The Golgi apparatus regulates cGMP-dependent protein kinase I compartmentation and proteolysis

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1Cardiovascular Research Center, Massachusetts General Hospital, Boston, Massachusetts; 2Departments of Anesthesia, Critical Care, and Pain Medicine, Massachusetts General Hospital, Boston, Massachusetts; 3Department of Medicine, Massachusetts General Hospital, Boston, Massachusetts; 4Department of Pediatrics, Massachusetts General Hospital, Boston, Massachusetts; 5Department of Pediatrics, Harvard Medical School, Cambridge, Massachusetts; 6Applied Video Research, Medford, Massachusetts

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Kato S, Chen J, Cornog KH, Zhang H, Roberts JD Jr. The Golgi apparatus regulates cGMP-dependent protein kinase I compartmentation and proteolysis. Am J Physiol Cell Physiol 308: C944–C958, 2015. First published April 8, 2015; doi:10.1152/ajpcell.00199.2014.—cGMP-dependent protein kinase I (PKGI) is an important effector of cGMP signaling that regulates vascular smooth muscle cell (SMC) phenotype and proliferation. PKGI has been detected in the perinuclear region of cells, and recent data indicate that proteoprotein convertases (PCs) typically resident in the Golgi apparatus (GA) can stimulate PKGI proteolysis and generate a kinase fragment that localizes to the nucleus and regulates gene expression. However, the role of the endomembrane system in PKGI compartmentation and processing is unknown. Here, we demonstrate that PKGI colocalizes with endoplasmic reticulum (ER), ER-Golgi intermediate compartment, GA cisterna, and trans-Golgi network proteins in pulmonary artery SMC and cell lines. Moreover, PKGI localizes with furin, a trans-Golgi network-resident PC known to cleave PKGI. ER protein transport influences PKGI localization because overexpression of a constitutively inactive Sar1 transgene caused PKGI retention in the ER. Additionally, PKGI appears to reside within the GA because PKGI immunoreactivity was determined to be resistant to cytosolic proteinase K treatment in live cells. The GA appears to play a role in PKGI proteolysis because overexpression of inositol 1,4,5-trisphosphate receptor-associated cGMP kinase substrate, not only tethered heterologous PKGI-β to the ER and decreased its localization to the GA, but also diminished PKGI proteolysis and nuclear translocation. Also, inhibiting intra-GA protein transport with monensin was observed to decrease PKGI cleavage. These studies detail a role for the endomembrane system in regulating PKGI compartmentation and proteolysis. Moreover, they support the investigation of mechanisms regulating PKGI-dependent nuclear cGMP signaling in the pulmonary vasculature with Golgi dysfunction.

cGMP-dependent protein kinase I; cGMP signaling; Golgi apparatus

Cyclic guanosine monophosphate (cGMP) plays an important role in regulating vascular smooth muscle cell (SMC) function (48). cGMP is produced in SMC by nitric oxide (NO)-stimulated soluble guanylate cyclase (sGC) and by natriuretic peptide-stimulated particulate guanylate cyclase, which is associated with the plasma membrane (20, 34). Subsequently, cGMP binds to cGMP-dependent protein kinase I (PKGI, also known as cGKI) and thereby induces conformational changes that activate the enzyme and stimulate the phosphorylation of protein targets by PKGI (25, 35). PKGI is expressed as two isoforms, PKGI-α and PKGI-β, that are translated from alternately spliced mRNA and differ in the first ~100 NH2-terminal amino acids. This variant portion of PKGI encodes a leucine zipper (LZ)-like domain that directs isoform-specific dimerization, autoinhibition, and interactions with select phosphorylation targets. The COOH-terminal portion of PKGI, which is identical in the PKGI isoforms, has cGMP- and ATP-binding domains and a catalytic region, which contains kinase and substrate recognition domains. In SMC, PKGI phosphorylation of protein targets regulates intracellular Ca2+ levels, cytoskeletal activity, and the activation of genes that control cell phenotype and proliferation (25, 49, 66).

Although PKGI is dispersed throughout the cytoplasm, it also localizes to subcellular compartments. This increases the proximity of PKGI with select phosphorylation targets and likely regulates some of its activities. In SMC, for example, PKGI is concentrated in focal adhesion centers of pseudopodia. There, PKGI has been colocalized with filamentous actin and vasodilator-stimulated phosphoprotein (9), which are PKGI phosphorylation targets that regulate the cytoskeleton (10, 80). PKGI isoforms also exhibit differential subcellular compartmentation in SMC (18, 21, 24, 94) and endothelial cells (39). This appears to be mediated in part by the selective binding of the variant PKGI LZ domains to different anchoring proteins or target proteins. For example, in SMC, the LZ domain causes PKGI-α interaction with the myosin-binding subunit of myosin phosphatase (MYPT1), and together they colocalize with actin-myosin stress fibers (84). PKGI-α phosphorylation of MYPT1 increases myosin light chain phosphatase activity and the dephosphorylation of the myosin light chain (84). Also, PKGI-α binds to the formin homology domain-containing protein 1 (FHOD1) through its LZ domain (94). This appears to concentrate PKGI-α together with FHOD1 in the perinuclear region of SMC. Compared with PKGI-α, PKGI-β is more generally dispersed throughout the SMC cytoplasm (21, 24). However, studies indicate that PKGI-β also localizes with some organelles through its interaction with anchoring proteins. For example PKGI-β binds to inositol 1,4,5-trisphosphate receptor (IP3R)-associated cGMP kinase substrate (IRAG, which is homologous to Mrvil) through a cluster of acidic amino acids in its LZ domain (12). In the presence of IRAG, PKGI-β localizes to the endoplasmic reticulum (ER) (13, 21, 28), where together with IRAG it forms a complex with IP3R that regulates intracellular Ca2+ levels (21). PKGI has been also observed to localize to the nucleus of several cell types including SMC (13, 18, 21, 32, 83). Although PKGI is detected throughout the nucleoplasm, our previous work sug-
gests that it is also anchored to subnuclear domains (83). In these studies, PKGI was found to localize to the nucleoli of interphase SMC, and PKGI immunoreactivity and enzyme activity were detected in purified SMC nucleoli. Moreover, PKGI-nucleolar localization was tightly associated with the integrity of that organelle. As shown by RNA polymerase II inhibitor studies, nucleolar assembly tightly controlled nuclear PKGI localization to that structure. Although PKGI nuclear compartmentation appears to be required for the regulation of gene expression by cGMP (13, 31, 32, 83), the pathways that process PKGI and control its nuclear activities are not fully understood.

