RhoA Expression Is Controlled by Nitric Oxide through cGMP-dependent Protein Kinase Activation*

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Vincent Sauzeau‡, Malvyne Rolli-Derkinderen‡§, Céline Marionneau, Gervaise Loirand¶, and Pierre Pacaud¶

From INSERM U-533, Faculté des Sciences, 44322 Nantes Cedex 3, France

The small G protein RhoA is a convergence point for multiple signals that regulate smooth muscle cell functions. NO plays a major role in the structure and function of the normal adult vessel wall, mainly through modulation of gene transcription. This study was thus performed to analyze in vitro and in vivo the effect of NO signaling on RhoA expression in arterial smooth muscle cells. In rat or human artery smooth muscle cells, sodium nitroprusside or 8-(2-chlorophenylthio)-cGMP induced a rise in RhoA mRNA and protein expression, which was inhibited by the cGMP-dependent protein kinase (PKG) inhibitor (R,)-8-bromo-β-phenyl-1,N6-ethenoguanosine 3′,5′-phosphorothioate. The NO/PKG stimulation of RhoA expression involved both an increase in RhoA protein stability and stimulation of rhoA gene transcription. Cloning and functional analysis of the human rhoA promoter revealed that the effect of NO/PKG involved phosphorylation of ATF-1 and subsequent binding to the cAMP-response element. Chronic inhibition of NO synthesis in Nω-nitro-L-arginine-treated rats induced a strong decrease in RhoA mRNA and protein expression in aorta and pulmonary artery associated with inhibition of RhoA-mediated Ca2+ sensitization. These effects were prevented by oral administration of the cGMP phosphodiesterase inhibitor sildenafil. These results show that NO/PKG signaling positively controls RhoA expression and suggest that the basal release of NO is necessary to maintain RhoA expression and RhoA-dependent functions in vascular smooth muscle cells.

Small G proteins of the Rho family function as tightly regulated molecular switches that govern a wide range of cell functions (1). A large body of evidence has now been obtained regarding the important functions of Rho proteins in the vascular, and RhoA has been shown to play a major role in vascular processes such as smooth muscle cell contraction, proliferation, and differentiation; endothelial permeability; platelet activation; and leukocyte migration (2–4). The activity of Rho is under the direct control of a large set of other regulators (1). In the inactive GDP-bound form, RhoA is locked in the cytosol by guanine dissociation inhibitors. The guanine nucleotide exchange factors catalyze the exchange of GDP for GTP to activate RhoA (5). Activation is then turned off by GTPase-activating proteins that hydrolyze GTP to GDP. Therefore, both the relative expression of these proteins (in particular, that of RhoA) and the fraction of active GTP-bound RhoA are key determinants of RhoA protein activity.

Data are now accumulating regarding the regulation of the amount of active GTP-bound RhoA. In vascular smooth muscle cells, several agonists of G protein-coupled receptors, including thrombin, thromboxane A2, endothelin, carbachol, angiotensin, α-adrenergic agonists, sphingolipids, and extracellular nucleotides, stimulate RhoA activity through the activation of guanine nucleotide exchange factors and increases in the fraction of GTP-bound RhoA. This RhoA activation is accompanied by the membrane translocation of GTP-bound RhoA (5–8). On the other hand, the NO, cGMP, and cGMP-dependent kinase (PKG)5 signaling pathway exerts inhibitory action on RhoA functions in cells stimulated by these G protein-coupled receptor agonists. We have previously demonstrated that PKG phosphorylates RhoA at Ser188 in vitro and that the effects of PKG activation on actin cytoskeleton are lost in cells expressing the non-phosphorylatable RhoA188 mutant, suggesting that inhibitory effects of PKG on RhoA-mediated contraction and actin organization are due to phosphorylation of RhoA at Ser188 (9). This effect involves inhibition of membrane translocation of GTP-bound RhoA. Several additional reports have now confirmed the inhibitory effect of the NO/cGMP/PKG signaling pathway on the RhoA-dependent component of agonist-induced contraction (10–13). Recently, it has also been shown that PKG inhibits RhoA-mediated serum response element (SRE)-dependent transcription (14). PKG inhibits SRE-dependent transcription induced by serum, constitutively active Gα12 or Gα13, constitutively active Rho exchange factor p115RhoGEF5 or constitutively active RhoA1635. This inhibition is associated with a decrease in the amount of active GTP-bound RhoA in cells stimulated with serum or constitutively active Gα12 or Gα13, but not in p115RhoGEF5 or RhoA1635-expressing cells, suggesting that PKG can act both upstream and downstream of RhoA. The effect on steps downstream of RhoA has been confirmed by showing that SRE-dependent transcription mediated by guest on July 28, 2018http://www.jbc.org/Downloaded from
by a constitutively active RhoA effector (ROK, PKN, or PRK-2) is inhibited by PKG activation (14). Therefore, the effects of the NO/cGMP/PKG signaling pathway on membrane-bound active GTP-bound RhoA in stimulated cells are beginning to be well documented.

