Published in final edited form as:
Arterioscler Thromb Vasc Biol. 2008 October ; 28(10): 1803–1810. doi:10.1161/ATVBAHA.108.165043.

PROTEIN KINASE G PHOSPHORYLATES SOLUBLE GUANYLYL CYCLASE ON SERINE 64 AND INHIBITS ITS ACTIVITY

Zongmin Zhou[1], **Nazish Sayed**[2], **Anastasia Pyriochou**[3], **Charis Roussos**[1], **David Fulton**[4], **Annie Beuve**[2], and **Andreas Papapetropoulos**[3]

[1]G.P. Livanos and M. Simou Laboratories, Evangelismos Hospital, Critical Care Department, University of Athens School of Medicine, Newark, New Jersey

[2]Department of Pharmacology, New Jersey Medical School, UDMNJ, Newark, New Jersey

[3]Laboratory of Molecular Pharmacology, Department of Pharmacy, University of Patras, Patras

[4]Vascular Biology Center, Medical College of Georgia, Augusta, Georgia

Abstract

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 ^{32}P incorporation into the α1 subunit. Serine to alanine mutation of putative 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 co-expressed with ΔPKG exhibited lower basal and NO-stimulated cGMP accumulation, while 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.

Keywords

cGMP; soluble guanylyl cyclase; PKG; nitric oxide; phosphorylation

Nitric oxide-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 nitric oxide (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

Address correspondence to: Andreas Papapetropoulos, Ph.D.; Laboratory of Molecular Pharmacology, Department of Pharmacy, University of Patras, Patras, Greece 26504 FAX +30-2610-997665, Tel: +30-2610-969337; apapapet@upatras.gr.

Disclosures: None

responsible for the majority of cGMP-mediated effects including smooth muscle relaxation and inhibition of platelet aggregation[4]. Several mechanisms that contribute to PKG I-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 post-transcriptional level, α1/β1 activity is dynamically regulated post-translationally 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 ^{32}P 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 due to Ser-dephosphorylation of the β1 subunit [10]. 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 and to study the 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 min in a volume of 50 μl containing 10 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, 5 μM cGMP, 0.1 mM ATP, 5 μCi [γ-^{32}P]-ATP, and 5 μg of GST-fusion protein. To initiate the reaction, 1000 units of bovine lung Protein Kinase G Iα were added. Incorporation of [^{32}P] 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 2×10^5 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 co-transfection experiments, equal amounts of DNA were used for each plasmid. Alternatively, A7r5 cells were infected with 5 MOI of each sGC subunit or 10 MOI of green fluorescent protein (GFP) as control. After 24–48hr 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 anti-myc antibody conjugated agarose beads overnight at 4 °C (throughout the study a N-terminally

myc-tagged version of α1 and a C-terminally V5/6xHis-tagged version of β1 was used). The beads were then washed five times with lysis buffer and immunoprecipitated proteins were subjected to SDS-PAGE. The membranes then were 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 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. After SDS-PAGE samples were transferred to 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 mM of the phosphodiesterase inhibitor IBMX for 15 min with or without a NO donor, as indicated. Media were aspirated and HCl was added to extract cGMP. After 30 min, HCl extracts were collected and cGMP was quantified using cGMP enzyme immunoassay kit. GC activity was determined as previously described[13]. K_m and V_{max} were determined with six different substrate concentrations ranging from 0.1 to 500 μM, in the absence of activator and in the presence of 100nM SNAP. Free magnesium was kept constant at 4mM. 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 one-way ANOVA followed by a *post-hoc* test (Newman-Keuls) or Student's t-test, as appropriate. An F-test that compares the NO concentration-response was used to determine the significance of difference between the EC_{50} obtained with wt and mutants. Statistical differences were considered significant when $p<0.05$.

Results

sGC is a PKG substrate

To determine if sGC is a direct PKG substrate, GST-fusion proteins of the α1 and β1 subunits were used in *in vitro* kinase assays. Incubation of full length GST-α1, but not GST-β1, with catalytically active PKG led to labeling with ^{32}P (Fig.1A&B). In order 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 1–360 incorporated ^{32}P after incubation with PKG *in vitro* (Fig.1A&B).

PKG phosphorylates sGC on Ser64

To test if PKG can phosphorylate sGC in intact cells, COSm6 were co-transfected with the sGC α1/β1 heterodimer and a constitutively active mutant of PKG (ΔPKG; Fig. 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 co-expressed with β1 in the presence or absence of a constitutively active PKG mutant (ΔPKG) in COSm6 and cells were labeled with [^{32}P] (Fig.1C). Autoradiograms demonstrated that mutation of Ser64 to the non-phosphorylatable analogue alanine abolishes the α1 labeling observed in wt sGC. Only a faint band can be seen that runs at a lower molecular weight than the α1 subunit and likely corresponds to autophosphorylated ΔPKG or an sGC interacting protein. It should be noted that PKG-Iα immunoreactivity was readily detectable in the α1 precipitates (supplemental Figure S1).