The studies described above indicate that PKGI localization plays a role in regulating its phosphorylation of targets. However, it is unknown how such compartmentation might also regulate PKGI posttranslational modifications and activation. Previously, we observed that PKGI can undergo proteolysis in SMC and cell lines and that this processing releases a COOH-terminal kinase fragment, PKGI-γ, that localizes to the nucleus and transactivates gene expression (14, 83). However, the mechanisms that regulate PKGI cleavage are incompletely understood. PKGI has been detected in the perinuclear region of a number of cells (18, 21, 24, 60, 65, 68, 73, 83, 94, 98) and in the particulate fraction of lysed aorta SMC (11), where it was concentrated in subcellular organelle fractions that are rich in ER and Golgi apparatus (GA) marker proteins (37). Moreover, recently we observed that SMC proprotein convertases (PCs) that typically reside in the Golgi increase PKGI proteolysis (41). Although these observations raise the possibility that PKGI localizes to the endomembrane system and undergoes processing in that organelle, a functional relationship between the PKGI and this system has not been established. Presently, we tested the hypothesis that the Golgi complex plays a role in regulating PKGI compartmentation and posttranslational modifications. After demonstrating that PKGI colocalizes with ER, GA, and trans-Golgi network (TGN) proteins and resides within the endomembrane system, we determined that decreasing heterologously expressed PKGI localization to the GA by tethering it to the ER and disrupting its transport within the Golgi complex reduces PKGI proteolysis. Together, these studies suggest a functional role of the Golgi complex in regulating PKGI localization and proteolysis. Moreover, they suggest that Golgi complex disruption, which has been observed in pulmonary vascular disease (38, 43, 55, 76), might play a role in inhibiting PKGI function.

**MATERIALS AND METHODS**

**Antibodies and reagents.** The anti-PKGI catalytic region (PKGIcr; KAP-PK005) antibody, which reacts with the common COOH-terminal portion of the PKGI isoforms, and anti-PKGI-β LZ domain (PKGI-β; KAP-PK002) and anti-protein disulfide isomerase (PDI, ADI-SPA-891) antibodies were obtained from Enzo Life Sciences. The anti-PKGI-α LZ antibody (PKGI-α) was obtained from Howard Surks and is described elsewhere (17). Anti-GM130 (610822) and GS28 (611184) antibodies were obtained from BD Biosystems; anti-GM130 (610822) and GS28 (611184) antibodies were obtained from BD Biosystems; anti-TGN38 (ab16059), nyc (ab9106), and furin (ab28548) antibodies, furin antigenic peptide (ab4398), control rabbit IgG (ab37415), and mouse IgG (ab18443) antibodies were obtained from Abcam. Anti-ER-Golgi intermediate complex (ERGIC)-53 (E1031) and FLAG (M2; F2921) antibodies and MOPC-21 (M5284) were purchased from Sigma. Biotinylated *Griffonia simplicifolia* lectin (GSL)-II (BK-3000) was purchased from Vector Laboratories. Alexa Fluor-labeled secondary antibodies and streptavidin and Zenon antibody labeling reagents from Life Technologies were used. Peroxidase-conjugated secondary antibodies (715-035-150) was purchased from Jackson Immuno-Research, and enhanced chemiluminescence (ECL) substrates (170-5061) were obtained from Bio-Rad. Poly-λ-lysine (0215017550) was obtained from Thermo Fisher Scientific. 8CPT-cGMP (CS5438), digoxigenin (D141), proteinase K (P2308), brefeldin A (BFA; B6542), monensin (M5273), and protease inhibitor cocktail solution (P8340) were obtained from Sigma.

**Cell culture and transfection.** Rat pulmonary arterial smooth muscle cells (PASMCs) were isolated using previously described methods (15) and used before the seventh passage. Human embryonic kidney 293 (CRL-1573), rat fetal lung (RFL)-6 (CCL-192), and baby hamster kidney (BHK) (CCL-10) cells were obtained from American Type Culture Collection. The RFL-6 cells were maintained in RPMI 1640 (no. 2633; Life Technologies); the other cells were cultured in DMEM (no. 11995; Life Technologies). To formulate complete media, 10% (vol/vol) heat-inactivated fetal bovine serum (no. SH3008803; HyClone), penicillin, and streptomycin were added to the media. The cells were passaged before becoming confluent using EDTA-trypsin. Cells were transfected using Lipofectamine 2000 reagent (no. 11668; Life Technologies) and Opti-MEM (no. 31983; Life Technologies) or Xfect (no. 631317; Clontech) using the manufacturer’s instructions. For immunofluorescence study of BHK cells, glass chamber slides were coated with poly-λ-lysine before the application of the cells.

**Plasmid construction and characterization.** pmTurquoise2-Golgi was constructed by Goedhart and colleagues (29). This plasmid encodes β-1,4-galactosyltransferase-mTurquoise2 (gaIT-mT2), which has the first 61 NH2-terminal amino acids of the long form of β-1,4-galactosyltransferase fused with mTurquoise2, a green fluorescent protein (GFP) mutant. pEGFP-Rab11 wild-type was made by Choudhury (16), and pcDNA3-GFP-golglin-84, which encodes NH2-terminal GFP fused with golgin-84, was constructed by Satoh (45, 71). These plasmids were purchased from Addgene, pMyC-IRAG, which encodes NH2-terminal myc epitope-tagged IRAG in pcDNA3, was a kind gift from Darren Castle and is detailed elsewhere (12, 13). pcDNA3 plasmids encoding mCherry (mCh) alone or fused with human Sar1 without (pcDNA3-Sar1) and with a T39N mutation (pcDNA3-Sar1[T39N]) were kindly provided by Jodene Eldstrom and David Fedida (93). pcDNA3-PKGI-α-FLAG and pcDNA3-PKGI-β-FLAG, which encode the indicated murine PKGI isoform with a COOH-terminal FLAG epitope tag, were used in the anti-PKGI LZ domain selectivity studies and previously constructed and characterized as described elsewhere (83). The authenticity of the plasmid constructs was confirmed with DNA sequencing or endonuclease mapping, as indicated.

