Protein Kinase G Phosphorylates Soluble Guanylyl Cyclase on Serine 64 and Inhibits Its Activity

Zongmin Zhou, Nazish Sayed, Anastasia Pyriochou, Charis Roussos, David Fulton, Annie Beuve, Andreas Papapetropoulos

Objective—Binding of nitric oxide (NO) to soluble guanylyl cyclase (sGC) leads to increased cGMP synthesis that activates cGMP-dependent protein kinase (PKG). Herein, we tested whether sGC activity is regulated by PKG.

Methods and Results—Overexpression of a constitutively active form of PKG (ΔPKG) stimulated 32P incorporation into the α1 subunit. Serine to alanine mutation of putatively active sites revealed that Ser64 is the main phosphorylation site for PKG. Using a phospho-specific antibody we observed that endogenous sGC phosphorylation on Ser 64 increases in cells and tissues exposed to NO, in a PKG-inhibitable manner. Wild-type (wt) sGC coexpressed with ΔPKG exhibited lower basal and NO-stimulated cGMP accumulation, whereas the S64A α1/β1 sGC was resistant to the PKG-induced reduction in activity. Using purified sGC we observed that the S64D α1 phosphomimetic /β1 dimer exhibited lower Vmax; moreover, the decrease in Km after NO stimulation was less pronounced in S64D α1/β1 compared to wild-type sGC. Expression of a phosphorylation-deficient sGC showed enhanced responsiveness to endothelium-derived NO, reduced desensitization to acute NO exposure, and allowed for greater VASP phosphorylation.

Conclusions—We conclude that PKG phosphorylates sGC on Ser64 of the α1 subunit and that phosphorylation inhibits sGC activity, establishing a negative feedback loop. (Arterioscler Thromb Vasc Biol. 2008;28:1803-1810)

Key Words: cGMP • soluble guanylyl cyclase • PKG • nitric oxide • phosphorylation

Nitric oxide (NO)-sensitive guanylyl cyclase (sGC) is a ubiquitously expressed heterodimer composed of an α and β subunit; the latter accommodates a heme prosthetic group that is capable of binding and responding to NO.1,2 Heme nitrosylation leads to conformational changes that are transmitted to the C-terminal catalytic domain, thereby stimulating the formation of cGMP; this in turn activates several intracellular pathways that include cGMP-dependent protein kinase (PKG), phosphodiesterases, and ion channels.1,3,4 From these downstream effectors of cGMP, PKG is the best characterized target and is responsible for the majority of cGMP-mediated effects including smooth muscle relaxation and inhibition of platelet aggregation.4 Several mechanisms that contribute to PKG-mediated vasorelaxation have been proposed, including decreases in intracellular free calcium concentrations, calcium desensitization, and thin filament regulation.5

The most abundant form of sGC is the α1/β1.2 Apart from its regulation at the transcriptional and posttranscriptional level, α1/β1 activity is dynamically regulated posttranslationally through protein–protein interactions and possibly through phosphorylation.2 However, the only definitively proven site for sGC phosphorylation is tyr-192 of the β1 subunit.6 Interestingly, tyr-192 phosphorylation does not affect sGC activity, but rather creates a docking site for SH2 domains recruiting the tyrosine kinases src and fyn.6 Studies with purified proteins in vitro had indicated that sGC is a substrate for protein kinase C and protein kinase A and that phosphorylation by either kinase increases enzyme activity.7,8 Protein kinase A was later shown to enhance NO-stimulated cGMP accumulation through phosphorylation of the α1 subunit in pituitary cells.9 More recently, using pharmacological activators for protein kinase G or phosphatase inhibitors it was proposed that sGC can be phosphorylated under certain conditions in living cells.10–12 Murthy demonstrated that exposure of gastric smooth muscle to sodium nitroprusside stimulates PKG-sensitive 32P incorporation into sGC and that this modification correlates with reduced activity of immunoprecipitated sGC.11,12 On the other hand, Ferrero et al have proposed that PKG activation inhibits sGC attributable to Ser-dephosphorylation of the β1 subunit.13 However, none of these reports have identified the exact site on the sGC subunit that becomes phosphorylated by PKG, nor have the effects of this modification on sGC activity been characterized in detail. Herein, we set out to identify the specific residue that is phosphorylated by PKG and to study the

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effect of this phosphorylation event on enzyme activity, as well as its biological significance.

**Methods**

**In Vitro sGC Phosphorylation**

Plasmid constructs encoding a chimeric protein consisting of glutathione S-transferase (GST) fused to the N terminus of sGC α1, β1 or fragments thereof were created by subcloning the rat cDNA into pGEX-Kg. GST-fusion proteins were expressed in *E. coli* and purified using glutathione-conjugated agarose beads. In vitro kinase assays were performed at room temperature for 30 minutes in a volume of 50 μL containing 10 mmol/L Tris-HCl (pH 7.4), 2 mmol/L MgCl₂, 5 μmol/L cGMP, 0.1 mmol/L ATP, 5 μCi [γ-³²P]-ATP, and 5 μg of GST-fusion protein. To initiate the reaction, 1000 U of bovine lung protein kinase G Iα were added. Incorporation of [³²P] into the sGC chimeric proteins was analyzed by SDS-PAGE followed by autoradiography.

