Smooth Muscle Cell Expression of Type I Cyclic GMP–dependent Protein Kinase Is Suppressed by Continuous Exposure to Nitrovasodilators, Theophylline, Cyclic GMP, and Cyclic AMP

Gerald A. Soff,* Trudy L. Cornwell,‡ Deborah L. Cundiff,* Stephen Gately,* and Thomas M. Lincoln‡

**Department of Medicine, Division of Hematology/Oncology, Northwestern University Medical School, Chicago, Illinois 60611; and ‡Department of Pathology, Division of Molecular and Cellular Pathology, University of Alabama at Birmingham, Birmingham, Alabama 35294*

Abstract

A key component of the nitric oxide–cyclic guanosine monophosphate (cGMP) pathway in smooth muscle cells (SMC) is the type I GMP-dependent protein kinase (PK-G I). Activation of PK-G I mediates the reduction of cytoplasmic calcium concentrations and vasorelaxation. In this manuscript, we demonstrate that continuous exposure of SMC in culture to the nitrovasodilators *S*-nitroso-*N*-acetylpenicillamine (SNAP) or sodium nitroprusside (SNP) results in ~ 75% suppression of PK-G I mRNA by 48 h. PK-G I mRNA and protein were also suppressed by continuous exposure to cGMP analogues 8-bromo- and 8-(4-chlorophenylthio) guanosine-3,5-monophosphate or the cAMP analogue dibutyryl cAMP. These results suggest that activation of one or both of the cyclic nucleotide–dependent protein kinases mediates PK-G I mRNA suppression. Using isoform-specific cDNA probes, only the PK-G Iα was detected in SMC, either at baseline or after suppression, while PK-G Iβ was not detected, indicating that isoform switch was not contributing to the gene regulation. Using the transcription inhibitor actinomycin D, the PK-G I mRNA half-life in bovine SMC was observed to be 5 h. The half-life was not affected by the addition of SNAP to actinomycin D, indicating no effect on PK-G I mRNA stability. Nuclear runoff studies indicated a suppression of PK-G I gene transcription by SNAP. PK-G I suppression was also observed in vivo in rats given isosorbide dinitrate in the drinking water, with a dose-dependent suppression of PK-G I protein in the aorta. PK-G I antigen in whole rat lung extract was also suppressed by administration of isosorbide or theophylline in the drinking water. These data may contribute to our understanding of nitrovasodilator resistance, a phenomenon resulting from continuous exposure to nitroglycerin or other nitrovasodilators. (*J. Clin. Invest.* 1997. 100:2580–2587.) Key words: cyclic GMP–dependent protein kinases • cyclic GMP • vascular smooth muscle • nitroglycerin • drug tolerance

Introduction

The nitric oxide (NO)–cGMP[1] pathway is a key regulator of vascular tone (1–4). In smooth muscle cells (SMC), NO, also identified as the endothelium-derived relaxing factor, activates soluble guanylyl cyclase, leading to an increase in levels of cGMP. Atrial natriuretic factor, another mediator of vasodilation, activates a membrane-bound guanylyl cyclase, also increasing cGMP levels. cGMP activates a cGMP-dependent protein kinase (PK-G), which mediates the vasorelaxant effect by phosphorylation of several proteins that regulate intracellular Ca^{2+} levels (2, 5–7). As a result, calcium is transported to the endoplasmic and sarcoplasmic reticulum and extruded from the cell, thus reducing cytoplasmic calcium concentrations and effecting vasorelaxation. Recent studies indicate that PK-G may also catalyze the phosphorylation of the inositol 1,4,5-triphosphate receptor and, therefore, regulate the mobilization of calcium stores by inositol triphosphate (8, 9). Additional data suggest that NO-induced vasorelaxation may be mediated in part by both cGMP-dependent and cGMP-independent activation of a K^+ channel (10, 11).

Two genes encoding mammalian PK-G have been identified, type I and type II. Type I PK-G exists as two isoforms referred to as type Iα and type Iβ (2, 7, 12, 13). The PK-G Iα and Iβ isoforms differ only in the initial coding region (89 amino acids for Iα and 104 for Iβ) (12, 13). Although the PK-G I isoforms have identical substrate specificity, the Iα isoform requires one-tenth the cGMP concentration for half-maximal activity as the Iβ isoform (7). In a recent study, the PK-G Iα isoform was found in the aorta, heart, kidneys, adrenal glands, cerebellum, and lung tissues (14). The PK-G Iβ isoform was only detected abundantly in the uterus (14). The type II PK-G has been identified in brain and intestinal epithelium (15–17).