Phosphorylation of endogenous sGC

To determine whether endogenously expressed sGC becomes phosphorylated on Ser64 under physiologically relevant conditions, we raised an antibody that recognizes the α1 subunit only when phosphorylated on Ser64. The presence of phospho-sGC could be observed in cells expressing wt sGC with a constitutively active form of PKG; no signal could be observed in the S64A mutant (Fig.2A). Interestingly, exposure of rat lung and aorta (Fig.2B) to sodium nitroprusside (SNP) led to an increase in phospho-sGC that reached a maximum at 10min in the case of the aorta that persisted for at least 30min (Fig 2C). The signal obtained could be blocked by the phospho-peptide used for immunization (unpublished data, 2007). Treatment of aortas with bradykinin enhanced Ser64 phosphorylation that was blocked by a NO synthase inhibitor (Fig.2D), providing evidence that endogenously produced NO can stimulate sGC phosphorylation. The NO-donor-induced phosphorylation of Ser64 of the α1 subunit could be blocked by DT3, a peptide inhibitor of PKG-I[14], in cultured A7r5 smooth muscle cells (Fig. 2E). Similar results were obtained with a small molecular weight PKG inhibitor, Rp-8pCPT-cGMPS, in both smooth muscle cells and lung tissue (supplemental Fig. S2A&B). In contrast, activation of PKG with 8pCPT-cGMP enhanced α1 phosphorylation (supplemental Fig. S2A)

Effect of sGC phosphorylation on cGMP accumulation and sGC activity

To test the effect of phosphorylation on cGMP accumulation, COSm6 cells were co-transfected with wt or S64A sGC and ΔPKG. Basal cGMP levels were similar in cells transfected with wt and non-phosphorylatable sGC (Fig.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 co-expression 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 (Fig.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 (Fig. 3D), S64D sGC produced lower levels of cGMP both under basal and NO-stimulated conditions (Fig.3C). The observed changes can not be attributed to alterations in heterodimer formation (unpublished data, 2007). Similar results to the ones obtained with the COSm6, where sGC phosphorylation resulted in reduced cGMP accumulation, were also observed in A7r5 (supplemental Fig. S3). A7r5 is a smooth muscle cell line that endogenously expresses PKG-I (supplemental Fig. S1), but lacks sGC; cells infected with S64A α1/β1 sGC accumulated greater amounts of cGMP when stimulated with

nM amounts of SNP. Reduced responsiveness to SNP was observed as early as 1min and lasted for at least 15min (supplemental Fig. S4).

To determine whether phosphorylation of sGC directly results in decreased sGC activity, sGC was first phosphorylated by pre-incubation with PKG-Iα then its activity measured under basal and SNAP(1μM)-stimulated conditions. Phosphorylation of sGC led to a significant inhibition (883.3±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±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 following incubation with PKG (159.4±12.6 vs 193.0±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 co-expressed with β1 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 1μM); in contrast, S64D sGC expressed lower activity throughout the concentration range tested (Fig.4A); moreover, the EC_{50} for SNAP was greater for the "constitutively phosphorylated" sGC compared to wt (3.64 ± 0.07 vs 3.02 ± 0.11 μM). Under basal conditions wt and S64D phosphomimetic mutant demonstrated similar affinity for the Mg^{+2}-GTP substrate, but exhibited a markedly reduced maximal velocity (Fig. 4B). Stimulation of wt α1/β1 with NO (100nM S-nitroso N-acetyl penicillamine, SNAP) resulted in a drop in Km that was less pronounced for the S64D mutant (Fig.4C). Moreover, although NO-stimulation increased Vmax in all three forms of sGC (wt and mutants), the S64D α1/β1exhibited approximately half the Vmax value of the wt enzyme (Table 1; supplementary Fig.S6).

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 non-phosphorylatable form of sGC and determined their ability to form cGMP with or without SNP pretreatment (supplemental Figure S5). Cells expressing the phosphorylation-deficient form of sGC and pre-treated 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 co-culture system[16]. Use of primary smooth muscle cells was not an option, as cells removed from the vasculature and cultured rapidly loose PKG expression[5]. A7r5 smooth muscle cells were infected with either wt α1/β1 or S64A α1/β1sGC. After 24hr, bovine pulmonary artery endothelial cells (BPAEC) 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 (Fig.5A). It should be noted that 1) inhibition of endothelial NO synthase abolishes the increase in cGMP brought about by the EC and 2) BPAEC are devoid of sGC ([16] and unpublished data, 2007). Results from these co-culture experiments revealed that while wt and S64 sGC were equally expressed in the respective cultures (Fig.5B), cGMP content of the latter was higher, suggesting that endothelium-derived NO activates the non-phosphorylatable 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 (Fig.5C).

To further study the biological significance of sGC phosphorylation on Ser64 we monitored vasodilator stimulated phosphoprotein (VASP) phosphorylation o n Ser-239, a site

preferentially phosphorylated by PKG. Expression of sGC increased the phosphorylation of VASP (Fig.5D&E). In addition, the amount of phosphorylated VASP after stimulation with a 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 (Fig.5E). It should be noted that no change in the α1, β1 or PKG Iα levels was observed between groups (Fig.5D). On the other hand, direct stimulation of PKG with 10μM 8pCPT-cGMP resulted in equal levels of VASP phosphorylation in cells expressing the S64A and the wt sGC (unpublished data, 2007). The above data taken collectively reinforce the notion that the phosphoylated, 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 Ca^{+2} 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 ^{32}P 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 ^{32}P incorporation in sGC[12].