**PKGI localization studies.** To colocalize endogenous PKGI immunoreactivity with gaIT-mT2 and EGFP-Rab11 fluorescence, 0.2 × 10^6^ PASMC/cm^2^ were seeded onto 1.7-cm^2^ chamber slides and then transfected with 1 μg of plasmid encoding the transgenes. Subsequently, the cells were incubated at 4°C and then treated with 20 μM digoxigenin in ice-cold PBS, fixed with 4% formalin in PBS, permeabilized with methanol, and then blocked with 1% goat serum in PBS. The cells were then reacted with the rabbit anti-PKGIcr, PKGI-α, or PKGI-β antibodies or control IgG and the Alexa Fluor 546-conjugated anti-rabbit antibody, with and without DNA-binding 4′,6-diamidino-2-phenylindole (DAPI) to identify the nuclei. To colocalize the PKGI and ERGIC-53 immunoreactivity, the PASMCs were seeded onto chamber slides, treated with digitonin, fixed with 4% formalin in PBS, permeabilized with 0.1% Triton X-100, and exposed to the rabbit anti-PKGI antibodies or control IgG and the Alexa Fluor 546-conjugated anti-rabbit secondary antibodies, as described above. Subsequently, the cells were exposed to rabbit anti-ERGIC-53 antibodies reacted with Alexa Fluor 488-conjugated Fab fragments before being fixed again and mounted. Serial z-stack images were collected using wide-field epifluorescence microscopy employing an integrated
microscope and cooled charge-coupled device (CCD) camera system (TIE Nikon and Andor Clara-E, respectively).

To colocalize PKGI immunoreactivity with Golgi- and TGN-resident proteins in RFL-6 cells, 0.5 × 10^5 cells/cm² were seeded onto the chamber slides, treated with digitonin, fixed, permeabilized, and blocked, as described above, and then reacted with rabbit anti-PKGI, or control IgG and Alexa Fluor 546-conjugated anti-rabbit antibody, biotinylated GSL-II, and Alexa Fluor 488-conjugated streptavidin, antibodies reactive to GM130 and GS28 and Alexa Fluor 488-conjugated secondary antibodies, or Alexa Fluor 488-conjugated Fab fragment-labeled anti-TGN38, furin, or nonreactive IgG antibodies. Subsequently, the cells were imaged using wide-field epifluorescence microscopy.

To test the effect of ER protein transport inhibition on PKGI Golgi complex localization, RFL-6 cells were seeded onto chamber slides, as described above, and transfected with 1.0 μg of pcDNA3-mCh, pcDNA3·Sar1, or pcDNA3·Sar1[3′TN3]. The following day, the cells were fixed, and fluorescent protein expression was examined, or the cells were treated with digitonin, and the PKGICR immunoreactivity was detected using methods described above.

To examine the influence of IRAG on overexpressed PKGI-β localization, 0.2 × 10^5 BHK cells/cm² were seeded on chamber slides and transfected with 0.5 μg of pcDNA3 PKGI-β-FLAG and up to 2 μg of pMyc-IRAG. pcDNA3 was used to balance the amount of plasmid used in the transfaction reactions. Subsequently, the cells were treated with digitonin, fixed, and probed with anti-FLAG, myc, and GM130 antibodies and fluorescent dye-labeled secondary antibodies, and then cell images were captured using laser-scanning confocal microscopy (LSCM).

**Antibody competition studies.** After diluting the antibodies with blocking solution to the concentration employed in the experiments described above, we reacted them overnight at 4°C with several molar excess of either recombinant PKGICRα or the furin immunogenic peptide, as indicated. Subsequently the antibodies were reacted with the digitonin-treated cells, and the immunofluorescence was detected using wide-field epifluorescence microscopy.

**PKGI localization analysis.** The analysis of the colocalization of PKGICR and Golgi marker protein expression and the cooccurrence of PKGICR- and Golgi marker protein-labeled organelle structures in cells was performed using the approach described by Dunn et al. (23) and van Steensel et al. (90). For these analyses, the cell images were first deblurred using deconvolution algorithms. For the wide-field 3D epifluorescence microscope images, following background subtraction and photobleaching correction (Bleach corrector, ImageJ) (58), a Richardson-Lucy deconvolution algorithm with total variation regularization (DeconvolutionLab, ImageJ) (92) was employed using a point-spread function (PSF). The PSF was generated using images of 0.175-μm fluorescent beads (PS-Speck beads, Invitrogen) that were collected using the same optical conditions employed in the cell-imaging experiments. The PSF was masked to remove noisy pixels and then smoothed using methods described by others (26, 27). For the 2D wide-field and LSCM images, deconvolution was performed using an estimated PSF and commercially available programs (NIS-Elements and Scientific Volume Imaging, respectively).

Pixel intensity spatial correlation analysis was employed to identify colocalized pixels. Image pixels corresponding to the PKGI immunoreactivity and Golgi marker protein reactivity exhibiting similar intensities were identified and mapped onto a merged image using an image-processing program (Colocalizer Finder, ImageJ) (47). The Mander’s coefficients were calculated using techniques described by others (6) after the images were thresholded to remove background fluorescence. In these studies, M1 refers to the proportion of the PKGI signal that is coincident with that of the Golgi marker protein, and M2 refers to the proportion of the Golgi marker protein signal that is coincident with the PKGI one. M1 is the ratio of the sum of intensities of colocalizing PKGI pixels divided by the sum of the intensities of all PKGI pixels. Similarly, M2 is the ratio of the sum of the intensities of Golgi marker protein pixels mapped to the PKGI immunoreactivity divided by the sum of intensity of all the Golgi marker pixels. The pixels in one channel are considered colocalized if the pixel at the same location in the other channel exceeds a threshold.

The cooccurrence analysis was performed using the following methods. First, arbitrary lines chosen to exemplify regions with and without PKGI immunoreactivity and GA protein marker cooccurrence were constructed, and then the relative signal intensity was determined along those vectors. Second, normalized cross-correlation analysis was performed using methods detailed by van Steensel and colleagues (90) although the object cooccurrence analysis was extended into a third dimension to take into account the structure of the GA. In this method, a volume of interest (VOI) corresponding to the gA1-tm2 fluorescence signal was constructed and used as a template. Subsequently, Pearson correlation coefficients (PCCs) were determined as the template was translated up to 1-μm distance from its original position in a search region across the three axes of the field image corresponding to PKGICR immunoreactivity. For contrast, a cross-correlation study was performed employing the same VOI and search method but using instead the field image rotated 90° about its center to form an orientation image. Dunn et al. (23) described this type of analysis in a control study previously. Two methods were used to represent the data from the later cooccurrence studies. A cross-correlation function was calculated showing the relationship between the PCCs and template localization across each axis in the test and control images. In addition, a surface plot of the PCCs relative to the x- and y-axis translations corresponding to the z-position with the maximum correlation was constructed.

