Direct Binding and Regulation of RhoA Protein by Cyclic GMP-dependent Protein Kinase Iα*

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Background: Regulation of vascular smooth muscle tone by cGMP-dependent protein kinase Iα (PKGIα) through RhoA inhibition is not understood.

Results: PKGIα binds RhoA, through cGMP-induced binding, and inhibition of RhoA requires PKGIα activity.

Conclusion: RhoA-PKGIα co-interaction regulates RhoA function.

Significance: Understanding RhoA regulation by PKGIα is important for understanding the regulation of vascular tone in vivo and in human diseases such as hypertension.

Vascular smooth muscle cell (VSMC) tone is regulated by the state of myosin light chain (MLC) phosphorylation, which is in turn regulated by the balance between MLC kinase and MLC phosphatase (MLCP) activities. RhoA activates Rho kinase, which phosphorylates the regulatory subunit of MLC phosphatase, thereby inhibiting MLC phosphatase activity and increasing contraction and vascular tone. Nitric oxide is an important mediator of VSMC relaxation and vasodilation, which acts by increasing cyclic GMP (cGMP) levels in VSMC, thereby activating cGMP-dependent protein kinase Iα (PKGIα). PKGI is known to phosphorylate Rho kinase, preventing Rho-mediated inhibition of MLC phosphatase, promoting vasorelaxation, although the molecular mechanisms that mediate this are unclear. Here we identify RhoA as a target of activated PKGIα and show further that PKGIα binds directly to RhoA, inhibiting its activation and translocation. In protein pulldown and immunoprecipitation experiments, binding of RhoA and PKGIα was demonstrated via a direct interaction between the amino terminus of RhoA (residues 1–44), containing the switch I domain of RhoA, and the amino terminus of PKGIα (residues 1–59), which includes a leucine zipper heptad repeat motif. Affinity assays using cGMP-immobilized agarose showed that only activated PKGIα binds RhoA, and a leucine zipper mutant PKGIα was unable to bind RhoA even if activated. Furthermore, a catalytically inactive mutant of PKGIα bound RhoA but did not prevent RhoA activation and translocation. Collectively, these results support that RhoA is a PKGIα target and that direct binding of activated PKGIα to RhoA is central to cGMP-mediated inhibition of the VSMC Rho kinase contractile pathway.

Vascular contraction and relaxation are highly regulated in vertebrates. Control of smooth muscle cell contractile state occurs primarily through regulation of the phosphorylation state of myosin light chains (MLCs)3 (1). In the vascular smooth muscle cell (VSMC), contraction is initiated by both calcium-dependent and -insensitive mechanisms. Increases in intracellular calcium activate the calcium/calmodulin-dependent myosin light chain kinase, leading to MLC phosphorylation and VSMC contraction (2). The calcium-insensitive pathway is mediated by G protein-coupled receptor activation of the small GTPase RhoA, which activates Rho kinase. Rho kinase then phosphorylates the regulatory subunit of myosin light chain phosphatase and inhibits its activity (3–6), leading to VSMC contraction and increased vascular tone (2, 6).

Conversely, relaxation of vascular smooth muscle results from a decrease in cytosolic Ca2+ concentration and/or reduced Ca2+ sensitivity of the contractile apparatus. Nitric oxide (NO), the most important endogenous vasodilator, regulates VSMC relaxation by increasing intracellular cGMP and activating cGMP-dependent kinase 1 (PKGI) (7, 8). The two isoforms of PKGI, PKGIα and PKGIβ, are identical except for their amino termini, which contain different leucine zipper (LZ) heptad repeat motifs that are critical for protein targeting (9). PKGIα and PKGIβ both regulate VSMC tone through interactions with key VSMC contractile proteins mediated by their respective LZ domains (8, 10–12). PKGI regulates both cytosolic Ca2+ concentration and contractile sensitivity to Ca2+. Although PKGIα decreases intracellular Ca2+ concentration by activating the regulator of G protein signaling 2 (RGS2) (13), PKGI also directly reduces the sensitivity of the contractile apparatus to Ca2+ through phosphorylation and activation of MLC phosphatase (10), as well as through inhibition of the Rho/Rho kinase contractile pathway (14–17).