In silico analysis demonstrated the existence of three 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 ^{32}P *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 a 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 utilized two 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, co-expression of PKG with the S64A non-phosphorylatable 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 three domains: an N-terminal regulatory domain, a middle dimerization domain and a C-terminal catalytic domain[1, 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 following 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[23, 24]. 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 phosphomimitic 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[25]. 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 1hr 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 to the notion that phosphorylation of sGC decreases its sensitivity to NO, we observed that the EC_{50} 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 −/− mice that lack endogenous NO production in the vasculature exhibit higher sGC activity in response to exogensouly 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 co-cultures. In agreement to what was observed with the low concentrations of NO donors, co-cultures 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 co-cultures 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 following 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 signalling 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 contributes to sGC desensitization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Dr. Franz Hofmann (Technical University of Munich) for the PKG Iα cDNA.

Sources of funding

This study was supported by grants from the Greek Secretariat of Research and Technology, the Greek Ministry of Education and by the Thorax Foundation (Athens, Greece) and by NIH RO1-GM067640 (A.B). We thank the European Social Fund (ESF), Operational Program for Educational and Vocational Training II (EPEAK II) and particularly the Program Pythagoras, for funding the above work.

References

1. Friebe A, Koesling D. Regulation of nitric oxide-sensitive guanylyl cyclase. Circ Res 2003;93(2):96–105. [PubMed: 12881475]
2. Pyriochou A, Papapetropoulos A. Soluble guanylyl cyclase: more secrets revealed. Cell Signal 2005;17(4):407–413. [PubMed: 15601619]
3. Lucas KA, Pitari GM, Kazerounian S, Ruiz-Stewart I, Park J, Schulz S, Chepenik KP, Waldman SA. Guanylyl Cyclases and Signaling by Cyclic GMP. Pharmacol Rev 2000;52(3):375–414. [PubMed: 10977868]
4. Hofmann F. The Biology of Cyclic GMP-dependent Protein Kinases. J. Biol. Chem 2005;280(1):1–4. [PubMed: 15545263]
5. Lincoln TM, Dey N, Sellak H. Signal Transduction in Smooth Muscle: Invited Review: cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression. J Appl Physiol 2001;91(3):1421–1430. [PubMed: 11509544]
6. Meurer S, Pioch S, Gross S, Muller-Esterl W. Reactive Oxygen Species Induce Tyrosine Phosphorylation of and Src Kinase Recruitment to NO-sensitive Guanylyl Cyclase. J. Biol. Chem 2005;280(39):33149–33156. [PubMed: 16079134]
7. Zwiller J, Revel MO, Malviya AN. Protein kinase C catalyzes phosphorylation of guanylate cyclase in vitro. J Biol Chem 1985;260(3):1350–1353. [PubMed: 2857174]
8. Zwiller J, Revel MO, Basset P. Evidence for phosphorylation of rat brain guanylate cyclase by cyclic AMP-dependent protein kinase. Biochem Biophys Res Commun 1981;101(4):1381–1387. [PubMed: 6118147]
9. Kostic TS, Andric SA, Stojilkovic SS. Receptor-Controlled Phosphorylation of {alpha}1 Soluble Guanylyl Cyclase Enhances Nitric Oxide-Dependent Cyclic Guanosine 5'-Monophosphate Production in Pituitary Cells. Mol Endocrinol 2004;18(2):458–470. [PubMed: 14630997]
10. Ferrero R, Rodriguez_Pascual F, Miras_Portugal MT, Torres M. Nitric oxide-sensitive guanylyl cyclase activity inhibition through cyclic GMP-dependent dephosphorylation. J Neurochem 2000;75(5):2029–2039. [PubMed: 11032892]