While much has been learned about the regulation of the genes for NO synthase (3), little is known about the regulation of the gene for PK-G I, despite its critical role for SMC function. Our recent studies have described the growth–state dependence of PK-G I expression in SMC in culture (18). We reported that PK-G I expression was reduced in subconfluent cultures of low passage aortic SMC and was increased at high density. These results are similar to other findings regarding the expression of smooth muscle–specific proteins (19–22). In a recent study, PK-G Iα was found to be suppressed by PDGF-

Address correspondence to Gerald A. Soff, M.D., Northwestern University Medical School, 320 East Superior St., Searle 3-565, Chicago, IL 60611. Phone: 312-503-5132; FAX: 312-503-8104; E-mail: gasoff@merle.acns.nwu.edu

Received for publication 30 September 1996 and accepted in revised form 20 September 1997.

J. Clin. Invest.

0021-9738/97/11/2580/08 $2.00
Volume 100, Number 10, November 1997, 2580–2587
http://www.jci.org

1. *Abbreviations used in this paper:* 8-Br-cGMP, 8-bromo-guanosine-3,5-monophosphate; 8-CPT-cGMP, 8-(4-chlorophenylthio) guanosine-3,5-monophosphate; DB-cAMP, dibutyryl cAMP; GAPDH, glyceraldehyde phosphate dehydrogenase; NO, nitric oxide; PK-A, protein kinase A; PK-G I, type I cGMP–dependent protein kinase; SMC, smooth muscle cells; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; TBS, Tris-buffered saline.

BB (14). In this study, suppression of PK-G I mRNA and protein has been demonstrated in vascular SMC in vitro and in vivo in response to continuous exposure to nitrovasodilators, theophylline, or analogues of cAMP and cGMP. These findings suggest that one mechanism that regulates PK-G I gene expression is the activation of one or both cyclic nucleotide–dependent protein kinases.

Methods

Materials. *S*-nitroso-*N*-acetylpenicillamine (SNAP) was from Calbiochem Corp. (San Diego, CA). Sodium nitroprusside (SNP), isosorbide dinitrate, 8-bromo-cGMP (8-Br-cGMP), and theophylline were obtained from Sigma Chemical Co. (St. Louis, MO). 8-(4-chlorophenylthio) guanosine-3,5-monophosphate (8-CPT-cGMP) was from Bio-Log, Inc. (La Jolla, CA). N^G-nitro-L-arginine methyl ester, hydrochloride (L-NAME) was obtained from Calbiochem Corp. All tissue culture reagents were from Life Technologies, Inc. (Gaithersburg, MD).

Preparation and culture of SMC. Primary bovine aortic SMC were isolated by a modification of the method of Gunther et al. (18, 23, 24). Rat aortic SMC were prepared from thoracic and abdominal aortae of Sprague-Dawley rats by a previously modified protocol of Smith and Brock (18, 25). Bovine and rat SMC were maintained in a standard humidified 5% CO_2 tissue culture incubator in DME and used within the first four passages. Bovine SMC were cultured with 20% calf serum, and rat SMC were cultured with 10% FBS. In all experiments in which the SMC were treated with test compounds (i.e., nitrovasodilators, cyclic nucleotides, etc.), the media and test reagents were replaced every 24 h with fresh test media or control media for the 48- and 72-h time points.

Northern blot analysis of cGMP kinase. Northern blot analysis using ^{32}P-labeled cDNA fragments as probes was performed as reported previously (18, 23). Probes included the full coding sequence of the bovine PK-G Iα isoform and glyceraldehyde phosphate dehydrogenase (GAPDH). PK-G Iα and Iβ isoform–specific cDNA probes were generated by PCR (see below). Northern blot results were quantified using a phosphorimager (Fujix BAS 2000; Fuji Photo Film Co., Tokyo, Japan).