PKGIα phosphorylation of RhoA on serine 118 inhibits RhoA activation and membrane translocation, which then inhibits Rho/Rho kinase-induced VSMC stress fiber formation.

3 The abbreviations used are: MLC, myosin light chain; 8-Br-cGMP, 8-bromo-cGMP; GDPβS, guanosine 5′-O-[(β-thio)diphosphate; GTPγS, guanosine 5′-[γ-(β-thio)triphosphate; Ki, kinase inactive; LPA, lysosphatidic acid; LZ, leucine zipper; LZM, LZ mutant; PKGI, cGMP-dependent protein kinase I; RGS2, regulator of G protein signaling 2; RhoGDI, RhoA GDP dissociation inhibitor; VSMC, vascular smooth muscle cell.

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and contraction (16, 17). We have shown previously that mice with selective mutations in the amino-terminal leucine zipper domain of PKG\textsubscript{1}\textalpha (leucine zipper mutant (LZM) mice) display both decreased Ser118 phosphorylation and increased activation of RhoA in their VSMCs (18). This dysregulation of RhoA contributes to VSMC contractile abnormalities, abnormal relaxation of large and resistance blood vessels, and increased systemic blood pressure (18), supporting the importance of PKG\textsubscript{1}\textalpha regulation of RhoA in maintaining normal VSMC function in vivo.

The mechanism by which PKG\textsubscript{1}\textalpha inhibits RhoA activation is not understood. Therefore, in the present study, we tested whether PKG\textsubscript{1}\textalpha regulates RhoA activation through a direct PKG\textsubscript{1}\textalpha-RhoA interaction, using lysophosphatic acid (LPA)-induced RhoA membrane translocation as an assay of RhoA activation. We further explored the specific PKG\textsubscript{1}\textalpha and RhoA domains required for co-interaction in vitro and in cellular lysates; and, using specific mutants of PKG\textsubscript{1}\textalpha, we determined the requirements of the PKG\textsubscript{1}\textalpha LZ domain for mediating PKG\textsubscript{1}\textalpha interaction with RhoA, and of PKG\textsubscript{1}\textalpha kinase activity for mediating inhibition of RhoA activation. Taken together, our findings define the mechanism by which PKG\textsubscript{1}\textalpha directly inhibits RhoA activity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The immortalized human aortic smooth muscle cell line Ao184 was established by infecting VSMCs, isolated from an explanted human aorta, with retroviral constructs containing the E6 and E7 human papillomavirus proteins as reported previously (13). COS-1 and Swiss 3T3 fibroblasts were obtained from American Type Culture Collection. Cells were cultured and passaged in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 \mu g/ml).

**Subcellular Fractionation**—VSMCs were grown at 37 °C in a 5% CO\textsubscript{2} humidified incubator. Cells were cultured and passaged in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 \mu g/ml). Cells were grown at 37 °C in a 5% CO\textsubscript{2} humidified incubator. Smooth muscle cells used in this study were passages 10–18. Cells were grown in 100-mm or 6-well dishes, respectively. Cells were ochem) in 100-mm or 6-well dishes, respectively. Cells were cultured and passaged in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 \mu g/ml). Cells were grown at 37 °C in a 5% CO\textsubscript{2} humidified incubator.