11. Murthy KS. Activation of phosphodiesterase 5 and inhibition of guanylate cyclase by cGMP-dependent protein kinase in smooth muscle. Biochem J 2001;360:199–208. [PubMed: 11696008]
12. Murthy KS. Modulation of soluble guanylate cyclase activity by phosphorylation. Neurochem Internatl 2004;45(6):845–851.
13. Sayed N, Baskaran P, Ma X, van den Akker F, Beuve A. Desensitization of soluble guanylyl cyclase, the NO receptor, by S-nitrosylation. Proc Natl Acad Sci U S A 2007;104Proc Natl Acad Sci U S A. .
14. Dostmann W, Taylor M, Nickl C, Brayden J, Frank R, Tegge W. Highly specific, membrane-permeant peptide blockers of cGMP-dependent protein kinase I inhibit NO-induced cerebral dilation. Proc Natl Acad Sci U S A 2000;97:14772–14777. [PubMed: 11121077]
15. Ruiz-Stewart I, Tiyyagura S, Lin J, Kazerounian S, Pitari G, Schulz S, Martin E, Murad F, Waldman S. Guanylyl cyclase is an ATP sensor coupling nitric oxide signaling to cell metabolism. Proc Natl Acad Sci U S A 2004;101:37–42. [PubMed: 14684830]
16. Papapetropoulos A, Marczin N, Snead M, Cheng C, Milici A, Catravas J. Smooth muscle cell responsiveness to nitrovasodilators in hypertensive and normotensive rats. Hypertension 1995;23:476–484. [PubMed: 7511569]
17. Butt E, Bernhardt M, Smolenski A, Kotsonis P, Frohlich LG, Sickmann A, Meyer HE, Lohmann SM, Schmidt HHHW. Endothelial Nitric-oxide Synthase (Type III) Is Activated and Becomes Calcium Independent upon Phosphorylation by Cyclic Nucleotide-dependent Protein Kinases. J. Biol. Chem 2000;275(7):5179–5187. [PubMed: 10671564]
18. Mullershausen F, Russwurm M, Thompson WJ, Liu L, Koesling D, Friebe A. Rapid nitric oxide-induced desensitization of the cGMP response is caused by increased activity of phosphodiesterase type 5 paralleled by phosphorylation of the enzyme. J. Cell Biol 2001;155(2):271–278. [PubMed: 11604422]
19. Zhou Z, Gross S, Roussos C, Meurer S, Móller-Ester lW, Papapetropoulos A. Structural and functional characterization of the dimerization region of soluble guanylyl cyclase. J Biol Chem 2004;279:24935–24943. [PubMed: 15037620]
20. Wagner C, Russwurm M, Jager R, Friebe A, Koesling D. Dimerization of Nitric Oxide-sensitive Guanylyl Cyclase Requires the {alpha}1 N Terminus. J. Biol. Chem 2005;280(18):17687–17693. [PubMed: 15749699]
21. Moncada S, Rees D, Schulz R, Palmer R. Development and Mechanism of a Specific Supersensitivity to Nitrovasodilators After Inhibition of Vascular Nitric Oxide Synthesis in vivo. PNAS 1991;88(6): 2166–2170. [PubMed: 1848694]
22. Brandes RP, Kim D-y, Schmitz-Winnenthal F-H, Amidi M, Godecke A, Mulsch A, Busse R. Increased Nitrovasodilator Sensitivity in Endothelial Nitric Oxide Synthase Knockout Mice : Role of Soluble Guanylyl Cyclase. Hypertension 2000;35(1):231–236. [PubMed: 10642303]
23. Lee Y-C, Martin E, Murad F. Human recombinant soluble guanylyl cyclase: Expression, purification, and regulation. PNAS 2000;97(20):10763–10768. [PubMed: 10995472]
24. Denninger JW, Schelvis JPM, Brandish PE, Zhao Y, Babcock G, Marletta MA. Interaction of Soluble Guanylate Cyclase with YC-1: Kinetic and Resonance Raman Studies. Biochemistry 2000;39:4191–4198. [PubMed: 10747811]
25. Papapetropoulos A, Go C, Murad F, Catravas J. Mechanisms of tolerance to sodium nitroprusside in rat cultured aortic smooth muscle cells. Br J Pharmacol 1996;117:147–155. [PubMed: 8825356]
26. Madhani M, Okorie M, Hobbs AJ, MacAllister RJ. Reciprocal regulation of human soluble and particulate guanylate cyclases in vivo. Br J Pharmacol 2006;149(6):797–801. [PubMed: 17016498]
27. Smolenski A, Poller W, Walter U, Lohmann SM. Regulation of Human Endothelial Cell Focal Adhesion Sites and Migration by cGMP-dependent Protein Kinase I. J. Biol. Chem 2000;275(33): 25723–25732. [PubMed: 10851246]
28. Kwiatkowski AV, Gertler FB, Loureiro JJ. Function and regulation of Ena/VASP proteins. Trends Cell Biol 2003;13(7):386–392. [PubMed: 12837609]
29. Ibarra-Alvarado C, Galle J, Melichar VO, Mameghani A, Schmidt HHHW. Phosphorylation of Blood Vessel Vasodilator-Stimulated Phosphoprotein at Serine 239 as a Functional Biochemical Marker of Endothelial Nitric Oxide/Cyclic GMP Signaling. Mol Pharmacol 2002;61(2):312–319. [PubMed: 11809855]