Generation of isoform specific cDNA probes. To allow for independent detection and quantification of the PK-G Iα and Iβ isoform mRNA species by northern blot analysis, isoform-specific cDNA fragments were synthesized. The two isoforms are both 7 kb, and differ only in the initial coding sequence (12, 13). A pair of oligonucleotide primers was generated to amplify cDNA of nucleotides 4–266 of the Iα isoform-specific domain (AACATGAGCGAGCTGGAGGAAGAC and GATTTGGTGAACTTCCGGAATGCC) and a second pair to amplify cDNA of nucleotides 1–472 of the Iβ isoform (GCAGACTGGGCATGCTCAGAAGCC and TGGACTCTTGGGGTAGAAGGGCAG). PCR was conducted under standard conditions, 94°C for 45 s, 60°C for 45 s, 72°C for 2 min for 30 cycles, followed by a 10-min extension at 72°C. The cDNA fragments were subcloned into plasmid Bluescript II (Stratagene Inc., La Jolla, CA) and sequenced using commercial reagents (GIBCO BRL, Gaithersburg, MD) to confirm the correct sequence.

Evaluation of PK-G mRNA stability and transcription. Early passage postconfluent bovine SMC were treated with 10 μg/ml of the transcriptional inhibitor actinomycin D (Life Technologies, Inc.) (26), 500 μM SNAP, or actinomycin D and SNAP together. Untreated SMC served as controls. PK-G I mRNA was quantified by Northern blot at 1, 6, and 24 h of treatment. PK-G I mRNA levels were normalized by reprobing the membrane with a labeled cDNA for the 18 S rRNA.

The effects of SNAP on transcription of the PK-G I gene were evaluated by the nuclear runoff technique, adapting a method reported previously (26, 27). The test cDNA species were PK-G I, α-actin, and GAPDH. The signal was quantified using the BAS 2000 phosphorimager. Background counts were subtracted from a region of control DNA (from herring sperm); these counts were equivalent to counts obtained at areas of the membrane without DNA loading.

Immunological quantitation of PK-G I from tissue culture. Primary cultures of rat aortic SMC were plated in 35-mm culture dishes and treated with 8-CPT-cGMP. PK-G I levels were determined from crude soluble fractions as reported previously (18). Samples (2 or 5 mg total protein in 0.5 ml) were diluted in Tris-buffered saline (TBS, 20 mM Tris-HCl, pH 7.5, 137 mM NaCl) and applied in triplicate to nitrocellulose membrane in wells of a slot-blot apparatus. Several concentrations of purified bovine lung PK-G Iα in TBS containing 10 mg/ml BSA were also applied to separate wells to construct a standard curve of band density (area units) versus nanograms of PK-G I protein. The primary antibody was affinity-purified rabbit anti–bovine PK-G I (1/100), and the secondary antibody was donkey anti–rabbit IgG conjugated to horseradish peroxidase (1/10,000). After enhanced chemiluminescence, data were analyzed by densitometry. Values have been expressed as nanograms of PK-G I per milligram of total protein.

Animal studies. Male rats of ~ 400 g, were given water supplemented with various doses of isosorbide dinitrate or theophylline for 4 d. The water was changed daily, and the volume of water consumed was recorded. The actual dose of drug consumed per animal was calculated based on volume of water consumed, as milligrams of isosorbide dinitrate or theophylline per gram of animal body weight per 24 h. Control animals were handled in a similar fashion, and were given untreated water. Animals were killed on the fourth day by CO_2 inhalation. Aortas were excised, then quickly snap-frozen at −85°C. Tissue lysates were prepared by homogenization in 0.5 ml 20 mM sodium phosphate, pH 6.8, 2 mM EDTA, 0.15 M NaCl, and 0.1 mM PMSF using an Ultraturax polytron (Tekmar Co., Cincinnati, OH) set at 50%. All animal care and handling were under the oversight and approval of the Northwestern University Animal Care and Use Committee.

Immunologic quantitation of PK-G from rat tissues. Crude soluble cell extracts were prepared from snap-frozen rat aortic and lung tissues from animals treated with isosorbide dinitrate or theophylline. Frozen tissue was pulverized with a mortar and pestle, cooled in liquid N_2, then homogenized by sonication in 0.4 ml of 50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1 mM PMSF, 10 mg/ml pepstatin A, and 10 mg/ml leupeptin. Homogenates were then centrifuged at 12,000 *g* for 10 min. The supernatants (75 μg protein) were analyzed by Western blot analysis for PK-G I using 7.5% SDS polyacrylamide gels. After electrophoresis, protein was transferred to nitrocellulose membrane. Blots were then incubated in a blocking buffer consisting of 0.5% nonfat dry milk in TBS for 1 h at 25°C and incubated overnight at 4°C with goat anti–bovine PK-G I (1/1,000). The blots were washed thee times for 7 min with TBS containing 0.05% NP-40, 0.125% Na deoxycholate, and 0.05% SDS (TBS wash buffer). Subsequently, blots were incubated with rabbit anti–goat IgG conjugated to horseradish peroxidase (1/10,000) for 1 h at 25°C. Blots were washed in TBS wash buffer five times for 5 min, and immunoreactive protein was visualized by enhanced chemiluminescence. Densities of PK-G I bands on autoradiographs were analyzed using a laser densitometer (Ultroscan XL; LKB, Uppsala, Sweden). The PK-G I signal was expressed as percentage of control sample. Protein was determined using the method of Bradford (28), using BSA as standard.