**Subcellular Fractionation**—VSMCs were grown on 100-mm dishes to ~80% confluence. The medium was then replaced with a low serum medium (DMEM containing 1% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin) for 16 h to allow the cells to become quiescent. The cells were then treated with serum-free medium (DMEM containing anti-oxidants) for 4 h prior to agonist stimulation. After 4 h, the cells were split into six-well dishes, and 8 h later the medium was replaced with a low serum medium overnight, followed by treatment with serum-free medium for 4 h. The cells were stimulated with 50 \mu M LPA (Sigma) for different durations as noted in the results. Cells were washed with ice-cold PBS two times and scraped in 0.5 ml or 0.3 ml of lysis buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 1 mM MgCl\textsubscript{2}, 2 mM EDTA supplemented with a protease inhibitor mixture (Calbiochem)) in 100-mm or 6-well dishes, respectively. Cells were lysed by two sequential freeze-thaw cycles. The lysate was first centrifuged at 500 \times g for 5 min to pellet the nuclear fraction and then centrifuged again at 120,000 \times g for 45 min to pellet the membrane fraction. The pellet was dissolved with solubilization buffer (1% Triton X-100, 3% glycerol in lysis buffer). The pellet and the supernatant were dissolved separately in 2 \times sample buffer (100 mM Tris-HCl, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, pH 6.8) and boiled for 5 min (19).

**Antibodies**—Antibodies were raised against GST-peptides corresponding to PKG\textsubscript{1}\textalpha or LZM-PKG\textsubscript{1}\textalpha amino-terminal 59 amino acids as described (10, 13). The rabbit polyclonal anti-PKG\textsubscript{1}\textalpha antibody was from Stressgen. Anti-RhoA-interacting protein RhoA GDP dissociation inhibitor (RhoGDI) antibody was from Cell Signaling. The mouse monoclonal anti-RhoA antibody, the goat polyclonal anti-PKG\textsubscript{1}\textbeta antibody, and the anti-goat peroxidase-conjugated secondary antibody were from Santa Cruz Biotechnology. Anti-mouse and anti-rabbit peroxidase-conjugated secondary antibodies were from Amersham Biosciences. The mouse monoclonal anti-human smooth muscle actin (1A4) antibody was from DAKO.

**Immunoblotting**—Protein concentrations were determined using the DC protein assay kit (Bio-Rad). Samples (20–40 \mu g of protein) were separated by SDS-PAGE (10 or 12.5% running, 4% stacking). The separated proteins were transferred by electrohoresis to a polyvinylidine difluore membrane (Millipore) using a Mini Trans-Blot cell (Bio-Rad). The blots were blocked for 1 h at room temperature in PBS-T buffer (0.02% Tween 20) containing 3% nonfat milk before incubation with the primary antibody overnight at 4 °C. After washing the membranes three times in PBS-T buffer, a peroxidase-conjugated secondary antibody was added for 1 h. Immunodetection was performed with an ECL Western blotting detection kit (Amersham Biosciences). For densitometric analysis, immunoblotted bands were quantitated using an Alpha Innotech image analyzer.

**Cloning and Expression Constructs**—Full-length RhoA cDNA and cDNA fragments encoding RhoA amino acids 1–44 and 1–147 were amplified by PCR from human aortic cDNA library (Clontech). The 5′-primer for RhoA was 5′-GCC GGA TCC ATG GCT GCC ATC CGG AAG-3′. The 3′-primers were 5′-CGC GTC GAC TCA CAA GAC AAG GCA CCC AGA TTG-3′, 5′-CGC GTC GAC TCA GCC ACA TAG TTC TCA AAC AC-3′, and 5′-CGC GTC GAC TCA CAT ATC TCT GCC TTC TTC AGG-3′ for full-length, RhoA amino acids 1–44, and 1–147, respectively. The PCR products were cleaved with BamH1-Sall and cloned into pGEX-4T-3 (Amersham Biosciences). To generate truncated PKG\textsubscript{1}\textalpha DNA fragments (amino acids 237–671, 360–379, and 620–671), cDNA fragments were amplified from the full-length pcDNA 3.1/PKG\textsubscript{1}\textalpha or pcDNA 3.1/PKG\textbeta DNA (20) by PCR. The following primers were designed for the investigation. The 5′-primer for PKG\textsubscript{1}\textalpha(237–671) and PKG\textsubscript{1}\textbeta(237–359) was 5′-GCC GAA TTC CTT GAT GAG ACC CAC TAT GAA-3′, for PKG\textsubscript{1}\textalpha(360–619) was 5′-GCC GAA TTC TTC AAT GAC TTT ACC ATT GACC CTT GG-3′, for PKG\textsubscript{1}\textbeta(620–671) was 5′-GCC CTC GAG ATG AGA CAG CCT GAT GTT GCC GTC-3′. The 3′-primer for PKG\textsubscript{1}\textalpha(237–671) and PKG\textsubscript{1}\textbeta(620–671) was 5′-GCC CTC GAG ATG AGA CAG CCT GAT GTT GCC GTC-3′. The PCR products were digested with EcoRI-XhoI and inserted into the plasmid DNA pGEX-4T-1. The inserts
were confirmed by sequencing. The catalytically inactive mutant of PKGI/H9251 was kindly provided by A. P. Broun (University of Calgary).