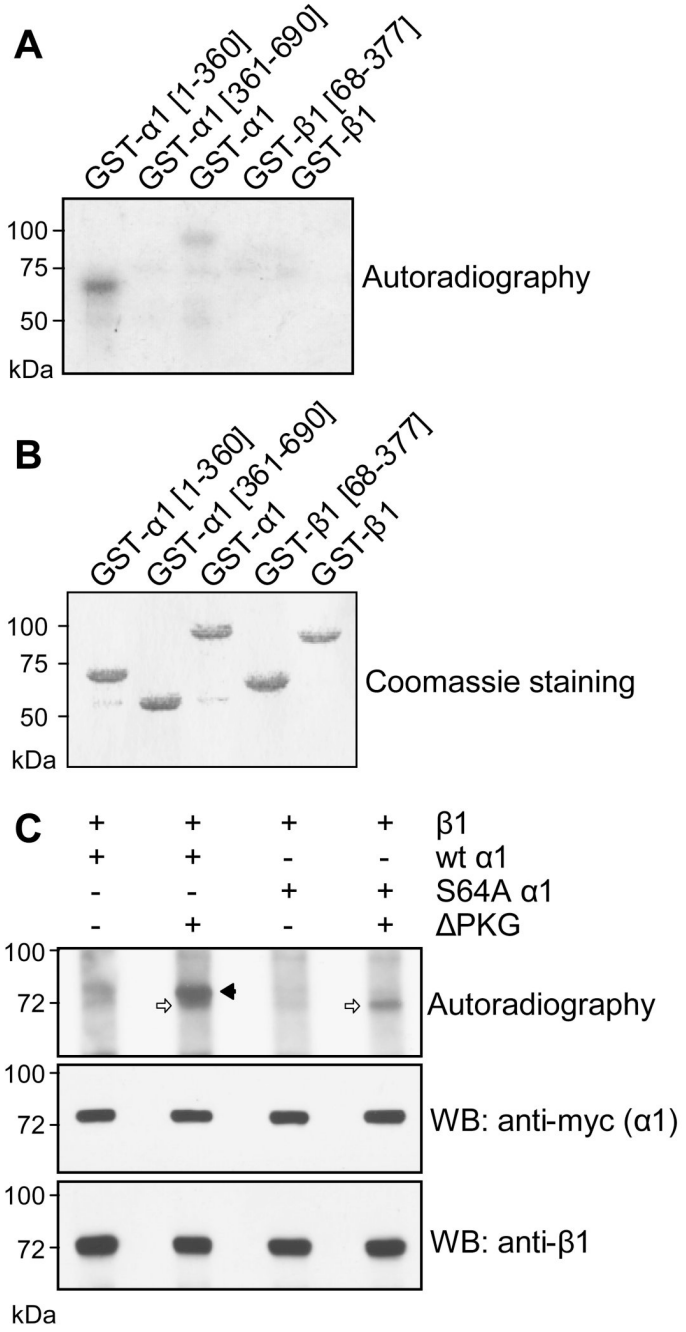

Fig.1. PKG phosphorylates sGC on Ser 64

(A) GST-sGC fusion proteins were labeled, precipitated and subjected to SDS-PAGE. Gels were then exposed to X-ray. (B) A SDS-PAGE gel with GST-fusion proteins was run in parallel with the one shown in A and stained with Coomasie Brilliant Blue. Numbers in brackets correspond to residues of the sGC subunits. (C) COSm6 cells were co-transfected with the indicated plasmids. After lysis, the α1 was immunoprecipitated, subjected to SDS-PAGE and transferred to PVDF membrane; X-ray film was then exposed to the membrane (top). Membranes were later blotted with anti-myc or a β1 antibody to ensure equal levels of expression for proteins in all the groups tested. White arrows pointing to the right indicate

autophosphorylated PKG or a sGC-interacting phospho-protein which is smaller than the α1; the black arrow pointing to the left indicates phosphorylated sGC.