Protein kinase A (PK-A) activation. The amount of active PK-A (free catalytic subunit) was estimated using the protein kinase activity ratio assay, modified from Forte et al. (29, 30). Postconfluent bovine aortic SMC were pretreated with DME containing 1 mg/ml BSA for 24 h and then treated for 5 min with the test reagents. Preparation of cell lysates and measurement of PK-A kinase activity were as per Cornwell et al. (30). The PK-A activity ratio (−cAMP/+cAMP) was determined by dividing PK-A activity in the absence of added cAMP by activity in the presence of saturating cAMP. Thus, a ratio of 1.0 indicates complete activation of PK-A by the respective test treatment. Forskolin served as a positive control for PK-A activation.

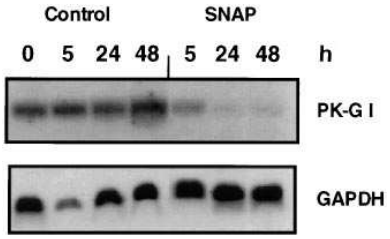

Figure 1. Northern blot of PK-G I mRNA. Continuous exposure of postconfluent aortic SMC to 500 μM SNAP resulted in a marked suppression of PK-G I mRNA, as illustrated in this representative Northern blot. GAPDH mRNA served as a control. The signals were quantified using a Fujix BAS 2000 phosphorimager.

Results

Suppression of PK-G I mRNA in SMC culture by nitrovasodilators. Early passage bovine aortic SMC (passage 4) were maintained in culture for 14 d postconfluence. Under these conditions, there is an abundance of PK-G I mRNA and protein (18). Medium was then changed to complete medium supplemented with the nitrovasodilators SNAP or SNP. At several time points beginning at 5 h, cellular RNA was harvested, and PK-G I mRNA was assayed by Northern blot. PK-G I mRNA levels were normalized to levels of GAPDH. As illustrated in Fig. 1 and Fig. 2 *A*, there was a significant time-dependent decrease in PK-G I mRNA after exposure to both nitrovasodilators in postconfluent cells. With exposure to 500 μM SNAP, PK-G I mRNA was suppressed to 61% of baseline by 5 h, with further suppression to 21–27% of baseline by 24–48 h. Similarly, exposure to 100 μM SNP suppressed PK-G I mRNA levels (Fig. 2 *A*). SNP could not be evaluated at doses of 500 μM or above, because of evidence of cellular toxicity; i.e., cells were lifting from the plate, and there was a loss in trypan blue exclusion. To determine whether the effect of SNAP and SNP is mimicked by cGMP, passage 4 bovine aortic SMC were exposed to various concentrations of 8-Br-cGMP, and PK-G I mRNA was quantified by Northern blot analysis. As illustrated in Fig. 2 *B*, continuous exposure to 8-Br-cGMP also suppressed PK-G I mRNA levels to ~ 60% (1 mM 8-Br-cGMP) and 31% (3 mM 8-Br-cGMP) of baseline by 48 h. Concentrations of 500 μM or lower of 8-Br-cGMP did not reproducibly suppress PK-G I mRNA levels.

To determine if inhibition of smooth muscle NO synthase results in altered PK-G I levels, early passage postconfluent bovine aortic SMC were treated with media supplemented with 50 μM of L-NAME, an NO synthase inhibitor. In six experiments, the mean PK-G I mRNA levels varied < 15% from baseline at 3, 24, and 48 h of treatment, not a significant change compared with control.