**Transient Transfection—**PKGI constructs and FLAG-RhoA proteins were overexpressed in COS-1 cells by using Lipofectamine 2000 (Invitrogen). Briefly, transfection was performed with 12 μg of DNA plasmid and 45 μl of Lipofectamine 2000 in 3 ml of Opti-MEM reduced serum medium (Invitrogen) in 100-mm dish plates (4 × 10⁵ cells/dish). The PKGI constructs were overexpressed in Swiss 3T3 cells using Lipofectamine LTX (Invitrogen). Briefly, transfection was performed with 15 μg of DNA plasmid, 30 μl of PLUS Reagent, and 90 μl of Lipofectamine LTX in 3 ml of Opti-MEM reduced serum medium in 100-mm dish plates, which contained 4 × 10⁵ cells/dish.

**GST Fusion Protein Expression in Escherichia coli—**GST fusion proteins of RhoA fragments and PKGI/H9251 fragments were expressed in E. coli BL-21DE3 (Stratagene) and transcription induced with 0.2 or 1 mM isopropyl-d-thiogalactopyranoside as described previously (13). The expressed GST fusion proteins were purified on glutathione-Sepharose beads (Novagen).

**In Vitro Pulldown Assay—**PKGIα protein purified from bovine lung (Promega) in binding buffer (50 mM Tris-HCl, 250 mM NaCl, 0.05% Nonidet P-40, 30 mM MgCl₂, pH 7.4) with protease inhibitor mixture (4 μg/ml) was mixed with 20–30 μl of GST-RhoA fragments bound to beads. Samples were rocked for 2 h at 4 °C (22). In control experiments, GST-alone beads were also tested for PKGI binding. For cell lysate assays, COS-1 cells were harvested in TLB buffer (20 mM Tris-HCl, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 25 mM β-glycerol phosphate, pH 7.4) 48 h after transfection. The cells were lysed by five passes through a 27-gauge needle on ice. The supernatant obtained by centrifugation (15,000 rpm, 30 min) was then mixed with 20–30 μl of GST fusion protein beads and rocked for 2 h at 4 °C. After a thorough washing with binding buffer, the bound proteins were solubilized in 2× SDS sample buffer and analyzed by 12.5% SDS-PAGE and immunoblotting. To indicate the amount of protein used in each pulldown assay, 5% of the input lysate was loaded in the input lane. Beads loaded with GST alone were included as negative controls in all pull-down experiments. Similar loading of GST fusion proteins was confirmed by Ponceau S staining.
GDPβS and GTPγS Binding Assay—GST–RhoA fusion beads were prepared as described above and incubated with 200 μM stable GTP/GDP analogue GTPγS or GDPβS for 15 min at room temperature. The reaction was stopped with MgCl₂. After centrifugation at top speed in a microcentrifuge, beads were washed three times with binding buffer followed by the addition of purified PKGIα (20 μg/ml) for 1 h at 4 °C. Samples were then centrifuged as above, washed three times, and eluted with SDS sample buffer followed by SDS-PAGE and Western blotting for PKGI with the PKGIcom antibody.