**Fluorescence protease protection assay.** To test whether PKGI resides within the endomembrane system, 0.2 × 10^5 RFL-6 cells/cm² were seeded onto 1.7-cm² chamber slides and then transfected with 1.5 μg of plasmid-encoding proteins localized to the GA with fluorescent protein domains within or outside of the lumen (topology detailed in RESULTS). Twenty-four hours later, the cells were incubated with or without 40 μg/ml digitonin in PBS for 5 min and then reacted with and without 1 μg/ml proteinase K in PBS for 3 min. Subsequently, the cells were fixed, permeabilized, exposed to the anti-PKGI, and Alexa 546-labeled secondary antibodies, and mounted in DAPI-containing media. Wide-field epifluorescence images were captured by a control-exposure paramo with the digitonin and proteinase K treatment groups. The fluorescent cell frequency and intensity data corresponding or mapped to the GA marker transgenes were collected using ImageJ while the investigator was masked with respect to the treatment groups.

**Inhibition of GA protein transport and examination of PKGI proteolysis.** To examine the effect of IRAG overexpression on PKGI proteolysis, 0.2 × 10^5 BHK cells/cm² were seeded onto 9.5-cm² tissue culture plates and subsequently transfected with 2 μg of pcDNA3·PKGI-β-FLAG with and without pMyc-IRAG or pcDNA3. After washing the cells with ice-cold PBS, we collected the protein in SDS-bicinchoninic acid (BCA) lysis buffer, which contains 50 mM Tris-HCl at pH 7, 4% (wt/vol) sodium dodecyl sulfate, and 10% (vol/vol) glycerol, and then sonicated it. Following centrifugation, the protein concentration in the supernatant was determined using a BCA-based protein assay method (Thermo Fisher Scientific). Subsequently, 1% (vol/vol) β-mercaptoethanol and 1% (vol/vol) bromophenol blue were added, and 30 μg of protein was resolved using SDS-PAGE and electrotransferred to a polyvinylidene fluoride (PVDF) membrane. After the blot was blocked with 5% nonfat milk in Tris-buffered saline, it was incubated with anti-FLAG and peroxidase-conjugated secondary antibodies. The antigen-antibody complexes were detected using ECL and a CCD camera-gel imaging system (Bio-Rad, ChemiDoc XRS+).

To examine the effect of GA protein transport inhibition on PKGI localization, 0.3 × 10^5 RFL-6 cells/cm² were seeded onto the chamber slides. Subsequently, the cells were exposed to 5 μg/ml BFA, 1 μM monensin, or diluent for 2 h before and during the 24 h following
transfection with 1 μg pmTurquoise2-Golgi. The cells were then washed with PBS, permeabilized with digitonin, and fixed, as described above. Following exposure to anti-PKGICR antibody, fluorophore-labeled secondary antibodies, and DAPI, wide-field epifluorescence images were obtained. To test the effect of GA protein transport inhibition on PKGI proteolysis, 0.5 × 10^6 RFL-6 cells/cm^2 were seeded onto 9.5-cm^2 tissue culture plates and then exposed to either 5 μg/ml BFA, 1 μM monensin, or diluent for 2 h before and 24 h during transfection with 3 μg pcDNA3-PKGICR-FLAG or pcDNA3. Subsequently, the cells were collected with SDS-BCA lysis buffer and then sonicated. After β-mercaptoethanol and bromphenol blue were added, as described above, 30 μg soluble protein was separated using SDS-PAGE, transferred onto PVDF membrane, and probed with anti-FLAG and peroxidase-conjugated secondary antibodies. The antigen-antibody complexes were then detected as described above.

Data analysis and statistical methods. Experiments were repeated at least three times, and representative images and data are shown. In the figure panels, the scale bar corresponds to 10 μm. The data were analyzed using R (36). For the fluorescence protease protection assay, the mean fluorescence signal intensity in the structure was normalized to the mean value of the digitonin-treated control group. The distribution of these data appeared to be skewed, the mean and 95% confidence interval values derived from the data were adjusted using methods described by Johnson (40). Moreover, the fluorescence data were compared using a Wilcoxon test. The relative PKGI proteolysis was determined by comparing the density of the bands with PKGI-γ immunoreactivity with the sum of all anti-PKGICR reactive bands. These data were compared using an unpaired t-test. Significance for the tests detailed above was determined at P < 0.05. In the experiments detailed above involving cell transfection with the control plasmid pcDNA3, antibody-mediated cell and protein blot reactivity were not observed (data not shown).

RESULTS

PKGI colocalizes with Golgi cisterna and TGN constituent proteins. To map PKGI to the Golgi complex, we tested whether PKGI colocalizes with proteins that reside in that organelle in cells using immunofluorescence. First, we examined whether PKGI immunoreactivity colocalizes with Golgi markers in rat PASMC. We used an antibody that reacts with the COOH-terminal portion of PKGI (PKGICR) so that we could survey the localization of the PKGI isoforms and PKGI-γ. Moreover, gaIT was used to identify the GA because others have used this protein as a marker for this organelle in cell fractionation studies (8). Although gaIT localizes to the ER, Golgi complex, and plasma membrane (74, 97), we employed a fragment of the long form of gaIT because it concentrates primarily in the trans-cisterna of the GA (63, 70). We overexpressed this gaIT fragment fused with mTurquoise2 because this GFP-mutant protein has been shown to resist photobleaching and works well in imaging studies (29). In this and subsequent subcellular localization studies, the cells were treated with digitonin before fixation and reaction with the antibodies. This is because digitonin is a detergent that preferentially adsors to the cholesterol-rich plasma membrane, where it forms complexes with hydroxysterols, permeabilizes the membrane, and allows soluble proteins to diffuse in and out of the cell. In contrast, the membranes of the ER, GA, and other vesicular structures, which contain low levels of cholesterol, are not disrupted by digitonin treatment, and the substances enveloped or attached to them remain intact (64, 67). Previously, we observed that digitonin treatment releases unbound PKGI from the cytosol and thereby enhances the detection of PKGI associated with cytoplasmic organelles and the nucleus (83). We studied PKGI localization in the PASMCs because PKGI expression is abundant in these cells and PKGI has an important role in regulating the proliferation and differentiation of these cells (5, 15, 42, 88, 99).