Modulation of RhoA expression has been less extensively described; however, a few recent studies reported that the RhoA mRNA level or expression of the RhoA protein is increased in arteries from hypertensive (15), aged (16), and diabetic (17) rats and in atherosclerotic lesions (18). The functional consequences of these changes in RhoA expression have been suggested by other observations showing that RhoA-dependent pathways are involved in excessive contraction, migration, and proliferation associated with arterial diseases such as hypertension and atherosclerosis (4). However, to our knowledge, the mechanisms of regulation of RhoA expression still have not been investigated.

In the normal adult vessel wall, vascular smooth muscle cells are continuously subjected to the action of the basal release of NO from endothelial cells, regulating arterial tone, but also smooth muscle cell expression (19). Here we analyzed the effect of the NO/cGMP/PKG signaling pathway on RhoA expression in arterial smooth muscle cells. We show that long-term stimulation of PKG positively controlled RhoA expression both in vitro and in vivo through an increase in RhoA protein stability and stimulation of rhoA gene transcription. Our data suggest that the tonic release of NO is absolutely necessary to maintain RhoA expression in vascular smooth muscle. This mechanism might thus provide an important regulation level of RhoA functions in vascular smooth muscle cells.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Drugs—** Mouse monoclonal anti-RhoA antibody (26C4) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The plasmid encoding PKG Iα and rabbit anti-PKG I antisem were provided by Dr. Suzanne Lehmann (Institute of Clinical Biochemistry and Pathobiology, Würzburg, Germany). (R)-8-Br-PET-cGMP-S and 8-pCPT-cGMP were from Biolog Life Science Institute (Bremen, Germany). Sildenafil was purchased from Pfizer (Sandwich, UK), and Y-27632 was a gift from the Institut International de Recherche Servier. All other reagents were purchased from Sigma (St. Louis, Missouri).

**Smooth Muscle Cell Culture—** Rat aortic and human internal mammary artery smooth muscle cells were isolated by enzymatic dissociation as previously described (20). Cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Secondary cultures were obtained by serial passages after the cells were harvested with 0.2 g/liter EDTA and reseeded in fresh Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and antibiotics. Only smooth muscle cells at passage 2 were used in this study. Stimulation of the NO/PKG pathway with sodium nitroprusside (SNP; 10 μM) or 8-pCPT-cGMP (50 μM) was performed in the absence of serum in the culture medium.

**Western Blot Analysis—** Endothelium-deendoed aortas or cells were rapidly frozen in liquid nitrogen and homogenized in lysis buffer containing 20 mM Hepes-NaOH, 10 mM KCl, 10 mM NaCl, 5 mM MgCl₂, 1 mM diethiothreitol, and Complete (one tablet/50 ml; Roche Molecular Biochemicals). Nuclei and unlysed cells were removed by low speed centrifugation at 10,000 × g for 15 min at 4 °C. The protein concentration of the supernatant was measured and adjusted; Laemmli sample buffer was added; and equal amounts of protein were loaded onto each lane of SDS-12% polyacrylamide gels, which were then electrophoresed and transferred onto nitrocellulose. The amounts of proteins were checked by reprobing the membrane with monoclonal anti-β-actin antibody.

For PKG I expression analysis, lysates were prepared from Swiss 3T3 cells transfected with PKG Iα and analyzed by Western blotting using rabbit anti-PKG I antisem (diluted 1:1000) to control PKG expression. The immunoreactive bands were detected by ECL and quantified using QuantityOne.

**Real-time Reverse-Transcription-PCR—** Total RNA was extracted using TRIzol reagent (Invitrogen, Cergy Pontoise, France), and reverse transcription was performed according to standard techniques. Quantitative real-time PCR assays were carried out with sequence-specific primer pairs on the iCycler iQ system (Bio-Rad) using intercalation of SYBR Green as a fluorescent probe. Results were evaluated using iCycler iQ real-time detection system software (Bio-Rad). The expression of glyceraldehyde-3-phosphate dehydrogenase mRNA was used to normalize the expression of RhoA mRNA. The primers used were as follows: glyceraldehyde-3-phosphate dehydrogenase, 5'-CCATGCCATCAGTCGACAC-3' (forward) and 5'-GGTTTCTCAGGTCACTCTGGG-3' (reverse); and RhoA, 5'-GGAGGTGAGTTGGCTTATATGG-3' (forward) and 5'-CTTGTGTGTCATCATCCGA3-3' (reverse).

**Northern Blotting—** Total RNA (20 μg) was separated on formaldehyde-agarose gels and blotted onto nylon membranes (Hybond TM-N, Amersham Biosciences, Orsay, France). Membrane filters were hybridized with 32P-labeled cDNA probes as a partial rhoA cDNA fragment amplified by PCR (sense primer, 5'-GACAGGATAGTGTGGCTTATG-3'; and antisense primer, 5'-CTTGTGTGTCATCATCCGA3-3'). Hybridized filters were washed under high stringency conditions (0.1× SSC, 70 °C) and analyzed by autoradiography. Equal loading of RNA was confirmed by staining of the ribosomal RNA with ethidium bromide.