**Transfection of COS Cells and Adenoviral Infection**

African green monkey kidney cells COSm6 or A7r5 rat smooth muscle cells were cultured in DMEM supplemented with 10% FCS. For the transfection experiments, COSm6 were plated in 6-well plates at a density of 2×10⁶ cells per well, grown overnight, and transfected with appropriate plasmids using a total of 3 μg DNA and 6 μL of jetPEI transfection reagent per well. For cotransfection experiments, equal amounts of DNA were used for each plasmid. Alternatively, A7r5 cells were transfected with 5 MOI c, the α1 sGC subunit or 10 MOI of green fluorescent protein (GFP) as control. After 24 to 48 hours cells were used for cGMP determinations or for the preparation of cell lysates.

**Immunoprecipitation and Western Blotting**

To precipitate sGC, lysates containing 200 to 250 μg of protein were incubated with antimyc antibody conjugated agarose beads overnight at 4°C (throughout the study a N-terminally myc-tagged version of sGC α1 was used). The beads were then washed 5 times with lysis buffer, and immunoiprecipitated proteins were subjected to SDS-PAGE. The membranes were then blocked and incubated with the primary and secondary antibodies. The phospho-specific sGC Ab was developed by Pacific Immunology, Inc (Ramona, CA, USA) against the following sequence SHPQRKTS*RNRVYLH. Immunoreactive proteins were detected using the SuperSignal chemiluminescence kit.

**Metabolic Labeling With [³²P]**

COSm6 cells were cotransfected with sGC subunits and a constitutively active PKG form lacking the first 64 amino acids (ΔPKG). Thirty hours after the transfection, cells were switched to phosphate-free DMEM medium with 10% FCS for 12 hours. [³²P] was then added into the phosphate-free medium (600 μCi/mL) and cultures incubated for an additional 6 hours. Cells were washed once with PBS and scraped in ice-cold lysis buffer. Cellular debris were pelleted at 12 000g for 15 minutes at 4°C and the supernatants were subjected to immunoprecipitation with antimyc conjugated agarose beads. After SDS-PAGE samples were transferred to polyvinylidene fluoride (PVDF) membranes and subjected to autoradiography. The same membranes were later subjected to Western blotting analysis.

**cGMP and GC Activity Assays**

Twenty-four or 48 hours after transfection or infection the cells were incubated in the presence 1 mmol/L of the phosphodiesterase inhibitor IBMX for 15 minutes with or without an NO donor, as indicated. Media were aspirated and HCl was added to extract cGMP. After 30 minutes, HCl extracts were collected and cGMP was quantified using cGMP enzyme immunoassay kit. GC activity was determined as previously described. Km and Vmax were determined with 6 different substrate concentrations ranging from 0.1 to 500 μmol/L, in the absence of activator and in the presence of 100 μmol/L SNAP. Free magnesium was kept constant at 4 mmol/L. All assays were performed in duplicate, and each experiment was repeated 3 times.

**Data Analysis and Statistics**

Results are presented as means±SEM of the indicated number (n) of observations. Statistical comparisons between groups were made using the 1-way ANOVA followed by a posthoc test (Newman-Keuls) or Student *t* test, as appropriate. An F-test that compares the NO concentration-response was used to determine the significance of difference between the EC₅₀ obtained with wt and mutants. Statistical differences were considered significant when *P*<0.05.

**Results**

**sGC Is a PKG Substrate**

To determine whether sGC is a direct PKG substrate, GST-fusion proteins of the α1 and β1 subunits were used in vitro kinase assays. Incubation of full length GST-α1, but not GST-β1, with catalytically active PKG led to labeling with [³²P] (Figure 1A and 1B). To determine the phosphorylation site(s) we generated an N-terminal fragment containing the regulatory and part of the dimerization domain and a C-terminal fragment that contained the remaining portion of the dimerization domain and the catalytic domain. From the
two fragments, only the α1 1 to 360 incorporated \(^{32}P\) after incubation with PKG in vitro (Figure 1A and 1B).