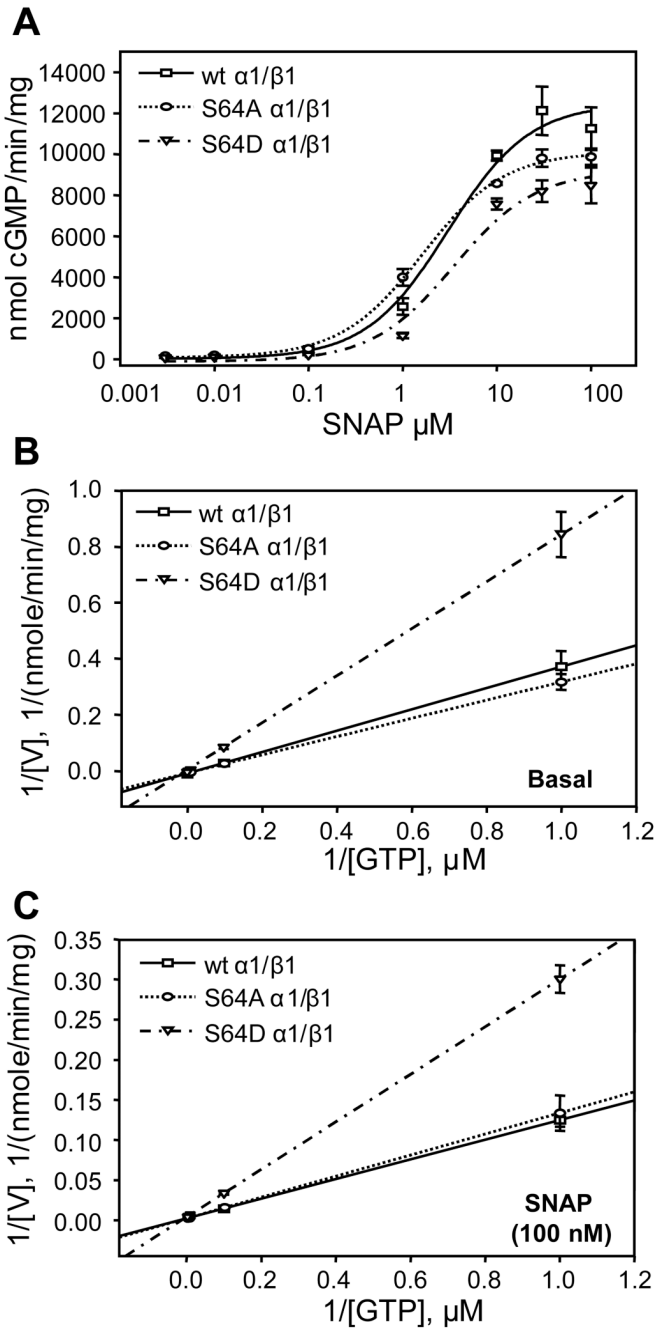

Fig.2. Phosphorylation of endogensouly expressed sGC by NO

(A) Transfected COSm6 cells were lysed and blotted with a phospho-Ser64 antibody raised against the α1 subunit or the indicated antibodies. (B). Rat aorta or lung was incubated for 10min with 100μM sodium nitroprusside (SNP), lysed and blotted with the phospho-specific sGC Ab. (C) Rat aortas were treated with 100μM SNP for the indicated time and analysed for the presence of phosphorylated sGC. (D) Rat aortas were treated with bradykinin (BK; 2 μM, 15min) in the presence or absence of L-nitroarginine methyl ester (100μM ; L-NAME) and sGC phosphorylation on Ser64 was determined with the phospho-specific S64 Ab. (E) A7r5 cells infected with wt α1/β1 were incubated for 1hr with a control peptide (AP; 50μM) or a PKG-I inhibitor peptide (DT3; 50μM). They were then stimulated with 10μM SNP for 10min,

lysed and Ser64 phosphorylation determined using the phospho-specific Ab. The blots shown are representative of experiments repeated two to three times with similar results.

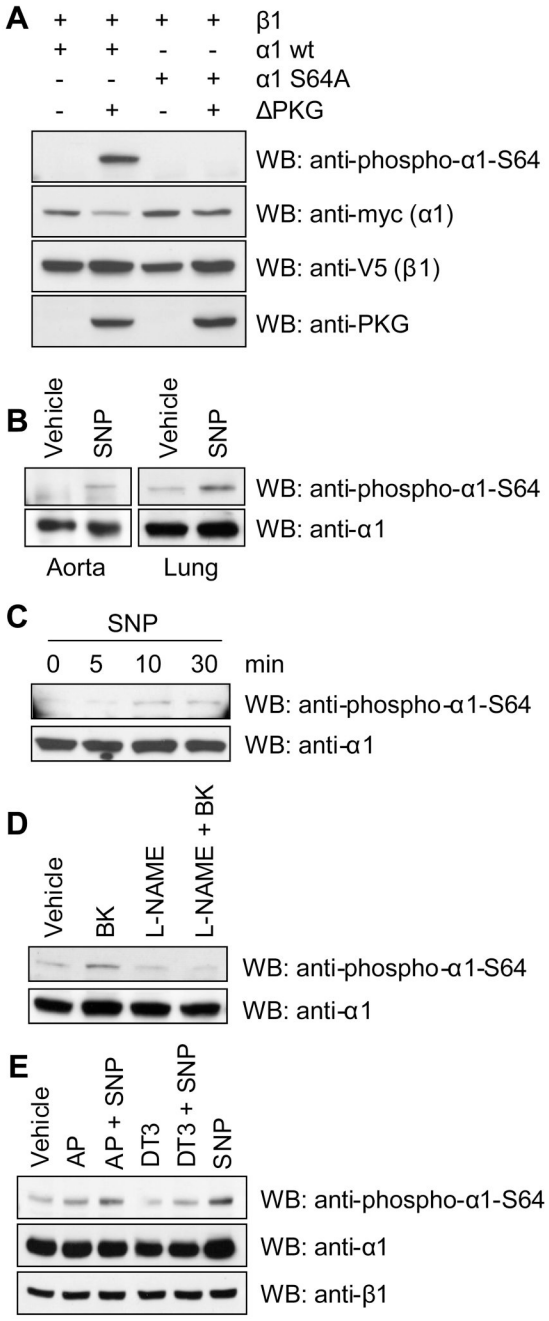

Fig.3. Phosphorylation of Ser 64 inhibits cGMP accumulation

COSm6 cells were transfected with the indicated plasmids. After 24 hr cells were exposed to vehicle or sodium nitroprusside for 15min in the presence of IBMX (1mM); cGMP was then extracted and measured by EIA. Means ± SEM, n=4 wells; $^{*}p<0.05$. B&D demonstrate expression of sGC and PKG proteins under the conditions used.