**Cloning and Site-directed Mutagenesis of the rhoA Promotor—** Sequences of 913 and 118 bp upstream of the ATG codon of the human rhoA gene were cloned into the pCR2.1 vector (Invitrogen) by PCR using the 5'-GGAGATCTCCAAATTAATCGCTCTTGCA-3' sense primer (1-97), the 5'-GGAGATCTCCAAATTAATCGCTCTTGCA-3' antisense primer (1-118) and p(dCRE) up, 5'-GCCCCATTCAACAAGCTGTCATGAAATCACGATCAC-3' (reverse) and the dCRE site-directed mutagenesis of the distal CRE (dCRE), the proximal CRE (pCRE), and the proximal SRE (pSRE).

**Transfections and Reporter Assays—** Swiss 3T3 fibroblasts were plated 24 h before transfection in 24-well plates to 60–70% confluence the day of transfection. The PKG Iα and RhoA plasmids and the different constructions of the rhoA promoter were transfected with jetPEI (PolyPlus Transfection, Illkirch, France) according to the manufacturer’s instructions. 24 h after transfection, 8-pCPT-cGMP (50 μM) or SNP (10 μM) was added to the medium for 6–24 h. Cells were then harvested and treated as described above for Western blot, Northern blot, and real-time reverse transcription-PCR analysis. For reporter assays, the pIREs-EGFP vector was always cotransfected to estimate the level of transfection fluorescence measure from lysates with Victor2 and to normalize the luciferase activity measured with the luciferase reporter reagent (Promega) in a LB96V luminometer (Berthold Technologies, Wildbad, Germany).

**Plasmids—** Full-length wild-type RhoA and RhoAΔNAs were cloned into the pSG5 vector (Stratagene), and full-length PKG Iα was cloned into pCDNA3 (Invitrogen, Groningen, The Netherlands). RhoA or PKG Iα plasmids were transiently transfected into Swiss 3T3 fibroblasts with jetPEI according to the manufacturer’s protocol. 48 h after transfection, cells were stimulated with 8-pCPT-cGMP (50 μM) and p(dCRE) up, 5'-GCCCCATTCAACAAGCTGTCATGAAATCACGATCAC-3' (reverse) and RhoA, 5'-GGAGGTGAGTTGGCTTATATGG-3' (forward) and 5'-CTTGTGTGTCATCATCCGA3-3' (reverse).
FIG. 1. Activation of the NO/PKG signaling pathway increases RhoA protein levels in vascular smooth muscle cells. A, stimulation of rat aortic (left panel) and lower panel, black bar) or human mammary artery (right panel and lower panel, open bars) smooth muscle cells with SNP (10 μM) for 0–24 h induced an increase in RhoA expression as analyzed by Western blotting. B, involvement of PKG was assessed by treatment of rat aortic smooth muscle cells with the cGMP analog 8-pCPT-cGMP (50 μM, for 24 h) and the selective PKG inhibitor R(+)8-Br-PET-cGMP-S (100 μM, pretreatment for 1 h). The RhoA protein level, normalized to β-actin expression, is expressed relative to the control taken as 1. Data shown are the means of four independent experiments; **, p < 0.01 versus control; ##, p < 0.01 versus SNP or 8-pCPT-cGMP stimulation.

FIG. 2. PKG expression is required for 8-pCPT-cGMP-mediated control of RhoA expression. Stimulation of 3T3 fibroblasts with 8-pCPT-cGMP (50 μM, 24 h) had no effect on RhoA expression (left panel); however, an increase in RhoA expression in response to 8-pCPT-cGMP (50 μM, 24 h) was induced by transfection of PKG I (right panel). The induction of the response correlated with the expression of PKG detected by Western blotting. The RhoA protein level, normalized to β-actin expression, is expressed relative to the control in the absence of 8-pCPT-cGMP taken as 1. Data shown are the means of three independent experiments. **, p < 0.01 versus control; ##, p < 0.01 versus unstimulated PKG-transfected cells.

Animal Model of Chronic Inhibition of NO Synthesis—Four groups of male Wistar rats (250 g) were used. The control group received untreated drinking water, and the sildenafil-treated control group received sildenafil orally (25 mg/kg/day) for 15 days. The third group was given 0.5 glitri N-nitro-l-arginine (l-NNA) in their drinking water for 2 weeks (l-NNA-treated rats), and the fourth group received both l-NNA (0.5 glitri) and sildenafil (25 mg/kg/day) for 2 weeks. At completion of this time, the main pulmonary artery and aorta were removed and dissected under binocular control, and the adventitial and medial layers were removed. Tissues were then prepared as indicated for tension measurements, RNA extraction, or Western blot analysis. NO synthesis inhibition was assessed by measurement of plasma NO concentration.