**PKG Phosphorylates sGC on Ser64**

To test whether PKG can phosphorylate sGC in intact cells, COSm6 were cotransfected with the sGC α1/β1 heterodimer and a constitutively active mutant of PKG (ΔPKG; Figure 1C). Immunoprecipitation of sGC followed by SDS-PAGE revealed that \(^{32}P\) is incorporated in the α1 subunit, suggesting that in vivo, too, sGC is phosphorylated by PKG. In silico analysis of the α1 primary structure revealed that Ser64, Thr210, and Ser360 conform to a PKG phosphorylation motif. Among these putative phosphorylation sites, Ser64 had the highest probability of being phosphorylated according to the algorithm used by NetPhos 2.0 and was present in all mammalian α1 subunits cloned so far; it was, thus, tested first as a PKG site. Wild-type or S64A α1 was coexpressed with β1 in the presence or absence of a constitutively active PKG mutant (ΔPKG) in COSm6 and cells were labeled with \(^{32}P\) (Figure 1C). Autoradiograms demonstrated that mutation of Ser64 to the nonphosphorylatable analogue alanine abolishes phosphorylation (supplemental Figure I). These differences could not be attributed to differences in expression of sGC, as α1 and β1 levels were equal in the presence and absence of ΔPKG for both wt and mutant sGC (Figure 1C). The effect of sGC phosphorylation on cGMP accumulation was also evaluated using a phosphomimetic mutant, in which Ser64 is mutated to aspartate (S64D); this accumulation was also evaluated using a phospho-specific sGC Ab. C, Rat aortas were treated with 100 μmol/L SNP for the indicated time and analyzed for the presence of phosphorylated sGC. D, Rat aortas were treated with bradykinin (BK; 2 μmol/L, 15 minutes) in the presence or absence of L-nitroarginine methyl ester (100 μmol/L; L-NAME), and sGC phosphorylation determined using the phospho-specific Ab. The blots shown are representative of experiments repeated 2 to 3 times with similar results.

**Effect of sGC Phosphorylation on cGMP Accumulation and sGC Activity**

To test the effect of phosphorylation on cGMP accumulation, COSm6 cells were cotransfected with wt or S64A sGC and ΔPKG. Basal cGMP levels were similar in cells transfected with wt and nonphosphorylatable sGC (Figure 3A). Stimulation of cells with SNP led to a more than 25-fold increase in cGMP in all groups; however, SNP-induced cGMP accumulation was inhibited in cells expressing ΔPKG, suggesting that when sGC is phosphorylated by PKG it exhibits reduced activity. This inhibition was greater in magnitude at low physiologically relevant NO concentrations. On the other hand, there was no reduction in the ability of S64A sGC to produce cGMP, irrespectively of the coexpression of PKG. These differences could not be attributed to differences in expression of sGC, as α1 and β1 levels were equal in the presence and absence of ΔPKG for both wt and mutant sGC (Figure 3B). The effect of sGC phosphorylation on cGMP accumulation was also evaluated using a phosphomimetic mutant, in which Ser64 is mutated to aspartate (S64D); negatively charged aspartate residues in some cases substitute functionally for phosphorylated residues, mimicking the action of covalently bound phosphate groups. In spite of similar expression levels (Figure 3D), S64D sGC produced lower...
levels of cGMP both under basal and NO-stimulated conditions (Figure 3C). The observed changes cannot be attributed to alterations in heterodimer formation (unpublished data, Zhou and Papapetropoulos, 2007). Similar results to the ones obtained with the COSm6, where sGC phosphorylation resulted in reduced cGMP accumulation, were also observed in A7r5 (supplemental Figure III). A7r5 is a smooth muscle cell line that endogenously expresses PKG-I (supplemental Figure I), but lacks sGC; cells infected with S64A and S64D mutants accumulated greater amounts of cGMP when stimulated with $\mu$mol/L amounts of SNP. Reduced responsiveness to SNP was observed as early as 1 minute and lasted for at least 15 minutes (supplemental Figure IV).

To determine whether phosphorylation of sGC directly results in decreased sGC activity, sGC was first phosphorylated by preincubation with PKG-I then its activity measured under basal and SNAP(1$\mu$mol/L)-stimulated conditions. Phosphorylation of sGC led to a significant inhibition (883.3$\pm$184.3 nmol cGMP/min/mg protein) of NO-stimulated sGC activity, as compared to the same reaction carried out in the presence of heat-inactivated PKG (1268.2$\pm$158.5 nmol cGMP/min/mg protein; n=4; P<0.05). There was also a significant, yet less pronounced, decrease in the basal sGC activity after incubation with PKG (159.4$\pm$12.6 versus 193.0$\pm$13.3 nmol cGMP/min/mg protein; n=4; P<0.05). To further study the effect of sGC phosphorylation on the kinetic properties of sGC, wt, S64A, and S64D mutants were coexpressed with in Sf21 insect cells and purified. This experimental set-up was preferred to the sGC/PKG as there was no need to add ATP to the reaction mixture, an agent that inhibits sGC activity.15 Recombinant sGC activity was indistinguishable between the S64A and wt sGC in physiologically relevant concentrations of the NO donor SNAP (up to 1 $\mu$mol/L); in contrast, S64D sGC expressed lower activity throughout the concentration range tested (Figure 4A); moreover, the EC50 for SNAP was greater for the “constitutively phosphorylated” sGC compared to wt (3.64$\pm$0.07 versus 3.02$\pm$0.11 $\mu$mol/L). Under basal conditions wt and S64D phosphomimetic mutant demonstrated similar affinity for the Mg$^{2+}$-GTP substrate, but exhibited a markedly reduced maximal velocity (Figure 4B). Stimulation of wt $\alpha1/\beta1$ with NO (100 nmol/L S-nitroso N-acetyl...
Penicillamine, SNAP) resulted in a drop in Km that was less pronounced for the S64D mutant (Figure 4C). Moreover, although NO stimulation increased Vmax in all 3 forms of sGC (wt and mutants), the S64D exhibited approximately half the Vmax value of the wt enzyme (Table; supplementary Figure VI).