Co-immunoprecipitation (CoIP) Assay—Cells were harvested in ice-cold TLB buffer 48 h after transfection (22). The cells were lysed by five passes through a 27-gauge needle on ice, and the supernatant was obtained by centrifugation (15,000 rpm, 30 min). The supernatant (200 μg of total protein) was mixed with target antibodies (3–4 μg) or mouse immunoglobulin as a negative control for 12 h rocking at 4 °C. Fresh protein G conjugated to agarose was then added, followed by 2–3-h rocking at 4 °C. Immunoprecipitates were centrifuged at 2,500 rpm for 2 min at 4 °C. The supernatant was then discarded, and the pellet was washed four times with binding buffer and then resuspended with the same volume of 2× SDS sample buffer.

cGMP-Agarose Affinity Assay—For the assay with purified protein, purified PKGI protein in TLB buffer with a proteinase inhibitor mixture at a concentration of 4 μg/ml was mixed with His–RhoA (4 μg/ml) and rocked overnight at 4 °C (22, 23). For the cell lysate assays, the supernatants were obtained by centrifugation (15,000 rpm, 30 min) and then rocked for 2 h at 4 °C. Fresh nonconjugated agarose, cGMP or Rp-8AET-cGMPS conjugated agarose (BIOLOG LIFE Science Institute) were then added with a further 2–3-h rocking. Complexes were centrifuged at 2,500 rpm for 2 min at 4 °C, the supernatant was discarded, and the pellet was washed four times with TLB buffer. The pellet was then resuspended in same volume of 2× SDS sample buffer, boiled for 5 min, and the supernatant was analyzed by 10% SDS-PAGE followed by immunoblotting. To indicate the amount of protein used in each pulldown assay, 5% of the input lysate was loaded in the input lane. Controls using agarose-only loaded beads were included as negative controls in all affinity assay experiments.

Statistics—All results are expressed as the mean ± S.E. Significance was tested by Student’s t test. A p value of <0.05 was considered significant.

RESULTS

PKGIα Regulates RhoA Translocation in Human Aortic Smooth Muscle Cells—In response to a variety of stimuli, GTP binding activates RhoA and stimulates its translocation to the plasma membrane, making this process a reliable assay for RhoA activation. In cultured human Ao184 VSMCs, treatment with the RhoA activator LPA (50 μM) induced RhoA translocation from the cytosolic to the membrane fraction in a time-dependent manner (0, 5, 15, 30 min) (data not shown), reaching a plateau at 30 min with membrane-associated RhoA increasing ~2-fold compared with vehicle-treated cells (Fig. 1A). Pretreatment with the cGMP analogue and direct PKG activator 8-Br-cGMPS (100 μM) completely inhibited LPA-stimulated RhoA membrane translocation (Fig. 1A). In each experiment, the quantities of RhoA in the cytosolic fractions and of PKGI in both the cytosolic and membrane fractions did not change (data not shown). Western blotting with PKGI isoform-specific antibodies confirmed that >90% of PKGI in Ao184 cells was PKGIα (data not shown). Furthermore, in Swiss 3T3 cells transfected with PKGIα, LPA-induced RhoA translocation was also inhibited by 8-Br-cGMPS treatment (Fig. 1B). However, in Swiss 3T cells transfected with leucine zipper mutant PKGIα (PKGIα LZM), the inhibitory effect of 8-Br-cGMPS was abolished (Fig. 1C). These data support a critical role of PKGIα, through its LZ domain, in regulating membrane translocation of RhoA.