NO3 Assay—Plasma NO3 concentration was determined by spectrophotometric analysis as described previously (23). Plasma samples were diluted 1:10 with phosphate buffer, 50 μl of NADPH stock solution (0.8 μg of NADPH/ml of phosphate buffer) and 10 μl of a nitrate reductase stock solution (5 units of nitrate reductase/ml of phosphate buffer) were added to 500 μl of diluted plasma. After incubation for 3 h at room temperature, Griess reagent was added and incubated for 10 min at room temperature. The absorbance of plasma samples was measured at 546 nm. A standard curve was prepared by addition of NaNO3 to phosphate buffer.

Tension Measurements in Intact Fibers—The aorta and pulmonary artery were collected in physiological saline solution (130 mM NaCl, 5.6 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 11 mM glucose, and 10 mM Tris, pH 7.4, with HCl), cleaned of fat and adherent connective tissue, and cut into rings. Smooth muscle rings were then suspended under isometric conditions and connected to a force transducer (Pioden Controls Ltd., Canterbury, UK) in organ baths filled with Krebs-Henseleit solution (118.4 mM NaCl, 4.7 mM KCl, 2 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, and 11 mM glucose), maintained at 37 °C, and equilibrated with 85% O2 and 5% CO2. The preparations were initially placed under a resting tension of 1500 mg, left to equilibrate for 1 h, and washed at 20-min intervals. Tension responses were induced by stimulation with KCl (60 mM) and the thromboxane A2 receptor agonist U46619 (1 μM). Rings were blotted dry and weighed (in milligrams) at the end of the experiments, and amplitude of the contraction
is expressed relative to the control (0 h) 

was expressed as milligrams/mg of tissue. Amplitude of the relaxation was expressed as percentage of the maximal amplitude of the contraction induced by phenylephrine recorded before carbachol or SNP application.

**Isometric Tension Measurement in Skinned Fibers**—Small muscle strips (~200 µm wide and 4 mm long) were isolated from the media of the pulmonary artery and tied at each end with a single silk thread to the tips of two needles, one of which was connected to a force transducer (AE 801, SensoNor, Horten, Norway). After measuring contraction evoked by high K+ solution, the strips were incubated in normal relaxing solution (85 mM KCl, 5 mM MgCl₂, 5 mM Na ATP, 5 mM creatine phosphate, 2 mM EGTA, and 20 mM Tris maleate, brought to pH 7.1 at 25 °C with KOH) for few minutes, followed by treatment with β-escin (50–70 µM) in relaxing solution for 35 min at 25 °C as previously described (24). The skinned muscle strip was then washed several times with fresh relaxing solution containing 10 mM EGTA. Calmodulin (1.5 mM) was added to the bathing solutions throughout the experiments. Tension developed by permeabilized muscle strips was measured in activating solutions containing 10 mM EGTA and a specified amount of CaCl₂ to give a desired concentration of free Ca²⁺ (24).

**Statistics**—All results are expressed as the means ± S.E. of sample size n. Significance was tested by Student’s t test. Probabilities <5% (p < 0.05) were considered significant.

**RESULTS**

**PKG Activation Increases RhoA Expression**—The effect of NO/cGMP/PKG signaling on the total levels of RhoA was examined by Western blot analysis. Stimulation of rat aortic smooth muscle cells with SNP (10 µM) for 0.1–24 h induced a time-dependent increase in RhoA expression (Fig. 1A). The rise in the amount of RhoA protein could be observed after 3 h of incubation with SNP, and there was a 3–3.5-fold increase in RhoA by 24 h. Similar results were obtained in human artery smooth muscle cells (Fig. 1A). The role of PKG activation in the increase in RhoA expression has been assessed using the cGMP analog 8-pCPT-cGMP and the PKG inhibitor (R₈p)-8-Br-PET-cGMP-S (Fig. 1B). Activation of PKG by 8-pCPT-cGMP (50 µM, 24 h) produced an increase in RhoA expression similar to that obtained in the presence of SNP (10 µM, 24 h). Under both conditions, the effect on RhoA expression was abolished in the presence of (R₈p)-8-Br-PET-cGMP-S (100 nM), whereas (R₈p)-8-pCPT-cAMP-S had no effect (data not shown), suggesting that cross-activation of cAMP-dependent protein kinase was not involved.

The involvement of PKG in cGMP-dependent regulation of RhoA expression was further examined in Swiss 3T3 cells transfected or not with an expression vector for PKG Iσ (Fig. 2). PKG Iσ overexpression induced a 2-fold rise in the basal level of RhoA expression. In addition, stimulation with the cGMP analog 8-pCPT-cGMP (50 µM) strongly increased RhoA expression in PKG Iσ-expressing cells and had no effect in untransfected cells (Fig. 2). The effect of 8-pCPT-cGMP was inhibited in the presence of (R₈p)-8-Br-PET-cGMP-S (100 nM) (data not shown). These data thus demonstrate the role of PKG in SNP/cGMP-mediated control of RhoA expression.