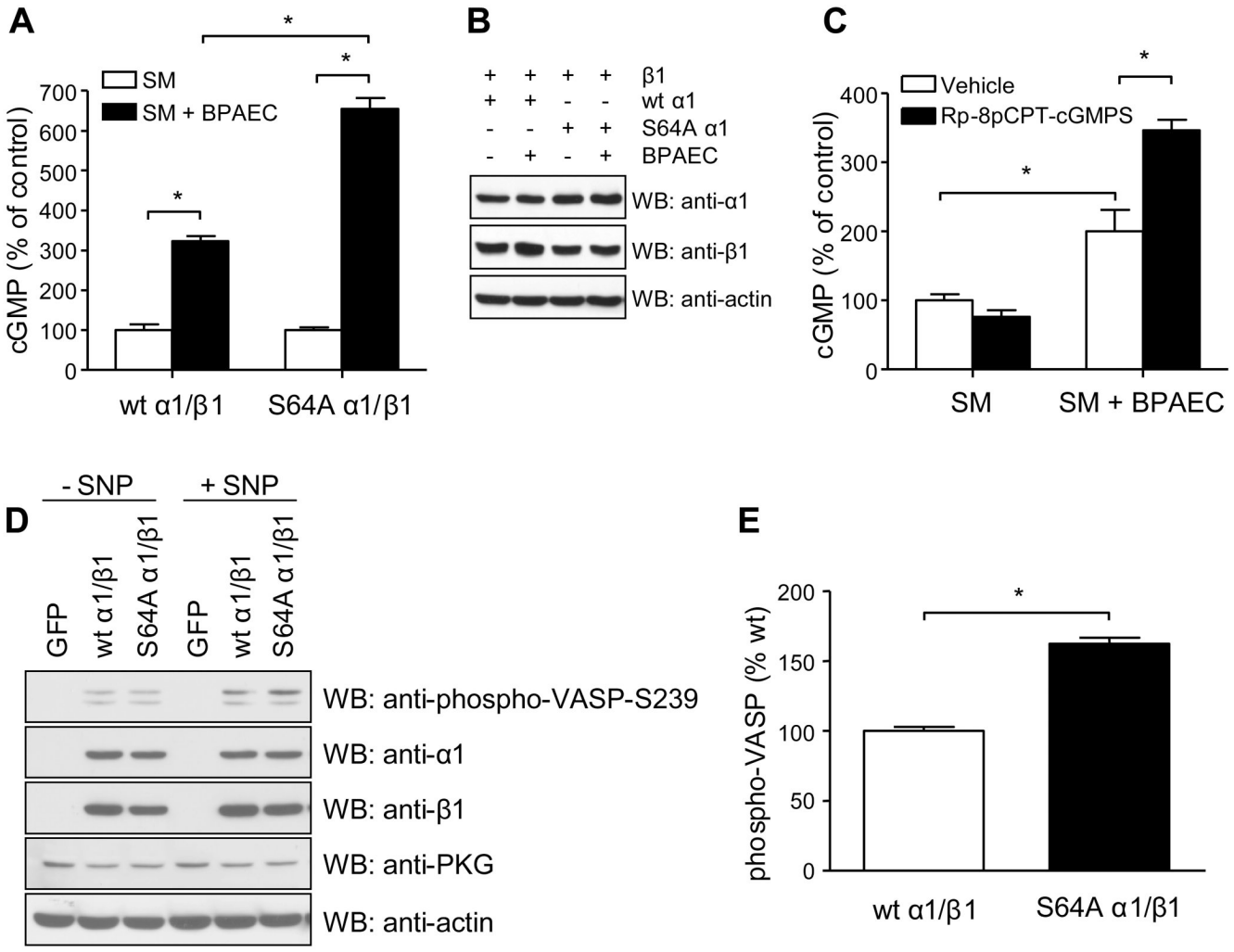

Fig.4. Effects of Ser64 phosphorylation on sGC activity

(A) Activity was measured in response to increasing concentrations of SNAP (0.1 to 100μM). (B and C): double reciprocal plots of sGC activity measurements done in the presence of varying concentrations of the substrate GTP (1 to 500μM) under basal (B) or SNAP (C) stimulated conditions. SNAP was used at 100nM. Means ± SEM of three independent experiments with each measurement done in duplicate.