PKGIα Interacts Directly with RhoA—Next, we tested for a direct interaction of PKGIα with RhoA. First, we performed pulldown experiments incubating GST–RhoA fusion proteins with lysates from COS-1 cells overexpressing PKGIα and demonstrated that PKGIα complexes with GST–RhoA, but not with GST alone (Fig. 2A). In vitro mixing assays using purified PKGIα and GST–RhoA demonstrated that this complex formation results from a direct interaction between PKGIα and RhoA (Fig. 2B). In separate experiments, CoIP studies using lysates of COS-1 cells overexpressing both FLAG-tagged RhoA and full-length PKGIα also demonstrated complex formation between PKGIα and RhoA (Fig. 3). Identical results were observed with in vitro immunoprecipitations using purified His-RhoA and purified PKGIα (data not shown). Because PKGIβ is also

FIGURE 2. GST-RhoA interacts directly with PKGIα. Immunoblots (B) from cell lysates (4 and C) or purified PKGIα (B) are shown. GST–RhoA fusion proteins were immobilized with GSH beads and incubated for 2 h at 4 °C with lysates from COS-1 cells transiently transfected with PKGIα (A), PKGIα purified from bovine lung (B), or lysates of COS-1 cells transiently transfected with PKGIβ (C). Co-interaction of PKGIα or β with GST fusion proteins was determined by immunoblotting with anti-PKGIcom antibody. Densitometry data are shown at the right of each panel. Summary data are representative of three or four independent experiments. Error bars, S.E. *, p < 0.05; **, p < 0.01 versus control (GST alone). PKGIcom antibody directed to common domain of PKGIα and PKGIβ. ADU, arbitrary densitometric units.
expressed in the VSMC we examined whether RhoA binds PKG\(\alpha\) in lysates from COS-1 cells transfected with the PKG\(\alpha\) isoform. GST-RhoA pulled down PKG\(\alpha\), but to a significantly lesser extent than PKG\(\beta\) (Fig. 2C). We next investigated the effect of RhoA nucleotide binding state on its interaction with PKG\(\alpha\). Purified PKG\(\alpha\) complexed similarly with GDP-treated GST-RhoA, compared with GTP-treated GST-RhoA, supporting that the PKG\(\alpha\)-RhoA interaction is not regulated by RhoA nucleotide binding (Fig. 4A). Furthermore, RhoGDI did not precipitate with either full-length GST-RhoA (Fig. 4B) or with PKG\(\alpha\) precipitated with cGMP-conjugated beads from Ao184 cells (Fig. 4C).

**RhoA Switch I Domain Interacts Directly with PKG\(\alpha\)**—To define the specific domains of RhoA required for binding to PKG\(\alpha\), GST fusion proteins were generated containing RhoA Switch I domain (residues 1–44); RhoA Switch I and Switch II domains (residues 1–147); full-length RhoA (residues 1–192); or GST alone (Fig. 5A). All three GST-RhoA constructs, but not GST alone, precipitated PKG\(\alpha\), supporting that residues 1–44, containing the Switch I domain, are sufficient for interaction with PKG\(\alpha\) (Fig. 5B).

**PKG\(\alpha\) LZ Domain Interacts Directly with RhoA**—We next investigated the domain(s) of PKG\(\alpha\) mediating the PKG\(\alpha\)-RhoA interaction. GST fusion proteins containing specific domains of PKG\(\alpha\) were generated as represented in Fig. 6A and incubated with purified His-RhoA, followed by immunoblotting with anti-RhoA antibody. RhoA was detected only in pull-downs containing the PKG\(\alpha\) LZ domain, using either the PKG\(\alpha\) LZ domain alone (residues 1–59) or the LZ-containing N-terminal portion of PKG\(\alpha\) (residues 1–236) (Fig. 6B).