As shown in Fig. 1A and B, PKGI(ER) immunoreactivity was observed in the PASMCs in an eccentric perinuclear pattern, in a fine vesicular pattern throughout the cytosol, and within the nucleus. Moreover, part of the perinuclear PKGI(ER) immunoreactivity resided in a ribbon-like pattern that strongly resembled the pattern exhibited by gaIT-mT2 fluorescence and is consistent with Golgi cisterna. Pixel intensity spatial colocalization analysis revealed several regions in this perinuclear area that exhibited similar levels of anti-PKGICR antibody reactivity and gaIT-mT2 fluorescence. However, not all the labeled perinuclear structures overlapped. As shown in the inset images, adjacent to some of the structures with signal colocalization were other ones that had either PKGI(ER) immunoreactivity or gaIT-mT2 fluorescence (compare the structures identified by the arrow heads in Fig. 1A). We also examined whether PKGI colocalizes with GM130 expression in the PASMCs. GM130 is a tether protein that resides primarily in the cis-cisterna of the GA and to a much lesser extent in the medial and trans-cisterna (2, 62, 81). In these experiments, a pattern of partial colocalization of PKGI(ER) and GM130 immunoreactivities was observed (Fig. 1B). The partial colocalization of PKGI(ER) with these Golgi cisternal markers is also supported by the modest values of the thresholded Mander’s coefficients. The specificity of the antibody used to identify PKGI(ER) reactivity is shown in Fig. 1C; preadsorption of the antibody with recombinant PKGI-α abolished the detection of PKGI(ER) in the perinuclear region of the cells and markedly reduced the abundant PKGI(ER) immunoreactivity detected in the nucleus. The specificity of the GM130 detection is suggested by the isotype antibody (MOPC-21) control study also shown.

Second, we tested whether the PKGI(ER) immunoreactivity spatially correlates with Golgi cisterna structures. As shown in Fig. 2A, only some of the perinuclear structures in PASMCs exhibiting PKGI(ER) immunoreactivity appear to be identical as those identified by gaIT-mT2. Moreover, inspection of the merged images in Fig. 2B suggests that some of the PKGI(ER) immunoreactive structures appear adjacent to those exhibiting gaIT-mT2 fluorescence. This pattern of partial cooccurrence is further exemplified by comparing the values of PKGI(ER) immunoreactivity intensity and the gaIT-mT2 fluorescence along lines transecting representative structures. In one example, in which the PKGI(ER) and gaIT-mT2 signals appear to cooccur across the transit line, the normalized intensity curves corresponding to each protein coincide over a length of the line. However, in another case, the signals do not appear to coincide, as the short distance of intensity signal overlap is less than the lateral resolving power of the microscope system. Cross-correlation analysis was next used to test whether the cooccurrence of structures exhibiting PKGI(ER) immunoreactivity and gaIT-mT2 fluorescence represents an occasional overlap of randomly distributed cytoplasmic organelles or a positive correlation of the structure localization. With this method, structures that cooccur are expected to exhibit decreasing overlap and, therefore, reduced signal correlation, as they are shifted a short distance with respect to each other. This analysis
indicated that the PKGICR and galT-mT2 signals coalesced within only a few micrometers in the image volume. As shown in Fig. 2C, shifting the galT-mT2 template <1 μm across the x-, y-, and z-axis with respect with the PKGICR immunoreactivity test image caused a reduction of the pixel correlation. In contrast, shifting the template across the 90°-rotated control image did not change the already low signal correlations. Together, these studies suggest that PKGICR immunoreactivity corresponds in part with that of the Golgi cisterna in PASMCs.

We also examined whether PKGI immunoreactivity correlates with the pre- and post-Golgi vacuolar compartments in PASMCs. As shown in Fig. 3A, PKGICR immunoreactivity was colocalized with ER resident PDI. This protein harbors a COOH-terminal KDEL motif that causes it to be concentrated within the ER. Moreover, PKGI also localizes with the ERGIC (Fig. 3B). PKGICR immunoreactivity colocalized with that of ERGIC-53, which appeared in a punctate, tubulovesicular, and perinuclear pattern described by others (4, 33). However, PKGI did not colocalize to a large degree with the structures that harbor Rab11. As shown in Fig. 3C, although transfected PASMCs express GFP-tagged Rab11 in a punctate pattern that is similar to that shown in fibroblasts by others (16), PKGICR immunoreactivity did not appear to colocalize with the signal.

Previous studies indicate that the PKGI isoforms localize to different compartments in SMC (18, 21, 24, 94). Therefore, we tested whether the endogenous PKGI isoforms exhibit differential Golgi localization in the PASMCs. Whereas resident PKGI-α immunoreactivity localized in an asymmetric perinuclear and in a reticular pattern in the cytosol of the PASMCs, the immunoreactivity of endogenous PKGI-β resided in a circumnuclear pattern and associated with more diffusely localized cytoplasmic structures (Fig. 4). Of interest, endogenous PKGI-α but not PKGI-β colocalized with galT-mT2 and with GM130 immunoreactivity. As expected, PKGI-α and PKGI-β LZ domain immunoreactivity was not detected in the nucleus; previously, we detected only the COOH-terminal PKGI-γ fragment of PKGI in the nucleus of SMC (14, 83). The anti-PKGI-β LZ domain antibody specificity was shown previously (83). The specificity of the anti-PKGI-α LZ domain antibody was demonstrated during preadsorption studies using recombinant PKGI-α (data not shown). The LZ domain selectivity of the PKGI isoform antibodies was characterized elsewhere (17). Moreover, we found that neither of the antibodies react with BHK cells, which do not express abundant PKGI, unless they were transfected with plasmids that encode the relevant PKGI isoform (data not shown). We tested whether PKGI expression maps also with Golgi protein markers in other cell types. RFL-6 cells are a fibroblast cell line that expresses soluble guanylate cyclase and PKGI.
As shown in Fig. 5A in RFL-6 cells, endogenous PKGI colocalizes with the reactivity of GSL-II, a tetrameric glycoprotein that binds to the α/β-linked N-acetylglucosamine residues of proteins that reside in the medial compartment and trans-cisternae of the GA in cells (30, 85, 86). Also, the PKGI immunoreactivity colocalized with that of GM130 and GS28,
an N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) protein that resides primarily in the medial Golgi cisternae (53, 61). Others have employed these GM130 and GS28 antibodies to delineate GA structures (77). Moreover, we observed that PKGI associates with TGN structures (Fig. 5B). In the RFL-6 cells, PKGICR immunoreactivity colocalizes with that of TGN38, which is a membrane-bound TGN-resident protein (52, 82). The specificity of the anti-TGN38 antibody was shown elsewhere (96). Importantly, we observed that PKGI immunoreactivity also colocalizes, in part, with that of furin in a perinuclear region of the RFL-6 cells. Recently we observed that furin, which is abundant within the GA and TGN (7, 57, 59, 91), but not PC7, which is a PC that localizes to the cell membrane (69), plays a role in regulating PKGI proteolysis (41). The perinuclear distribution of furin that we observed is similar to that reported by other investigators (7, 54, 59, 87). The specificity of the anti-furin antibody used in this study was demonstrated in preadsorption immunofluorescence studies using the antigenic peptide (data not shown). Together, these results indicate that PKGI localizes to the Golgi complex and TGN of cells.