**PKG-mediated Increase in RhoA Expression Involves Stabilization of the RhoA Protein**—Regulation of protein stability has emerged as an important mechanism for controlling biological functions (25). To address the stability of RhoA in the context of PKG stimulation, we examined its fate in vascular smooth muscle cells in the presence and absence of SNP using cycloheximide (10 µg/ml) to abrogate new RhoA synthesis. At the times indicated, extracts of total protein were prepared and analyzed by RhoA immunoblotting. As shown in Fig. 3A, when protein neosynthesis was blocked by cycloheximide, RhoA expression gradually decreased from 0 to 10 h. In contrast, in the presence of SNP and cycloheximide, RhoA protein expression was maintained up to 6 h and then decreased and reached a
PKG activation increases RhoA mRNA expression. A, relative expression of RhoA mRNA assessed by real-time PCR. Stimulation of rat aortic smooth muscle cells with SNP (10 μM) or 8-pCPT-cGMP (50 μM) induced an increase in the amount of RhoA mRNA, which was inhibited by the PKG inhibitor (Rb)-8-Br-PET-cGMP-S (100 nM). Results, normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA expression, are expressed relative to the control taken as 1. Data shown are the means of four independent experiments. **, p < 0.01 versus control at the same time; ##, p < 0.01 versus 8-pCPT-cGMP stimulation. B, time-dependent effect of 5,6-dichlorobenzimidazole (DRB; 50 μM) alone (●) or in combination with SNP (10 μM; □) on steady-state RhoA mRNA levels after 0, 2, 4, and 10 h as analyzed by virtual Northern blotting. Band intensities were normalized to control levels at 0 h and plotted semilogarithmically as a function of time. The corresponding ethidium bromide-stained 28 S and 18 S band intensities were used to check loading conditions. Results displayed are representative of three separate experiments.

level similar to that observed in the absence of SNP at 10 h. This observation suggests that PKG-mediated up-regulation of RhoA involved change in the rate of RhoA protein degradation. The rate of small G protein degradation has been shown to be regulated by the phosphorylation state of the protein (26), leading to the hypothesis that PKG-mediated phosphorylation of RhoA at Ser188 (9) could slow down the degradation of RhoA. To assess this hypothesis, PKG-expressing Swiss 3T3 fibroblasts were transfected with RhoA or the phosphorylation-resistant RhoA mutant (RhoAAl188). RhoA expression was then assessed in cycloheximide-treated cells in the presence and absence of 8-pCPT-cGMP as described above (Fig. 3B). Stimulation with 8-pCPT-cGMP decreased RhoA degradation in RhoA-expressing cells, but not in RhoAAl188-expressing cells, suggesting that the PKG-mediated increase in RhoA stability depended on the phosphorylation of RhoA at Ser188.

RhoA mRNA Is Up-regulated by the NO/cGMP/PKG Pathway—To address the possibility of increases in RhoA mRNA levels in response to PKG stimulation, the abundance of RhoA mRNA was examined by quantitative reverse transcription-PCR in arterial smooth muscle cells incubated with or without SNP or 8-pCPT-cGMP for the indicated times. Both agents stimulated RhoA mRNA expression, with a maximal 2.5-fold increase after 9 h of incubation (Fig. 4A). This effect was inhibited in the presence of the PKG inhibitor (Rb)-8-Br-PET-cGMP-S. This result suggests that the observed modulation of RhoA protein expression induced by PKG stimulation also involved an increase in the amount of RhoA mRNA.

To determine whether the observed changes in RhoA mRNA levels were due to stimulation of gene transcription or involved post-transcriptional RhoA mRNA stabilization, the RhoA mRNA half-life was determined using the RNA polymerase inhibitor 5,6-dichlorobenzimidazole in the absence and presence of SNP. The half-life of RhoA mRNA was 3.4 ± 0.5 h under control conditions and 3.6 ± 0.4 h (n = 3; p > 0.05) in the presence of 10 μM SNP, indicating that there was no significant difference in post-transcriptional regulation of RhoA mRNA after treatment of aortic smooth muscle cells with SNP (Fig. 4B). Therefore, PKG-induced stimulation of RhoA mRNA expression results from PKG-mediated stimulation of rhoA gene transcription.

PKG Induction of RhoA mRNA Expression Does Not Require de Novo Protein Synthesis—PKG is known to directly control gene expression through phosphorylation of transcription factors such as members of the CREB/ATF family and/or indirectly by controlling expression of immediate-early genes such as c-fos, which are themselves transcription factors (19, 27). Therefore, to determine whether PKG-mediated increases in rhoA gene transcription required de novo protein synthesis, the effect of SNP on RhoA mRNA was analyzed by Northern blotting in the presence of cycloheximide. As shown in Fig. 5, cycloheximide alone slightly decreased RhoA transcript levels after 3 h, but did not affect SNP-induced rises in RhoA mRNA, suggesting that PKG-dependent stimulation of rhoA gene transcription does not require new protein synthesis.