cGMP-activated PKG\(\alpha\), but Not Inactive PKG\(\alpha\), Binds RhoA Directly—We next investigated whether RhoA-PKG\(\alpha\) binding requires cGMP binding of PKG\(\alpha\). Lysates of COS-1 cells transfected with both PKG\(\alpha\) and FLAG-RhoA were incubated with purified His-RhoA, followed by immunoblotting with anti-RhoA antibody. RhoA was detected only in pull-downs containing the PKG\(\alpha\) LZ domain, using either the PKG\(\alpha\) LZ domain alone (residues 1–59) or the LZ-containing N-terminal portion of PKG\(\alpha\) (residues 1–236) (Fig. 6B).
bated with agarose beads conjugated with either cGMP or with the cGMP antagonist Rp-8AET-cGMPS to isolate activated or inhibited PKGI/H9251, respectively. Agarose beads were used as controls. cGMP-bound PKGI/H9251 formed a complex with RhoA, but Rp-8AET-cGMPS-bound (inactivated) PKGI/H9251 failed to complex with RhoA (Fig. 7, A and B). Similar results were observed using purified His-RhoA and purified PKGI/H9251 rather than cell lysates (Fig. 7, C and D). cGMP-conjugated agarose did not directly bind with RhoA (data not shown). To test whether binding of cGMP-bound PKGI/H9251 requires a functional LZ domain, COS-1 cells transfected with FLAG-RhoA and with either wild type PKGI/H9251 or with PKGI/H9251-LZM were incubated with cGMP-conjugated agarose or with agarose alone. Whereas cGMP-bound PKGI/H9251 interacted with RhoA, cGMP-bound LZM-PKGI/H9251 precipitated substantially less RhoA (Fig. 8).

PKGIα Kinase Activity Is Required for RhoA Inhibition by PKGIα, but Not for PKGIα–RhoA Binding—We next examined whether PKGIα kinase activity is required for PKG-mediated inhibition of RhoA (as assayed by RhoA membrane translocation). Swiss 3T3 cells, which lack detectable endogenous PKG (17), were transfected with mutant PKGIα lacking kinase activity (kinase inactive; KI-PKGIα) (21) and were treated with LPA with or without 8-Br-cGMP. In the KI-PKGIα-transfected cells, LPA induced RhoA membrane translocation, but 8-Br-cGMP failed to inhibit LPA-induced RhoA membrane translocation (Fig. 9A), supporting that PKGIα requires intact kinase activity to inhibit RhoA. Next, we investigated whether PKGIα kinase activity is also required for PKGIα–RhoA interaction. cGMP-agarose or agarose-alone binding assays were performed on lysates from COS-1 cells transfected with FLAG-RhoA and with either KI-PKGIα or full-length PKGI/H9251, followed by Western blotting for FLAG-RhoA. We observed no decrease in RhoA interaction with cGMP-bound KI-PKGIα compared with wild type PKGIα (Fig. 9C). These results indicate that PKGI kinase activity is required for inhibition of RhoA activation, but not for cGMP-induced interaction with RhoA.

DISCUSSION

In the present study, we define the mechanism by which PKGIα inhibits RhoA activation in vitro and in cultured cells. First, we have demonstrated that the 1α isoform of PKGI inhibits LPA-induced RhoA membrane translocation in both aortic
smooth muscle cells and in 3T3 cells. We show further that PKGIα and RhoA form a complex using both GST pulldown and CoIP assays. In vitro mixing experiments support direct binding between these two proteins. Furthermore, this interaction requires both an intact PKGIα LZ domain and cGMP activation, but complex formation does not require intact PKGIα kinase activity. In contrast to this, intact kinase activity is required for PKGI-mediated inhibition of RhoA activation and membrane translocation. Taken together, these data support a mechanism by which PKGIα binds directly to RhoA through an interaction involving the PKGIα LZ domain and inhibits RhoA activation through an event requiring PKGIα kinase activity. Our prior work has demonstrated that homozygous knock-in mice harboring discrete mutations in the PKGIα LZ
domain (LZM mice) develop hypertension and derangements in vascular relaxation, accompanied by decreased RhoA phosphorylation in the VSMCs and increased RhoA activity (18). Therefore, the current study clarifies that this PKGI\(\text{a}\)/H9251 regulation of RhoA occurs via direct binding and defines a mechanism of PKGI\(\text{a}\)/H9251-RhoA regulation of vascular relaxation.