Phosphorylation of Ser64 Desensitizes sGC and Dampens NO Signaling

Exposure of cells or tissues to SNP or other nitrovasodilators leads to the rapid desensitization of sGC and impaired cGMP formation. To study the contribution of Ser64 sGC phosphorylation to the desensitization of sGC in response to SNP, we infected A7r5 with wt or the nonphosphorylatable form of sGC and determined their ability to form cGMP with or without SNP pretreatment (supplemental Figure V). Cells expressing the phosphorylation-deficient form of sGC and pretreated with SNP accumulated increased amounts of cGMP after the second SNP challenge as compared to cells expressing wt sGC, demonstrating resistance of the S64A sGC to the desensitizing action of SNP.

To determine the biological relevance of the reduction in sGC activity observed after Ser64 phosphorylation of α1, we used an endothelium–smooth muscle coculture system. Use of primary smooth muscle cells was not an option, as cells removed from the vasculature and cultured rapidly loose PKG expression. A7r5 smooth muscle cells were infected with either wt α1/β1 or S64A α1/β1 sGC. After 24 hours, bovine pulmonary artery endothelial cells (BPAECs) were seeded onto the A7r5 and incubated for an additional day. cGMP content in response to endothelial-derived NO was then measured in the presence of the phosphodiesterase inhibitor IBMX (Figure 5A). It should be noted that (1) inhibition of endothelial NO synthase abolishes the increase in cGMP brought about by the ECs and (2) BPAEC are devoid of sGC (16 and unpublished data, Zhou and Papapetrou).

**Table.** Kinetic Parameters of Recombinant Rat sGC wt and Mutants

<table>
<thead>
<tr>
<th></th>
<th>Basal (nmol/min/mg protein)</th>
<th>SNAP (100 nmol/L) (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km</td>
<td>Vmax</td>
</tr>
<tr>
<td>wt α1/β1</td>
<td>118.7±16.4</td>
<td>351.6±14.7</td>
</tr>
<tr>
<td>S64A α1/β1</td>
<td>116.1±16.2</td>
<td>328.0±39.5</td>
</tr>
<tr>
<td>S64D α1/β1</td>
<td>107.7±3.2</td>
<td>118.3±9.0*</td>
</tr>
</tbody>
</table>

The apparent Km and Vmax values were determined by double reciprocal plots analysis, in the absence or presence of 100 nmol/L SNAP (see Figure 4B and 4C). Values are mean±SEM of 3 experiments, with each measurement done in duplicate. Free MgCl₂ was kept constant at 4 mmol/L. *P<0.05 from wt.
Results from these coculture experiments revealed that while wt and S64 sGC were equally expressed in the respective cultures (Figure 5B), cGMP content of the latter was higher, suggesting that endothelium-derived NO activates the nonphosphorylatable form of sGC to a greater extent, compared to the wt sGC. Moreover, inhibition of PKG enhanced the responsiveness of wt sGC to endothelium-derived NO (Figure 5C).

To further study the biological significance of sGC phosphorylation on Ser64 we monitored vasodilator stimulated phosphoprotein (VASP) phosphorylation on Ser-239, a site preferentially phosphorylated by PKG. Expression of sGC increased the phosphorylation of VASP (Figure 5D and 5E). In addition, the amount of phosphorylated VASP after stimulation with an NO donor was increased in cells expressing S64A α1/β1 as compared to wt sGC, reflecting the lack of sGC inhibition in the S64A phosphorylation deficient mutant (Figure 5E). It should be noted that no change in the α1, β1, or PKG ια levels was observed between groups (Figure 5D).

On the other hand, direct stimulation of PKG with 10 μmol/L 8pCPT-cGMP resulted in equal levels of VASP phosphorylation in cells expressing the S64A and the wt sGC (unpublished data, Zhou and Papapetropoulos, 2007). The above data taken collectively reinforce the notion that the phosphorylated, wt sGC forms reduced amounts of cGMP, leading to attenuated downstream signaling responses.