Fig. 4. Endogenous PKGI-α but not PKGI-β colocalizes with Golgi complex organelles in PASMCs. A: following digitonin treatment and fixation, anti-PKGI-α antibody reactivity was detected in PASMCs using a fluorescently labeled secondary antibody. As described in previous figures, Golgi apparatus (GA) trans-cisternae were identified in cells using the galT-mT2 transgene and the cis-cisterna were detected using an anti-GM130 antibody. Median-intensity z-projections of the image stacks were derived, and colocalized pixels were identified as described before. PKGI-α immunoreactivity colocalizes with the Golgi cisternae. B: PKGI-β immunoreactivity was detected in PASMCs using an anti-PKGI-β antibody and methods described for A. PKGI-β immunoreactivity did not localize with Golgi cisternal structures.

Fig. 5. PKGI colocalizes with GA and trans-Golgi network (TGN) organelles in rat fetal lung (RFL)-6 cells. A: cells were treated with digitonin, fixed, and then exposed to antibodies that detect PKGI and to either *Griffonia simplicifolia* lectin-II (GSL-II) or antibodies that identify GM130 and GS28. B: cells that were permeabilized and fixed as above and reacted with antibodies that detect PKGI and the trans-Golgi resident proteins TGN38 or furin. Colocalized pixels were identified using intensity spatial correlation analysis, as described previously, and are shown in white.
ER protein transport plays a role in regulating PKGI distribution in the endomembrane system. To investigate whether ER-to-Golgi protein transport regulates PKGI localization, we tested whether inhibiting protein export from the ER alters the distribution of endogenous PKGI in the endomembrane system. For these studies, we expressed Sar1 with a T39N mutation in the cells. This is because others demonstrated that this Sar1 mutant is locked in a GDP-bound, inactive state and that its overexpression competitively inhibits Sar1-mediated COPII-coated vesicle formation at ER exit points (46). Sar1[T39N] expression effectively decreases ER-to-Golgi protein transport. To identify cells in which ER protein export was modulated, cells were transfected with plasmids that encode mCh fused to the Sar1 genes, as described by others (93). The study was conducted using RFL-6 cells because, as shown above, they exhibit PKGI immunoreactivity in the Golgi structures. In contrast with the cells overexpressing wild-type Sar1 or mCh, we observed that the cells harboring the Sar1 mutant exhibited PKGICR immunoreactivity retention in an ER exit site pattern (Fig. 6). This study suggests that ER-GA protein transport plays a role in regulating PKGI distribution in the endomembrane system.

Although these studies indicate that PKGI localizes to GA, they have not determined whether PKGI is luminal or is bound to the outside of this structure. Therefore, we adopted a fluorescent protein protection assay method described by others (51) to test whether PKGI resides within the endomembrane system. This method uses selective plasma membrane permeabilization of live cells with digitonin to permit the introduction of proteases into the cytoplasm that cleave proteins that are not protected by digitonin-resistant membranes, such as those that bound the endomembrane system (67) and nucleus (1). The schema of the method used in our study is shown in Fig. 7A. We validated this system in RFL-6 cells during pilot studies. We observed that, although digitonin and proteinase K treatment, together, decreased the fraction of cells exhibiting fluorescence from a Golgi membrane-spanning transgene with a fluorescent protein tag projecting into the cytosol (GFP·golgin-84), these treatments did not diminish the proteinase K treatment, together, decreased the fraction of cells exhibiting fluorescence from a Golgi membrane-spanning transgene with a fluorescent protein tag projecting into the cytosol (GFP·golgin-84), these treatments did not diminish the

Fig. 6. ER protein exit disruption causes PKGI retention in the ER of RFL-6 cells. Cells were transfected with plasmids that encode GDP-bound inactive Sar1 (mCh·Sar1[T39N]) or wild-type Sar1 (mCh·Sar1) fused with mCherry (mCh), or mCh alone, and, on the following day, after digitonin treatment, anti-PKGICR immunoreactivity was detected using epifluorescence microscopy. Expression of the constitutively inactive Sar1 mutant was associated with increased retention of PKGICR immunoreactivity in the ER.

Fig. 7. PKGI resides within the endomembrane system and nuclei of PASMCs. A: schema of the method used to examine protein topology using a fluorescence protease assay. Following selective permeabilization of plasma membranes with digitonin, proteases added to the media, such as proteinase K, diffuse into the cytosol and cleave proteins that are not bounded by protective digitonin-resistant membranes, such as the ER, GA, and the nuclear membranes. In such an assay, following protease treatment, cells expressing galT-mT2, a GA-localizing fusion protein that has an mTurquoise2 fluorescent protein domain residing within the Golgi lumen, are expected to exhibit fluorescence, whereas those harboring GFP·golgin-84, which is a GA-associated protein that has a GFP tag that projects into the cytosol, are expected to have reduced fluorescence. B: PASSMCs transfected with plasmids that encode the fluorescent protein-tagged transgenes described in A were treated with digitonin without and with proteinase K and then fixed. Subsequently, the cells were exposed to antibodies that detect PKGICR, and images were obtained using wide-field epifluorescence microscopy and identical exposure conditions. Whereas the GA-like and nuclear PKGI immunoreactivity and galT-mT2 fluorescence patterns appeared to be protected from cytosolic proteinase activity, the fluorescence signal of GFP·golgin-84 was diminished.
fraction of fluorescent cells expressing a transmembrane GA protein encoding a fluorescent protein tag residing within the lumen (galT-mT2, data not shown). Subsequently, we determined that GA PKGI immunoreactivity in digitin-treated RFL-6 cells was protected from proteinase K activity (typical result is shown in Fig. 7B; the fluorescence data are summarized in Table 1). Also, as expected, the nuclear immunoreactivity of PKGI was protected from proteinase K treatment by the digitin-resistant nuclear membrane. In control studies, we observed that the digitin or proteinase K treatment, alone, did not change the frequency of RFL-6 cells exhibiting the fluorescent proteins and the mean fluorescence of those proteins or immunoreactivity of PKGI in the cells. These data suggest that PKGI resides within the lumen of the endomembrane system.

PKGI compartmentation regulates PKGI proteolysis. The functional role of PKGI localization in the endomembrane system is unknown. However, because we observed previously that PCs that are localized and activated in the GA, such as furin, regulate PKGI proteolysis (41), and that PKGI immunoreactivity colocalizes with furin in the TGN (Fig. 5B), we tested whether the GA might influence PKGI cleavage. In these studies, we examined whether inhibiting the localization of heterologously expressed PKGI to the GA by overexpressing an anchoring protein that binds it to the ER structures or inhibiting intra-GA protein transport regulates PKGI proteolysis.