Other groups have previously observed that PKGI can phosphorylate RhoA in vitro (16) and that cGMP regulates RhoA effects on VSMC structure and function (16–18). However, our findings represent the first demonstration of the requirement for direct binding of PKGI\(\text{a}\) and RhoA for regulation of RhoA function. Moreover, we have observed co-precipitation of these proteins by GST pulldown assays, immunoprecipitations, and through cGMP-conjugated agarose affinity assays.

Our GST binding studies demonstrate that LZ-containing domains of PKGI\(\text{a}\) mediate PKGI\(\text{a}\)-RhoA binding. Our GST pulldown experiments support that the LZ domain of PKGI\(\text{a}\) alone is sufficient to bind RhoA. LZM PKGI\(\text{a}\) was unable to be purified, thus precluding comparison of the in vitro binding of WT and LZM PKGI\(\text{a}\) to RhoA (data not shown). However, our
experiments in cultured cells with the full-length PKGIα-LZM protein demonstrate that the normal domain is necessary for full binding of RhoA-PKGIα. Additionally, we also detected increased RhoA pulldown from the PKGIα(1–236) domain compared with the PKGIα(1–59) domain alone. Although this analysis is admittedly qualitative and measuring true $K_d$ of this interaction is beyond the scope of this study, these findings do suggest that additional domains of PKGIα between amino acids 59 and 236 may contribute to the PKGIα–RhoA interaction. In previous work, we have identified other LZ-mediated binding partners of PKGIα, including RGS2 (13), formin homology domain protein (FHOD1) (22), and MLC phosphatase (10). Our finding of an LZ-mediated interaction between PKGIα and RhoA therefore reveals RhoA as a new cGMP-regulated PKGIα target in the VSMCs of potential importance in vascular function and regulation.

Our GST pulldown studies also demonstrate that PKGIα binds primarily to the conserved Switch I domain of RhoA. Importantly, the Switch I domain is highly conserved throughout a number of small GTPase molecules, including RGS2, Ras, and CDC42 (24), suggesting a possible shared region of PKGIα binding. These data therefore suggest potential mechanisms of regulation of other Rho family GTPases by PKGIα.

We have also observed, from the cGMP-conjugated bead experiments, that PKGIα binding by cGMP increases PKGIα–RhoA interaction, but that PKGIα kinase activity is not required for PKGIα–RhoA binding. One potentially confusing finding is that Rp-8AET-cGMPS, which inhibits PKGI kinase activity by preventing disengagement of the kinase and autoinhibitory sites, also disrupts normal LZ-dependent PKGIα–RhoA binding. Others have shown, however, that liberation of the PKIα kinase domain from the autoinhibitory site leads to increased solubility of the LZ domain (25). Therefore, we interpret our findings to support that cGMP-mediated positioning of the PKGIα LZ domain, as well as its kinase activating effect, mediates PKGIα RhoA binding. These findings clarify the importance of cGMP in regulating RhoA activation; and our observations that kinase-inactive PKGIα binds RhoA identically to WT PKGIα, but fails to inhibit RhoA activation, represent the first demonstration of a direct requirement for PKGI kinase activity in the regulation of RhoA function. Together, these data define a mechanism by which cGMP-bound PKGIα binds RhoA in a LZ domain-dependent manner, presumably leading to RhoA phosphorylation and inhibition of RhoA activity.

An understanding of the molecular mechanism of cGMP and PKGIα regulation of RhoA is of particular importance to the study of hypertension. Current dogma for human hypertension states that all causes of hypertension arise from renal-mediated derangements in salt handling. However, a number of mouse models, including the LzM model described above (18), support that primary vascular abnormalities are sufficient to induce hypertension. The current findings elucidating the mechanism by which PKGIα regulates RhoA activation in VSMCs therefore have potentially important implications for our understanding of the homeostatic mechanisms that maintain normal systemic blood pressure.

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