rhoA Promoter Activity Is Stimulated by PKG Stimulation—To gain additional insight into the mechanism of regulation of rhoA gene transcription by PKG, functional analysis of the rhoA gene promoter was performed. A 913-bp fragment upstream of the ATG codon of the rhoA human gene sequence
was cloned into pGL2-Basic (pRhoA-Luc) as described under "Experimental Procedures." PKG-expressing Swiss 3T3 fibroblasts were transiently transfected with this pRhoA-Luc construct and stimulated with 8-pCPT-cGMP for 6 h. Stimulation of PKG induced a 2.5-fold increase in rhoA-driven luciferase activity. Stimulation of Swiss 3T3 fibroblasts that do not express PKG with SNP or 8-pCPT-cGMP had no effect on rhoA-driven luciferase activity (data not shown). Based on the partial sequence analysis of the rhoA promoter, diverse consensus sequences for the binding of transcription factors were identified, including CRE (one distal and one proximal), SF1 (steroidogenic factor-1), MEF2 (myocyte enhancer factor-2), and SRE (Fig. 6). To analyze the role of these sequences in the effect of PKG, diverse rhoA promoter constructs containing mutations or deletions were generated. As shown in Fig. 6, mutational ablation of the dCRE or the proximal SRE had a minimal effect on PKG stimulation, indicating that these elements do not play a significant role in PKG regulation of rhoA. In contrast, mutational ablation of the pCRE abolished the effect of PKG. Consistent with a major role of the pCRE, the 118-bp fragment containing the CRE and SRE was sufficient for activation of rhoA transcription by PKG. As expected, point mutation of the SRE did not modify this effect (data not shown), whereas it was abolished by mutational ablation of the pCRE (Fig. 6). Neither the nonsense (-913) rhoA promoter nor the pGL2-Basic construct responded to 8-pCPT-cGMP stimulation.

NO/cGMP-induced Phosphorylation of ATF-1 and Its Binding to the pCRE of the rhoA Gene Promoter—Phosphorylation of CREB/ATF-1 transcription factors is necessary for CREB/ATF-1-mediated transcriptional activation via binding to CRE (28). Using a phospho-CREB/ATF-1-specific antibody, we found that 8-pCPT-cGMP stimulation of PKG-expressing 3T3 fibroblasts or aortic smooth muscle cells induced phosphorylation of CREB and ATF-1, which was inhibited by (Rp)-8-Br-PET-cGMP-S (Fig. 7A). To determine whether CREB and/or ATF-1 binds to the CRE of the rhoA promoter, we next performed EMSA.

Nuclear proteins from 8-pCPT-cGMP-stimulated aortic smooth muscle cells were incubated with labeled oligonucleotide probes corresponding to the pCRE and dCRE of the rhoA promoter. The EMSA results shown in Fig. 7B demonstrate a significant increase in pCRE binding for nuclear extract from 8-pCPT-cGMP to the wild-type pCRE (wt pCRE), the mutant pCRE (m pCRE), or the wild-type dCRE (wt dCRE) of the rhoA promoter as described under "Experimental Procedures." Lane 1, labeled oligonucleotide; lane 2, labeled oligonucleotide + nuclear extract; lane 3, labeled oligonucleotide + nuclear extract + 200-fold molar excess of unlabeled oligonucleotide; lane 4, labeled oligonucleotide + nuclear extract + anti-CREB antibody; lane 5, labeled oligonucleotide + nuclear extract + anti-ATF-1 antibody.

The NO/cGMP Pathway Regulates RhoA Expression in Vivo—We next assessed the existence of NO-mediated regulation of RhoA in rat arteries in vivo. For this purpose, we used L-NNA-treated rat aortas and pulmonary arteries. Alteration of NO/PKG signaling was confirmed by a 53 ± 2% (n = 4) reduction of the NO⁻ concentration in the plasma of rats treated with L-NNA for 15 days compared with control rats. In addition, the NO/PKG-dependent relaxation of phenylephrine-induced contraction in response to muscarinic receptor activation was reduced by 77 ± 6% (n = 4) in aortas from L-NNA-treated rats compared with control rats, without modification of SNP-induced relaxation. Real-time PCR and Western blot analysis revealed decreases of 71 ± 1% (n = 4; p < 0.01) and 51 ± 2% (n = 6; p < 0.01) for RhoA mRNA and for RhoA expression in aortas from L-NNA-treated rats, respectively (Fig. 8, A and B). A similar down-regulation of RhoA mRNA (65 ± 7%, n = 4; p <
Fig. 8. Effect of chronic inhibition of NO synthesis on RhoA expression and RhoA-dependent function in vivo. RhoA mRNA (A) and protein (B) expression was examined in aortas and pulmonary arteries of control and L-NNA-treated rats (15 days) and analyzed by real-time PCR (A) and Western blotting (B). L-NNA treatment induced a decrease in RhoA mRNA and protein expression, which was prevented by the treatment of rats with sildenafil (25 mg/kg/day) for 15 days. RhoA mRNA expression was normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA (A), and RhoA protein expression was normalized to β-actin (B). Results are expressed relative to control rats taken as 1. Data shown are the means of four independent experiments. **, p < 0.01 versus control rats. pCa-tension relationships were measured under control conditions (●) and in the presence of 10 μM GTPγS without (▲) and with (●) 10 μM Y-27632 in β-escin-permeabilized pulmonary artery smooth muscle cells from control rats (C), L-NNA-treated rats (D), and L-NNA-treated rats receiving sildenafil (E). Data shown are the means of four independent experiments.