Previous work indicates that IRAG is an ER membrane-associated protein (21, 78) that interacts with the LZ portion of the PKGI-β isoform (3, 12) and thereby regulates PKGI-β intracellular compartmentation (13, 21, 28). IRAG does not bind the LZ domain of PKGI-α. Moreover, although PKGI-β overexpression, alone, increases perinuclear, ER, and nuclear PKGI localization in BHK cells, PKGI-β and IRAG cooverexpression was found to concentrate cytosolic PKGI-β at the ER and inhibit PKGI nuclear localization (13). On the basis of this information, we first examined whether IRAG could be used as a tool to regulate the localization of overexpressed PKGI-β in the cytosol and thereby test the role of the GA in PKGI posttranslational modifications. In agreement with observations by others (13, 21, 28), and shown in Fig. 8A, IRAG colocalizes with heterologous PKGI-β to an ER-like pattern in cells. In BHK cells transfected with plasmids that encode PKGI-β-FLAG and myc-IRAG and treated with digitin, IRAG appeared to localize with PKGI-β in a typical ER pattern, as described by others (13, 21, 28). Moreover, we observed that, in the absence of IRAG overexpression, the PKGI-β transgene colocalized, in part, with the GA marker GM130 (Fig. 8B). In the absence of IRAG, overexpressed PKGI-β has been localized to a perinuclear, Golgi-like pattern by others (13). However, with IRAG overexpression, PKGI-β-FLAG immunoreactivity was also observed to be decreased in the perinuclear region highlighted by this Golgi marker. These studies support the notion that the relative amount of PKGI and anchoring protein expression influences the distribution of PKGI in cellular compartments. We then tested whether the decrease in PKGI-β localization to the GA with IRAG overexpression is associated with diminished PKGI proteolysis. As shown in Fig. 9A, overexpression of IRAG in BHK cells was associated with a decrease in the immunodetection of ~60-kDa COOH-terminal PKGI fragments in cell lysates, which is consistent with a decrease in PKGI proteolysis, compared with cells that expressed less IRAG. This regulation of PKGI proteolysis by IRAG was supported by densitometric quantification of the PKGI fragments detected in the immunoblots. Furthermore, in agreement with work by others (13, 21), the overexpression of IRAG was associated with decreased nuclear PKGI immunoreactivity (Fig. 9B), which we previously showed is dependent on PKGI proteolysis (83).

In additional studies, we tested whether inhibiting GA protein transport also decreases PKGI proteolysis. For these studies, we used the fungal macrocyclic lactone BFA because it decreases ER-to-GA protein transport (44). Monensin was also used because this ionophore inhibits intra-Golgi protein transport by inducing H⁺/Na⁺ exchange across GA membranes and causing vesicular swelling (22, 89). These experiments were performed in RFL-6 cells because these cells exhibit endogenous PKGI and PKGI localization to the Golgi (Fig. 4A), and our previous work shows that RFL-6 cells exhibit PKGI proteolysis (83). In pilot studies, we noted that BFA and monensin treatments decrease galT-mT2 and PKGI expression in RFL-6 and other cells. Therefore, for the localization studies, the galT-mT2 and PKGI-FLAG immunoreactivity signals in the BFA- and monensin-treated cells were boosted so that the overall fluorescence intensity matched that of the control ones. Moreover, the amount of protein resolved using SDS-PAGE was balanced so that the levels of full-length PKGI-α-FLAG detected by immunoblotting were similar in the experiments.

As shown in Fig. 10A, BFA and monensin treatment disrupted Golgi cisternal organization in RFL-6 cells. BFA caused the galT-mT2 fluorescence to assume a perinuclear cytoplasmic pattern consistent with the ER and to acquire anastomosing tubular extensions (arrow head) that are similar to those described by others (44, 50). In contrast, monensin caused the galT-mT2 fluorescence to accumulate in swollen perinuclear

Table 1. Fluorescence protease protection assay

<table>
<thead>
<tr>
<th>Probe</th>
<th>Treatment</th>
<th>Mean Fluorescence</th>
<th>95% CI</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKGI immunofluorescence</td>
<td>digitin</td>
<td>1.03</td>
<td>0.90–1.16</td>
<td>0.655</td>
</tr>
<tr>
<td></td>
<td>+ proteinase K</td>
<td>1.07</td>
<td>0.96–1.19</td>
<td></td>
</tr>
<tr>
<td>GFP-golgin-84</td>
<td>digitin</td>
<td>1.06</td>
<td>0.80–1.32</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>+ proteinase K</td>
<td>0.22</td>
<td>0.11–0.33</td>
<td></td>
</tr>
<tr>
<td>galT-mT2</td>
<td>digitin</td>
<td>1.09</td>
<td>0.88–1.30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ proteinase K</td>
<td>1.36</td>
<td>1.07–1.65</td>
<td>0.323</td>
</tr>
</tbody>
</table>

Mean fluorescence was normalized to the mean value for the digitonin treatment alone control group; *comparison with cells treated with digitonin alone; α = 25 for cGMP-dependent protein kinase I (PKGI) immunoreactivity and 10 for the green fluorescent protein (GFP)·golgin-84 and the β1,4-galactosyltransferase-mTurquoise2 (galT-mT2) transgene groups.

 Downloaded from http://ajpcell.physiology.org/ on 10.22013.36 on April 13, 2017
structures (arrow). Others have shown that monensin causes a similar accumulation of galT in distended cisternae, which appear to be derived from the TGN (72). Of note, BFA and monensin treatments also altered the intracellular pattern of PKGI<sub>cx</sub> immunoreactivity. BFA and monensin treatments also increased PKGI<sub>cx</sub> detection in a perinuclear pattern consistent with the ER. Moreover, as shown in Fig. 10A, BFA treatment was associated with a minor decrease in PKGI proteolysis. In contrast, a large decrease in PKGI cleavage was detected in the lysates of cells treated with monensin. The decrease in PKGI proteolysis associated with BFA and monensin cell treatments is quantified in Fig. 10C. Together, these results support a link between PKGI compartmentation in the GA and PKGI proteolysis in cells.