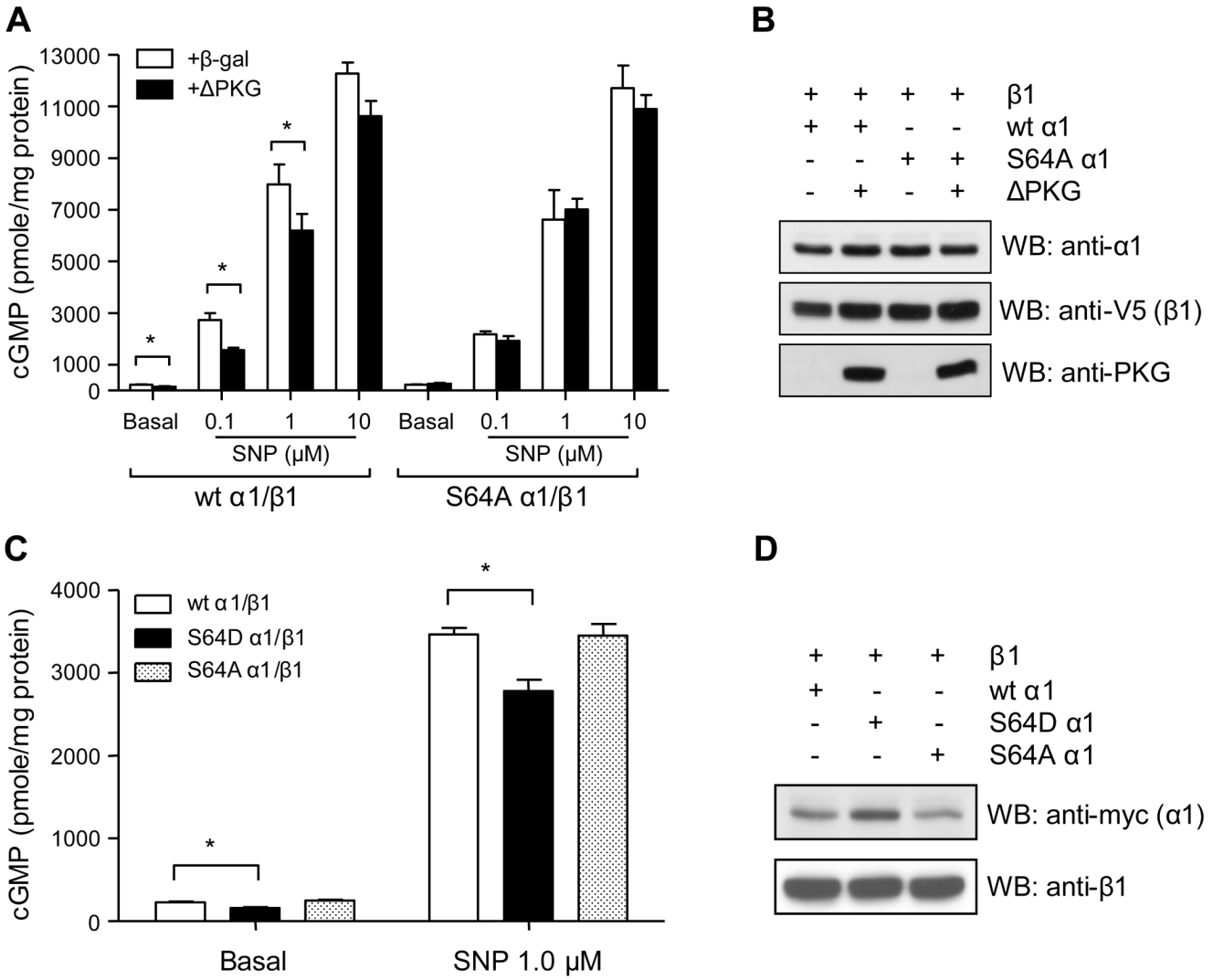

Fig.5. Downregulation of NO/cGMP signaling by Ser64 phosphorylation

(A) A7r5 smooth muscle cells (SM) were co-infected with wt α1 or S64A α1 and β1 sGC (5 MOI each). Twenty four hours later, BPAEC cells (60,000/C-24 well) were added to the A7r5 for an additional 24 hr. Cells were then incubated with IBMX (1mM) for 15min. cGMP was extracted and measured. Means ± SEM, n=4 wells; * $p<0.05$. (B) Equal sGC expression in the co-cultures is confirmed. (C) A7r5/BPAEC co-cultures were established as in A. Prior to the 15min exposure to IBMX, cells were incubated with vehicle (DMSO) or 10 μM Rp-8pCPT-cGMPS for 1hr. cGMP was then extracted and measured as in A. Means ± SEM, n=4 wells; * $p<0.05$. (D) A7r5 smooth muscle cells were infected with 5 MOI of wt α1, S64A α1 and β1 sGC or 10 MOI of an adenovirus expressing GFP. Forty eight hours later, cells were incubated with vehicle (−SNP) or 1μM SNP (+SNP) for 10min. Lysates were then prepared, subjected to SDS-PAGE and blotted with the indicated antibody.(E) pVASP and corresponding actin autoradiograms of wt and S64A sGC infected cells exposed to SNP were scanned and quantitated using image analysis software. Means ± SEM, n=4 wells $^{*}p<0.05$.

Table I

Kinetic parameters of recombinant rat sGC wt and mutants

	Basal		SNAP (100 nM)	
	Km	**Vmax (nmol/min/mg protein)**	**Km**	**Vmax (nmol/min/mg protein)**
wt α1/β1	118.7 ± 16.4	351.6 ± 14.7	58.5 ± 5.7	442.7 ± 20.6
S64A α1/β1	116.1 ± 16.2	328.0 ± 39.5	49.8 ± 4.3	440.3 ± 31.3
S64D α1/β1	107.7 ± 3.2	118.3 ± 9.0*	70.6 ± 5.1*	253.3 ± 8.4*

The apparent K_m and V_{max} values were determined by double reciprocal plots analysis, in the absence or presence of 100nM SNAP (see Fig.4B and C). Values are mean ± SEM of three experiments, with each measurement done in duplicate. Free $MgCl_2$ was kept constant at 4 mM.

* $p<0.05$ from wt.