Discussion

Many signaling pathways possess negative or positive regulatory feedback loops which allow them to adapt to changes in the intensity or frequency of activation. Components of the NO/cGMP pathway have been shown to be regulated by cGMP-dependent protein kinase G. The endothelial isoform of NO synthase is phosphorylated on ser-1177 by PKG rendering the enzyme more active at lower Ca2+ concentrations acting in a feed-forward fashion.17 In contrast, PKG phosphorylates the cGMP-specific phosphodiesterase 5, increasing its activity and leading to diminished activation of the NO/cGMP pathway.11,18 To determine whether sGC, the NO receptor, is also phosphorylated by PKG, GST-fusion proteins of the α1 or the β1 subunit were used as substrates for in vitro kinase reactions. These experiments revealed that the N-terminal half of the α1 is indeed phosphorylated by PKG. Our observations were confirmed in vivo, as PKG was found to interact with the sGC heterodimer and promote 32P incorporation in the large sGC subunit. These observations are in line with those of Murthy who demonstrated that precipitated sGC can be phosphorylated by PKG in vitro and that exposure of cells to increasing amounts of NO lead to concentration-dependent 32P incorporation in sGC.12

In silico analysis demonstrated the existence of 3 putative phosphorylation sites within the first 360 residues of α1. We focused on Ser64 as it is conserved in all mammalian species variants of the α1 subunit cloned so far. Site directed mutagenesis experiments showed that substitution of Ser with Ala abolished 32P in vivo labeling of sGC in cells expressing a constitutively active PKG. Further experiments with endogenously expressed sGC revealed that α1 can be phosphorylated on Ser64 in vivo. Exposure of rat tissues to an NO donor led to a time-dependent appearance of phospho-sGC. Moreover, stimulation of endothelium-intact aortas with bradykinin enhanced sGC phosphorylation in a NOS inhibitor–sensitive manner, suggesting that endothelium-derived NO is capable of promoting phosphorylation of sGC on Ser64. Proof that PKG mediates the NO-stimulated phosphorylation on α1 Ser64 was offered from experiments in which PKG inhibition blocked the appearance of phosphorylated sGC.

To determine the effect of Ser64 phosphorylation on sGC activity in a cellular context we used 2 heterologous expression systems, transfecting wt sGC or the phosphorylation-deficient sGC mutant in COS or A7r5 cells. COS cells expressing wt sGC exhibited reduced basal and SNP-stimulated cGMP accumulation in the presence of a constitutively active PKG. In contrast, coexpression of PKG with the S64A nonphosphorylatable form of sGC did not result in reduced cGMP synthesis. In A7r5, S64A sGC responded by accumulating higher levels of cGMP compared to wt sGC. Moreover, the constitutively “phosphorylated” S64D mutant behaved similarly to wt sGC expressed in presence of PKG, generating lower amounts of cGMP. Soluble GC subunits consist of 3 domains: an N-terminal regulatory domain, a middle dimerization domain, and a C-terminal catalytic domain.19 As Ser64 lies within a region that has been proposed to be critical for heterodimerization with the β1,20 we tested the possibility that the S64D mutant exhibited altered dimerization properties. Data from immunoprecipitation experiments suggested that both mutants bind the β1 subunit with comparable avidity to the wt α1. In line with our data from cGMP accumulation experiments, catalytic activity of wt sGC incubated with PKG or purified S64D sGC was lower compared to the wt enzyme. These observations taken together suggest that activation of the NO/cGMP axis is subject to negative feedback regulation, with PKG, a downstream NO effector, inhibiting the activity of the NO receptor through covalent modification of Ser64. In line with our findings, blood vessels exhibit an increased sensitivity to exogenous NO and enhanced sGC activity after exposure to NO donors after inhibition of endogenous NO production, endothelial denudation, or disruption of the eNOS gene locus.21,22

To determine the impact of phosphorylation on the kinetic properties of sGC, we expressed wt α1/β1 or a constitutively “phosphorylated” mutant of sGC in which Ser64 was mutated to aspartate in insect cells. In line with previous reports, exposure of sGC to the NO donor SNAP lead to a drop in Km consistent with a rise in the substrate binding affinity, as well as an increase in Vmax. Mutating Ser64 to Ala did not significantly alter the above mentioned parameters for sGC. In contrast, exposure of the S64D α1/β1 mutant to NO led to a moderate drop in Km and a more conservative increase in Vmax as compared to the wt enzyme, in line with the lower cGMP forming ability observed by this phosphomimetic mutant after NO stimulation in COSm6 overexpression experiments.

