localizes to the nucleus of cells. The CFP τ_m heat map images show that PKGI γ but not PKGI γ -NLS increases the NLS-ICAP CFP τ_m , particularly in the nuclear compartment, suggesting that PKGI γ but not PKGI γ -NLS regulates nuclear CREB activation. (C) The photon decay curves in the BHK cells expressing the indicated transgenes confirm the modulation of NLS-ICAP CFP photon relaxation time by PKGI γ but not PKGI γ -NLS. The distribution of the weighted residuals

supports the validity of a bi-exponential decay curve employed here in modeling NLS-ICAP CFP τ_m .

Fig. 5.

Nuclear PKGI γ regulates phospho-CREB biosensor activation through a typical CREB KID PKA phosphorylation site. (A) BHK cells were transfected with plasmids encoding NLS-ICAP without and with a mutant serine corresponding to CREB S¹³³ (NLS-ICAP- S¹³³), the indicated PKGI γ transgenes, or pcDNA3, were fixed and then subjected to FLIM and the mean nuclear τ_m was obtained. Cells expressing PKGI γ exhibited increased NLS-ICAP CFP τ_m compared with those expressing PKGI γ - NLS or transfected with pcDNA3. Importantly, PKGI γ expression did not change the CFP τ_m of NLS-ICAP- S¹³³ indicating that PKGI γ modulates NLS-ICAP CFP τ_m through targeting the S¹³³ in CREB. N = 15 cells per group. (B) The PKGI γ - NLS transgene exhibited typical cytoplasmic PKGI kinase activity *in vivo*. BHK cells transfected with plasmids that encode the indicated PKGI γ transgenes or pcDNA3 and FLAG-VASP, a cytosolic PKGI target, were lysed and the soluble proteins were resolved using SDS-PAGE, electro-blotted onto a charged membrane, and antigens were detected with the indicated antibodies, peroxidase labeled secondary antibodies, and enhanced chemiluminescence. FLAG-VASP exhibited phosphorylation and a typical increase in apparent molecular weight in lysates of cells expressing the PKGI γ transgenes but not transfected with the control plasmid.

Fig. 6. PKGI γ kinase activity is required for nuclear phospho-CREB biosensor activation. (A) PKGI γ -DK lacks PKGI kinase activity. BHK cells were transfected with plasmids encoding NLS-ICAP, PKGI γ , PKGI γ harboring a mutation previously shown to inhibit PKGI β activity (PKGI γ -DK), or pcDNA3, and FLAG-VASP were lysed and the soluble proteins were resolved using SDS-PAGE, electro-blotted onto a charged membrane, and antigens were detected with the indicated antibodies, peroxidase labeled secondary antibodies, and enhanced chemiluminescence. The lysates of cells transfected with the plasmid that encodes PKGI γ -DK did not exhibit FLAG-VASP phosphorylation or the typical shift of FLAG-VASP migration in SDS-PAGE associated with this post-translational modification. (B) BHK cells were transfected with plasmids encoding NLS-ICAP, the indicated PKGI γ transgenes, or pcDNA3, were fixed and then subjected to FLIM. The PKGI γ -DK expressing BHK cells did not exhibit changes in the nuclear CFP τ_m consistent with NLS-ICAP phosphorylation and CREB activation. N = 15 cells per group.

Fig. 7.

PKGI γ regulates nuclear phospho-CREB biosensor activation in BHK cells through mechanisms that do not require PKA. The nuclear CFP t_m values are shown of BHK cells transfected with plasmids expressing the indicated transgenes, or with pcDNA3, and treated with or without 40 μ M forskolin and 10 μ M myristoylated PKA inhibitor 14–22 amide (myr-PKI), as indicated. Myr-PKI did not inhibit the increase in NLS-ICAP CFP t_m associated with PKGI γ although it did prevent the increase the activation of this phospho-CREB biosensor associated with forskolin treatment. N=15 each group.

Fig. 8.

Cytosolic PKGI γ regulates nuclear phospho-CREB biosensor activation in aortic SMC in part by modulating MAPK signalling. (A) Rat aortic SMC were transfected with plasmids encoding NLS-ICAP and either the indicated PKGI γ transgenes or pcDNA3. Subsequently, they were fixed and nuclear CFP τ_m was determined using FLIM. Both PKGI γ and PKGI γ -NLS increased the activation of NLS-ICAP. (B) Rat aortic SMC were transfected as described above, treated with 10 μ M U0126 or DMSO, the diluent, fixed, and then nuclear CFP τ_m was determined using FLIM. Although U0126 abolished the increase in the nuclear CFP τ_m observed in SMC expressing PKGI γ -NLS, it only partially decreased the increased nuclear CREB activation observed in the cells expressing PKGI γ . Both A and B, N=15 in each group.

Fig. 9.

PKGI γ but not PKGI γ - NLS increases SMC differentiation marker expression levels in SMC. (A) Rat aortic SMC were transfected with bicistronic plasmids encoding mCherry2 in the second open reading frame (ORF), without (pCP2) or with FLAG-tagged PKGI γ transgenes encoded, as indicated, in the first ORF. Subsequently, the cells were fixed, permeabilized, and exposed to anti-FLAG and Alexa 488 anti-mouse IgG antibodies and subjected to epifluorescence microscopy. In these merged images of representative cells, the red, green, and blue colors represent mCherry2, anti-FLAG reactivity, and DAPI fluorescence signals, respectively. SMC transfected with the FLAG-PKGI γ , FLAG-PKGI γ - NLS, and mCherry2-encoding plasmids exhibited anti-FLAG immunoreactivity and mCherry2 fluorescence indicating that mCherry2 expression effectively marks the transfected cells. (B and C) SMC were transfected with the plasmids noted above or pcDNA3. Subsequently they were dissociated from the plates, fixed, permeabilized, exposed to Alexa 488-labeled antibodies detecting calponin or MHC, and analyzed using flow cytometry. Following gating to include cells exhibiting mCherry2 fluorescence, here represented by the red data points in the scatter gram, it was determined that cells transfected with the plasmid encoding PKGI γ but not PKGI γ - NLS exhibited increased expression of the SMC differentiation markers. The histogram shows the distribution of cells exhibiting the calponin and MHC fluorescence signal levels in the mCherry2-expressing

SMC; the bar graph represents the mean fluorescence values related to the immunoreactivity of these SMC protein markers in the cells. A typical result of three independent experiments is shown.