Ca2⁺ and RhoA protein expression (56 ± 7%, n = 6; p < 0.01) also occurred in pulmonary arteries. The involvement of cGMP-dependent signaling in the down-regulation of RhoA detected in arteries from L-NNA-treated rats was assessed using the phosphodiesterase-5 inhibitor sildenafil (29). Administration of sildenafil (25 mg/kg/day) for the entire duration of L-NNA treatment completely prevented the down-regulation of both RhoA mRNA and protein expression in aortas and pulmonary arteries despite a reduction of the plasma NO concentration similar to that measured in rats treated with L-NNA alone (57 ± 4%, n = 4; p > 0.5) (Fig. 8A and B).

To establish whether these changes in RhoA expression correlate with alteration of RhoA-dependent functions, we next analyzed contractile responses induced by KCl and U46619 in pulmonary arteries from control and L-NNA-treated rats. The maximal amplitude of KCl-induced contraction was not significantly altered in L-NNA-treated arteries (550 ± 41 mg/mg versus 611 ± 32 mg/mg in controls, n = 4; p > 0.5), suggesting that Ca²⁺-dependent contraction is not modified by chronic inhibition of NO production. On the contrary, responses to U46619 were significantly decreased in L-NNA-treated arteries (87 ± 11 mg/mg versus 808 ± 31 mg/mg in controls, n = 4; p < 0.001). Contraction mediated by U46619 is essentially due to the activation of RhoA/Rho kinase-mediated Ca²⁺ sensitization (3). These results thus suggest an alteration of RhoA/Rho kinase-mediated Ca²⁺ sensitization in L-NNA-treated rats, which has been directly assessed by tension measurements in permeabilized smooth muscle.

Ca²⁺-dependent contractions and Ca²⁺ sensitization of contractile proteins could be independently evoked in β-escin-permeabilized smooth muscle strips. Ca²⁺-dependent contractions were induced by gradual increases in Ca²⁺ concentrations (submaximal pCa (−log[Ca²⁺]) = 8) to maximal pCa (4.5), and Ca²⁺ sensitization was evoked by addition of 10 μM GTPγS. The Ca²⁺ sensitization appeared as a leftward shift of the pCa-tension relationship. In β-escin-permeabilized pulmonary artery strips from control rats, GTPγS induced an increase in the Ca²⁺ sensitization of contractile proteins, illustrated by an increase in the pC₅₀ from 6.05 ± 0.01 (n = 4) to 6.67 ± 0.02 (n = 4; p < 0.01) (Fig. 8C). This shift in the pC₅₀-tension relationship was completely abolished in the presence of the Rho kinase inhibitor Y-27632 (10 μM), indicating that the GTPγS-induced Ca²⁺ sensitization in the pulmonary artery was mediated by the RhoA/Rho kinase pathway (Fig. 8C). The pC₅₀-tension relationship in permeabilized pulmonary artery strips from L-NNA-treated rats was similar to that in controls (pC₅₀ = 6.03 ± 0.03, n = 4; p > 0.5), but the GTPγS-induced Ca²⁺ sensitization was completely lost (pC₅₀ = 6.10 ± 0.03, n = 4; p > 0.5), and Y-27632 had no effect (Fig. 8D). The GTPγS-induced Ca²⁺ sensitization was restored in sildenafil-treated L-NNA rats (pC₅₀ = 6.69 ± 0.02 in the presence of GTPγS versus 6.07 ± 0.03 in its absence, n = 4; p < 0.01), and the effect of GTPγS was blocked by Y-27632 (pC₅₀ = 6.10 ± 0.03, n = 4) (Fig. 8E). These results suggest that chronic inhibition of NO synthesis-induced loss of RhoA expression is associated with impairment of the RhoA/Rho kinase signaling pathway. Decreases in both RhoA expression and RhoA/Rho kinase-dependent functions are prevented by sildenafil.

DISCUSSION

The data presented in this work show that the NO/cGMP/PKG pathway positively controls RhoA expression in vascular smooth muscle cells both in vitro and in vivo. The small G protein RhoA is clearly identified as a convergence point for multiple signals that regulate cell functions. However, little is known about the regulation of RhoA protein expression and rhōA gene transcription. Here we show that the basal release of NO is absolutely necessary to maintain RhoA expression and RhoA-dependent contractile functions in vascular smooth muscle. Our results provide the first evidence that the control of RhoA expression by physiological signaling pathways represents a new mechanism that regulates the ability of cells to respond to chemical or physical stimuli.