Acute exposure of smooth muscle cells to NO donors reduces their responsiveness to subsequent challenges with NO.23 To investigate the relevance of Ser64 phosphorylation to these events, A7r5 were infected with wt or S64A sGC.
Pretreatment of wt sGC-expressing cells to SNP for 1 hour led to desensitization as evidenced by the decline in their responsiveness to a second exposure to SNP. On the other hand, S64A sGC-expressing cells were more resistant to desensitization, suggesting that phosphorylation of Ser64 contributes to the development of tachyphylaxis to nitrovasodilators. In agreement with the notion that phosphorylation of sGC decreases its sensitivity to NO, we observed that the EC<sub>50</sub> for SNAP was greater in the purified phosphomimetic form of sGC compared to wt. Additional mechanisms contributing to desensitization to the action of NO at the level of sGC have been described and include changes in the redox status of the enzyme and S-nitrosylation.13 Our observation for reduced sGC sensitivity to NO after Ser64 phosphorylation is in agreement with the finding that vessels from eNOS<sup>−/−</sup> mice that lack endogenous NO production in the vasculature exhibit higher sGC activity in response to exogenously administered NO compared to those of wt mice, in the absence of significant differences in sGC subunit expression.22 Our findings that PKG induces phosphorylation and inhibits sGC activity might also explain the cross tolerance between NO donors and natriuretic peptides observed under certain conditions and the reciprocal regulation of sGC and pGC.10,26

To determine the biological significance of sGC phosphorylation, the responsiveness of wt or S64A sGC to endothelium-derived NO was determined in smooth muscle–endothelium cocultures. In agreement with what was observed with the low concentrations of NO donors, cocultures of smooth muscle cells expressing S64A sGC generated more cGMP than those expressing wt sGC, suggesting that phospho-sGC is less sensitive to endogenously produced NO. It should be noted that inhibition of PKG in cocultures of smooth muscle expressing wt sGC resulted in an enhanced response to NO produced by the endothelium, similarly to what has been described to occur after NOS inhibition.21 To study the impact of Ser64 phosphorylation on downstream signal transduction events we monitored changes in VASP phosphorylation. VASP, a PKG substrate, is associated with the actin cytoskeleton, regulating cell spreading and movement.27,28 Several phosphorylation sites exist on VASP; Ser-239 is preferably phosphorylated by PKG and has been used as a readout for the activity of the NO/cGMP pathway.29 In our experiments, phospho-VASP content of cells expressing a phosphorylation-deficient form of sGC was higher as compared to that observed in wt sGC expressing cells, reflecting the fact that sGC activity is reduced after sGC phosphorylation and reinforcing that notion that a negative feedback loop exists in the NO signaling pathway.

In summary, we have shown that sGC is directly phosphorylated by PKG on Ser-64 of the α1 subunit and that this modification leads to inhibition of sGC activity and limits sGC responsiveness to endogenously produced NO. Given the key regulatory role of sGC in cGMP-dependent pathways, acute regulation of its activity at the posttranslational level allows for the plasticity required by signaling pathways and contributes to the fine-tuning of cGMP generation. The PKG-mediated inhibition in cGMP production by sGC could dampen excessive stimulation of the NO/cGMP pathway and contribute to sGC desensitization.

Acknowledgments

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Disclosures

None.

References


Protein Kinase G Phosphorylates Soluble Guanylyl Cyclase on Serine 64 and Inhibits Its Activity