**DISCUSSION**

Previous studies suggest that PKGI compartmentation has an important role in regulating its phosphorylation of select targets. However, it is unknown whether such mechanisms might also regulate PKGI posttranslational modifications that are important for some of its activities. Previously, we observed that cGMP stimulates PKGI proteolysis and the generation of a COOH-terminal kinase fragment, PKGI-γ, that localizes to the nucleus of SMC and transactivates gene expression (83). Moreover, we recently showed that PKGI-γ nuclear localization plays an important role in regulating SMC differentiation because mutant PKGI-γ that does not enter the nucleus does not increase the expression of SMC contractile proteins (14). On the basis of insights from the NH<sub>2</sub>-terminal amino acid sequence of immunopurified nuclear PKGI-γ and the putative structure of protease recognition domains, we examined whether PCs might contribute to PKGI cleavage and nuclear PKGI-γ localization (41). During these studies, we observed that PC overexpression increased PKGI proteolysis and rescued PKGI cleavage in cells with a dominant-negative mutant PC form. Moreover, PC inhibitors were observed to decrease PKGI proteolysis and nuclear localization. Unexpectedly, we found that PCs that typically reside within the Golgi complex of SMC, such as furin, increase PKGI proteolysis, whereas those that do not fail to cleave this kinase. Therefore, we tested whether PKGI localizes to the GA and whether this compartmentation plays a role in regulating PKGI proteolysis in vascular SMC. We determined that PKGI immunoreactivity colocalizes with constituent Golgi cisternae and TGN proteins, including PC-resident furin, and with the endomembrane structures. In addition, although endogenous PKGI-α colocalized with the GA, PKGI-β did not. Moreover, in agreement with our past studies (83), the LZ domains of PKGI-α and PKGI-β were not detected in the nucleus using selective antibodies, whereas the COOH-terminal domain of PKGI was found in the nucleus. We also adapted a fluorescent protein protection assay developed by others (51) to define the topology of PKGI. Using this...
method, we determined that GA-localizing PKGI immunoreactivity was resistant to a protease introduced into the cytosol of living cells, indicating that it resides in part within this endomembrane system. Moreover, studies detailed here suggested that the Golgi complex has a role in regulating PKGI
proteolysis. This is because decreasing PKGI-β migration to the Golgi complex, by tethering exogenous PKGI-β to ER-bound IRAG, and decreasing Golgi protein transport with monensin was observed to inhibit PKGI proteolysis. Together, these studies implicate the GA as an organelle that has a role in regulating PKGI proteolysis.

Although a majority of PKGI resides within the soluble fraction of the cytosol (11), PKGI has been observed to localize with intracellular structures that harbor phosphorylation substrates that regulate cytoskeletal kinetics and function (reviewed in Ref. 25). For example, PKGI has been observed to colocalize with actin filaments in the pseudopodia of migrating cells. Moreover, PKGI has been detected in microsomal compartments of the cytosol and observed to interact with proteins that are integral members of intracellular organelles. Ives et al. (37) and Casnellie et al. (11) observed that a small fraction of cellular PKGI resides in microsomal fractions of SMC that exhibited enzyme activity normally found in ER and Golgi structures (11, 37). In addition, protein interaction studies suggest that PKGI can associate with the ER because PKGI-α has been observed to immunoprecipitate with IP3 and IRAG, which are associated with this organelle.

Previous studies suggested that PKGI might localize to the Golgi complex. For example, PKGI immunoreactivity has also been observed in the perinuclear region of SMC in culture and in tissue sections (18, 24, 60). Moreover, PKGI immunoreactivity has been detected in the perinuclear region of pulmonary endothelial cells (39) and macrophages (68). However, this area also includes the ER as well as the GA, and no formal colocalization study of PKGI with Golgi complex structures has been performed. In one study, however, PKGI-α was observed to interact with an anchoring protein (GKAP42) that is expressed primarily in the Golgi complex of male germ cells (98). Previously, we observed that cGMP increased perinuclear as well as nuclear localization of PKGI. In these studies, PKGI colocalized with isoelectin IB4-bound α-d-galactoside-rich proteins and disulfide isomerase, which are abundant in the GA and rough ER, respectively (83). Moreover, we observed that disruption of ER-Golgi architecture using BFA or nocodazole inhibited nuclear PKGI-γ localization. These studies pointed to the potential importance of the GA in PKGI-γ nuclear localization. In the present work, to more precisely map PKGI immunoreactivity to Golgi cisterna and examine possible colocalization of PKGI with TGN markers, we used digitonin to introduce pores in the cytoplasmic membrane and deplete soluble PKGI immunoreactivity to highlight the PKGI bound to cytoplasmic organelles. Analysis of PKGI immunoreactivity in ~1-μm-thick optical z-stack sections of the perinuclear region and quantitative techniques employing objective thresholding techniques (19) confirmed PKGI localization with TGN marker proteins TGN38 and furin, suggesting a role for the GA in posttranslational modifications of PKGI.

The studies performed here also suggest that GA has an important role in regulating PKGI proteolysis. Pilz et al. (66) previously postulated that PKGI anchoring to cytosolic proteins might regulate PKGI nuclear localization. This hypothesis was supported by studies demonstrating that PKGI-β interaction with ER-bound IRAG decreases nuclear PKGI translocation (13, 21) and transcriptional activity (13). Our present observations indicate that increased IRAG, not only inhibited overexpressed PKGI-β from associating with Golgi cisterna, but also decreased PKGI-β from undergoing proteolysis. However, it is also possible that increased anchoring of PKGI-β to the ER by IRAG also decreased PKGI-β from localizing to other compartments where it undergoes proteolysis. The detection of decreased PKGI proteolysis in cells treated with BFA and monensin further supports the notion that the GA plays a functional role in regulating PKGI cleavage. It is interesting to note that, whereas monensin caused a large inhibition of PKGI proteolysis, the reduction in PKGI cleavage induced by BFA treatment was minor. This might be due to a BFA-mediated redistribution of furin into the ER, as observed by others (59), and the cleavage of PKGI by furin in that structure.

Our current results implicate the GA as an important organelle that regulates PKGI proteolysis. These results suggest that cellular pathologies that disrupt ER-GA homeostasis, such as those that have been associated with pulmonary vascular injury (38, 56, 75), might have an important effect in decreasing PKGI proteolysis and nuclear PKGI-γ compartmentation and cGMP signaling.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: S.K. and J.D.R. conception and design of research; S.K., J.C., K.H.C., and J.D.R.prepared figures; S.K., J.C., K.H.C., and J.D.R. analyzed data; S.K., J.C., K.H.C., and J.D.R. interpreted results of experiments; S.K., J.C., K.H.C., and J.D.R. prepared figures; S.K., K.H.C., H.Z., and J.D.R. drafted manuscript; S.K., J.C., K.H.C., and J.D.R. performed experiments; S.K., J.C., K.H.C., H.Z., and J.D.R. approved final version of manuscript.

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