Nitric oxide is a unique lipophilic, diffusible, short-lived messenger that modulates a variety of functions, including growth, differentiation, and apoptosis in many different cell types. A large number of NO effects are mediated through changes in gene expression essentially through modulation of gene transcription. NO can directly control the activity of transcription factors such as NF-xB, AP-1, and c-Jun by S-nitrosylation (30–
However, a large number of NO effects are mediated through the activation of PKG (19). Several sequence elements have been proposed to mediate the transcriptional effect of PKG, notably the SRE, AP-1-binding site, and CRE (27, 33–35).

In this study, we have shown that the NO donor SNP or the cGMP analog 8-pCPT-cGMP positively controls RhoA expression and that this process is strictly dependent on PKG activity. Part of this effect was mediated by an increase in rhoA gene transcription that did not require de novo protein synthesis. The rhoA reporter gene containing 913 bp 5′ of the transcription start site responded to PKG stimulation in PKG-expressing cells, but not in untransfected cells, thus confirming the role of PKG. Mutation of the pCRE in the rhoA promoter or in the short promoter construct (−118) abolished the effect of PKG, showing that the pCRE is the response element that mediates the PKG regulation of rhoA transcription. Both CREB and ATF-1 were phosphorylated by PKG in smooth muscle cells. Furthermore, EMSA experiments strongly suggest that ATF-1/ATF-1 homodimer binding to the pCRE is responsible for the PKG-mediated regulation of rhoA transcription.

The cycloheximide experiments (Fig. 3) reveal that activation of the NO/PKG pathway increased the stability of the RhoA protein. Although this observation could be due to a PKG-dependent mechanism that regulates protein degradation, transfection experiments strongly suggest that this stabilization is related to PKG-mediated phosphorylation of RhoA at Ser188. We have previously shown that PKG phosphorylates RhoA at Ser188, causing its translocation from membranes to the cytosol (9). Such regulation of the cellular location of small G proteins through phosphorylation of serine residues in the C-terminal domain seems to be shared by several different subtypes of small G proteins. cAMP-dependent protein kinase has been shown to phosphorylate Rap1 at Ser180 Rap1B at Ser179, and RhoA at Ser188, causing their translocation in the cytosol (36–38). Our present results suggest that, in addition to this effect, phosphorylation of RhoA at Ser188 by PKG increases the stability of the protein. There are only very limited published data on the regulation of stability or production/degradation of the Rho proteins. The phosphorylated form of Ras has been shown to be more stable in cells (26), and carboxyl methylation of RhoA and Cdc42 has been reported to increase their half-lives (39). Our results suggest that phosphorylation of Rho proteins in the C-terminal domain could also protect these proteins from degradation.

The question is then to understand whether the transcriptional/post-translational regulation of RhoA by PKG occurs under physiological basal conditions. This modulation of RhoA through the NO/PKG pathway is of particular interest in vascular smooth muscle cells that are normally submitted to a low and continuous basal release of NO from endothelial cells. The decrease in RhoA mRNA and protein expression associated with the decrease in NO production in arteries from L-NNA-treated rats suggests that RhoA expression in vascular smooth muscle is indeed controlled by the continuous NO release from endothelial cells in vivo. This is further supported by the observation that elevation of cGMP levels by sildenafil treatment completely prevented the down-regulation of RhoA induced by L-NNA treatment. Although mechanisms regulating RhoA expression in smooth muscle cells have never been studied, variations of RhoA levels have already been observed in several systems. In myometrial smooth muscle cells, up-regulation of RhoA expression at the end of pregnancy is involved in the mechanisms underlying the enhanced uterine contractility at term (40). In bronchial smooth muscle cells, the RhoA protein level is increased in airway hyper-responsive rats (41), and a rise in RhoA expression has been detected in aortic smooth muscle from spontaneous hypertensive rats (15). Modulation of RhoA expression thus appears to be a regulatory mechanism that controls the capability of cells to respond to external stimuli, and our results suggest that the basal activity of NO/PKG exerts a tonic regulation of RhoA expression in vascular smooth muscle cells. Indeed, functional analysis clearly shows that the decrease in RhoA expression induced by the chronic inhibition of NO synthesis is associated with a reduction of RhoA/Rho kinase-dependent Ca2+ sensitization, indicating that the level of RhoA expression is a limiting factor of RhoA-dependent functions. The NO-dependent regulation of RhoA expression therefore appears to be a crucial component of the determinant action of NO in the structure and function of the vessel wall under normal conditions (42).

In summary, the data presented herein indicate that RhoA expression is regulated by NO through PKG-dependent mechanisms. Therefore, NO-mediated regulation of the RhoA signaling pathway is more complex than one would expect, and NO could not be solely considered as a negative regulator of RhoA activation. Given the importance of both RhoA and NO/PKG signaling pathways in the regulation of vascular smooth muscle cell functions, further studies are now required to identify the functional roles of the NO-mediated regulation of RhoA expression in normal vessels and in vascular diseases.

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RhoA Expression Is Controlled by Nitric Oxide through cGMP-dependent Protein Kinase Activation

Vincent Sauzeau, Malvyne Rolli-Derkinderen, Céline Marionneau, Gervaise Loirand and Pierre Pacaud

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