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SUPPLEMENT

Materials and Methods

Materials — Dulbecco’s Modified Eagle Medium (DMEM) and fetal calf serum (FCS) were obtained from GIBCO-BRL (Paisley, UK). Cell culture plastic wares were from Greiner (Frickenhausen, Germany). The QuickChange™ Site-Directed Mutagenesis Kit and BL21-codon plus strain of *E. coli* were from Stratagene (La Jolla, CA, USA). The pcDNA3.1 Directional TOPO Expression kit, monoclonal anti-V5 antibody and platinum *Pfx* DNA polymerase were from Invitrogen (Paisley, UK). The dNTPs were purchased from Fermentas (St. Leon-Rot, Germany) and the restriction enzymes were obtained from New England Biolabs (Frankfurt, Germany). The purified bovine lung Protein kinase G I was from Calbiochem (San Diego, CA, USA). The nucleospin plasmid kits for the isolation of plasmid DNA and polyvinylidene difluoride (PVDF) membrane were from Macherey-Nagel (Düren, Germany). cGMP enzyme immunoassay kits were from Assay Designs (Ann Arbor, MI, USA) and jetPEI transfection reagent was from Polyplus-transfection (Illkirch, France). SuperSignal West Pico chemiluminescent substrate was from Pierce (Rockford, IL, USA). DC Protein assay kit, Tween 20 and other immunoblotting reagents were from BioRad (Munich, Germany). The anti-β1 antibody was from Cayman Chemicals (Ann Arbor, MI, USA). The anti-actin antibody was from Chemicon (Temecula, CA, USA). The anti-Protein kinase G I α (anti-cGK Iα) and the horseradish peroxidase (HRP) conjugated goat anti-rabbit and rabbit anti-goat antibodies were from Santa Cruz (Santa Cruz, CA, USA). The anti-phospho-VSAP-Ser239 and HRP conjugated horse anti-mouse IgG antibodies were from Cell Signaling (Beverly, MA, USA). All other reagents including anti-α1 antibody, anti-myc antibody conjugated agarose beads, penicillin, streptomycin, isobutylmethylxanthine (IBMX), sodium nitroprusside (SNP), phenylmethylsulfonyl fluoride (PMSF), aprotinin, EGTA, EDTA, pepstatin and glutathione-conjugated agarose beads were from Sigma (St. Louis, MO, USA). [$γ$-$^{32}$P]-ATP and $^{32}$PO$_4$ were purchased from ICN Biomedicals (Irvine, CA, USA). The phospho-specific sGC Ab was developed by Pacific Immunology, Inc (Ramona, CA, USA) against the following sequence (SHPQRKT*$\alpha$RNVYLH).
Construction of mammalian expression plasmids and recombinant adenoviruses —N-terminally myc-tagged rat α1, C-terminally V5/His-tagged rat β1, and deletion mutants of bovine cGMP-dependent protein kinase G Iα (PKG) with or without an N-terminal myc-tag and/or a C-terminal V5/His tag were generated by PCR and cloned into the pcDNA3.1/V5-His TOPO vector. The Quickchange™ kit was used to produce the serine to alanine or serine to aspartate mutants of the α1 subunit according to the manufacturer’s instructions. The recombinant adenoviruses expressing the N-terminally myc-tagged rat sGC α1 wild-type, S64A, S64D, or C-terminal V5/His tagged β1 were generated as previously described (J. Pharmacol. Exp. Ther.; 2006, 319:663-671). All cDNA constructs used in this study were sequenced prior to use.

Baculovirus construction, expression and purification in a Sf21 system of sGC and mutants—

The baculovirus transfer vectors pBacPAK8 containing the rat cDNAs α1, α1S64A, α1S64D and β1-Histag were separately co-transfected with BacPAK6 viral DNA (Bsu36I digest) to generate viruses by homologous recombination. Sf21 cells (500 ml; 1.5 x 10⁶/ml) were co-infected with α1 (or mutants) and β1-Hisα1 containing viruses (ratio 3:1, respectively) and grown for 48 hr. Cells were harvested by centrifugation at 1,500 × g for 10 min. All subsequent steps of purification using cobalt column and FPLC were done as previously described (J. Biol. Chem.; 2005, 280:11513-9). Fractions from the major peak of absorption at 431nm were pooled in 10% glycerol and 5mM DTT, flash frozen and stored at -80 ºC.

Immunoprecipitation and western blotting— Cells washed with ice-cold PBS once and lysed in a lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 0.1 mM EGTA, 1 mM Na₃VO₄, 5 mM glycerophosphoric acid, 0.5% deoxycholic acid, 0.1% SDS, 10 μg/ml aprotinin, 10 μg/ml pepstatin, and 20 mM PMSF. Cellular debris was pelleted at 12,000 × g for 10 min, the supernatants were collected and their protein concentrations determined. Cell lysates containing 200 to 250 μg of protein were
incubated with anti-myc antibody conjugated agarose beads overnight at 4 °C. The beads were then washed five times with lysis buffer and immunoprecipitated proteins or cell lysates were subjected to SDS-PAGE on 10% polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked with 5% dry milk in TBS-T (10 mM Tris-HCl, pH 7.5; 100 mM NaCl; 0.1% Tween-20) for 1 hr at room temperature, rinsed and incubated overnight at 4 °C with primary antibody diluted in TBS-T. Subsequently, the blots were incubated with secondary antibody for 2 h at room temperature. Immunoreactive proteins were detected using the SuperSignal chemiluminescence kit.

In vitro phosphorylation and sGC activity measurements- Purified recombinant PKG Iα (100 units, Calbiochem) was incubated with 50 ng of semi-purified sGC in a reaction buffer (50 mM HEPES, pH 8, 1 mM DTT) in the presence of 10 mM MgCl₂, 100 μM cGMP and 200 μM ATP, for 10 min at 33 °C. sGC activity was then determined by the formation of [α-³²P]-cGMP from [α-³²P]-GTP in the absence or presence of SNAP 1 μM: reaction was performed for 5 min at 33 °C in a final volume of 100 µl, in 50mM HEPES (pH 8.0) reaction buffer containing 500µM GTP, 1mM DTT and 5mM MgCl₂. As control, PKG was inactivated by heat (75 °C for 5min) prior to the phosphorylation reaction and incubation with sGC. These experiments were repeated independently 4 times, with each measurement done in duplicate. When the S64D and S64A forms of sGC were used in activity assays this was done as follows. Reactions were performed in a volume of 100 µl for 10 min at 30 °C. The final concentration of GTP was 500µM in a reaction buffer containing 50mM HEPES pH 8.0, 1mM DTT and 4.5 mM MgCl₂, unless otherwise stated. For NO dose-response curves, the NO-donor SNAP was used at seven different concentrations from 10 nM to 100 μM. All concentration-response experiments were performed in duplicate, and each experiment was repeated three times and with two independently purified WT and mutant enzymes. The EC₅₀ values were calculated from the dose-response curves and corresponded to the concentration of SNAP that half-maximally activates the enzyme. Kₘ and Vₘₐₓ were determined with
substrate concentrations ranging from 0.1 to 500 μM, in the absence of activator and in the presence of 100 nM SNAP. Free magnesium was kept constant at 4 mM. All assays were performed in duplicate, and each experiment was repeated 3 times.

Metabolic labelling with $^{32}$P — COSm6 cells were co-transfected with sGC subunits and a constitutively active PKG form lacking the first 64 amino acids (ΔPKG). Thirty hours after the transfection, cells were switched to phosphate-free DMEM medium with 10% FCS for 12 hr. $^{32}$P was then added into the phosphate-free medium (600 μCi/ml) and cultures incubated for an additional 6 hr. Cells were washed once with PBS and scraped in ice-cold lysis buffer. Cellular debris were pelleted at 12,000 × g for 15 min at 4 °C and the supernatants were subjected to immunoprecipitation with anti-myc conjugated agarose beads. To reduce non-specific binding, the antibody conjugated beads were pre-incubated with non-labeled normal COSm6 cell lysate for 1 hour at 4 °C. The immunoprecipitated proteins were subjected to SDS-PAGE on 10% polyacrylamide gels and transferred to PVDF membranes prior to autoradiography. The same membranes were later subjected to Western blotting analysis.
**Fig. S1. PKG interacts with sGC in vivo.** A7r5 smooth muscle cells were infected with 5 MOI of myc-tagged α1 and 5MOI β1 sGC or 10 MOI of an adenovirus expressing GFP. Twenty four hours later, sGC was immunoprecipitated using an anti-myc antibody. The precipitates and lysates were blotted with an α1 or PKG Iα antibody. Experiment was performed twice with similar results.
**Fig. S2.**  
A. A7r5 smooth muscle cells were co-infected with wt α1 or S64A α1 and β1 sGC (5 MOI each). Forty-eight hours later, cells were serum starved for 4 hr and pre-treated with vehicle or 10 μM Rp-8pCPT-cGMPS for another 1 hr. At the end of this time, cells were exposed to 10 µM SNP or 10 µM 8pCPT-cGMP for 10 min. Cell lysates were blotted with an α1 S64 phospho-specific antibody and anti-α1 or β1 antibodies.  
B. Mouse lung tissues were pre-incubated with vehicle or 10 μM Rp-8pCPT-cGMPS in DMEM for 1 hr at 37 °C and were then exposed to SNP (10 µM) for 10 min. Tissues were homogenized and lysates were blotted with appropriate antibodies. Experiments were performed twice with similar results.
**Fig. S3.** A7r5 smooth muscle cells were co-infected with wt α1 or S64A α1 and β1 sGC (5 MOI each). Forty-eight hours later, cells were washed twice and stimulated with different concentrations of SNP for 15 min in the presence of the phosphodiesterase inhibitor IBMX (1mM). cGMP was then extracted with 0.1N HCl and measured by EIA. Means ± SEM, n=4 wells, *p<0.05 from wt α1/β1. B depicts expression of sGC subunits.
**Fig. S4.** A7r5 smooth muscle cells were co-infected with wt α1 or S64A α1 and β1 sGC (5 MOI each). Forty-eight hours later, cells were washed twice and stimulated with 10 µM SNP for the indicated time in the presence of the phosphodiesterase inhibitor IBMX (1mM). cGMP was then extracted with 0.1N HCl and measured by EIA. Means ± SEM, n=4 wells; * p<0.05 from wt α1/β1. B depicts expression of sGC subunits.
**Fig. S5. Ser-64 phosphorylation contributes to the sGC desensitization.** A7r5 smooth muscle cells were co-infected with wt α1 or S64A α1 and β1 sGC (5 MOI each). Forty eight hours later, cells were pre-incubated with vehicle (control) or 1 µM SNP for 1hr. At the end of this time, cells were washed twice and stimulated with 1µM SNP for 10min in the presence of the phosphodiesterase inhibitor IBMX. cGMP was then extracted with 0.1N HCl and measured by EIA. B demonstrates equal expression of sGC subunits. Means ± SEM, n=4 wells *p<0.05 from control; # p<0.05 from α1/β1.
**Fig. S6.** The data in this figure are the same as the data shown in Fig.3 B and C; they are plotted in a different way, to allow for easier comparisons between Vmax values of the sGC enzymes.