Suppression of PK-G I protein in SMC culture. Studies were extended to analyze the levels of PK-G I protein in rat aortic SMC. Early passage cells were grown until 3 d postconfluence and treated with 8-CPT-cGMP at 10 or 100 μM for 48 or 72 h. Unlike 8-Br-cGMP, 8-CPT-cGMP is highly phosphodiesterase resistant and cell permeant. These properties allow one to use significantly lower concentrations to elicit effects through activation of cyclic nucleotide–dependent protein kinases. PK-G I antigen was measured by Western blot analysis as reported previously by our group (18), and the results were reported as nanograms of PK-G I per milligram of total cellular protein. As illustrated in Fig. 3, PK-G I protein levels were suppressed to 50% of baseline with 100 μM 8-CPT-cGMP.

Evaluation of PK-G I isoforms, and generation of isoform-specific cDNA probes. To determine if the suppression of PK-G I mRNA levels was associated with alteration of one or both of the PK-G I isoforms, isoform-specific cDNA fragments were synthesized by PCR. Oligonucleotide pairs were synthesized to yield a fragment of nucleotides 4–266 for bovine PK-G type Iα, and 1–472 for bovine PK-G type Iβ (12, 13). Initial PCR used bovine genomic DNA, as it was hypothesized that the isoform-specific domains were single exons. After

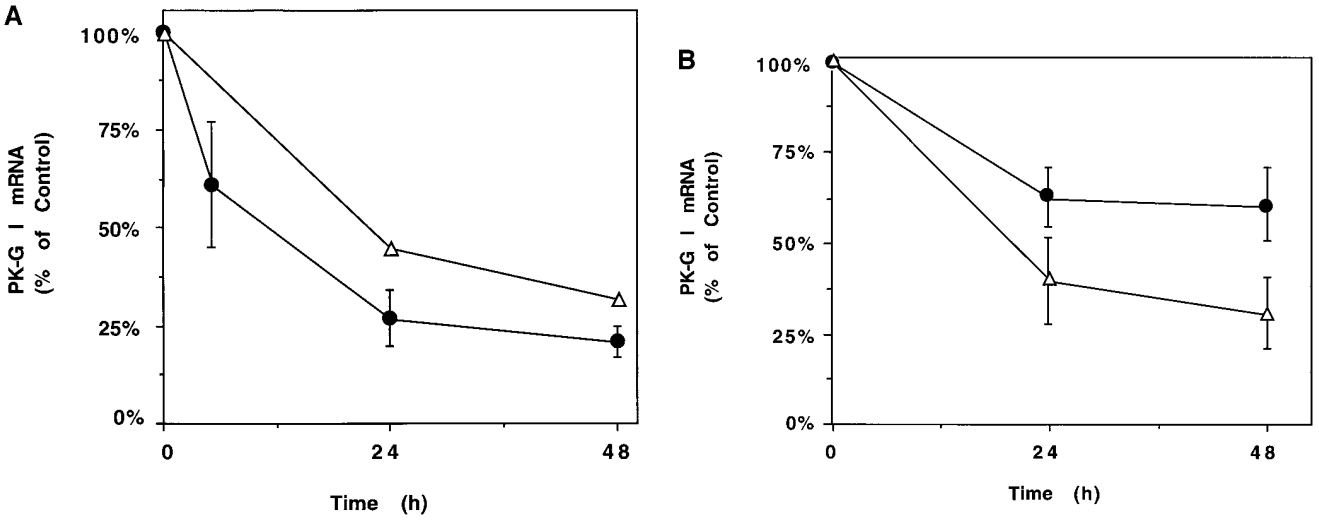

Figure 2. (*A*) Suppression of PK-G I mRNA by nitrovasodilator exposure. Postconfluent bovine SMC were treated with 500 μM SNAP (●) or 100 μM SNP (△), and PK-G I and GAPDH mRNA were quantitated by Northern blot. For quantification, the PK-G I signal was normalized to the GAPDH signal. The data are the mean±SEM of five experiments for SNAP, and a single experiment for SNP ($P < 0.001$ for SNAP at 24 and 48 h). (*B*) cGMP analogues suppress PK-G I mRNA. PK-G I mRNA in postconfluent bovine SMC was suppressed by exposure to 1 mM (●) and 3 mM (△) 8-Br-cGMP. The data represent the mean±SEM of four experiments for 1 mM and three experiments for 3 mM 8-Br-cGMP ($P < 0.05$ for 1-mM exposure and $P < 0.01$ for 3 mM 8-Br-cGMP).

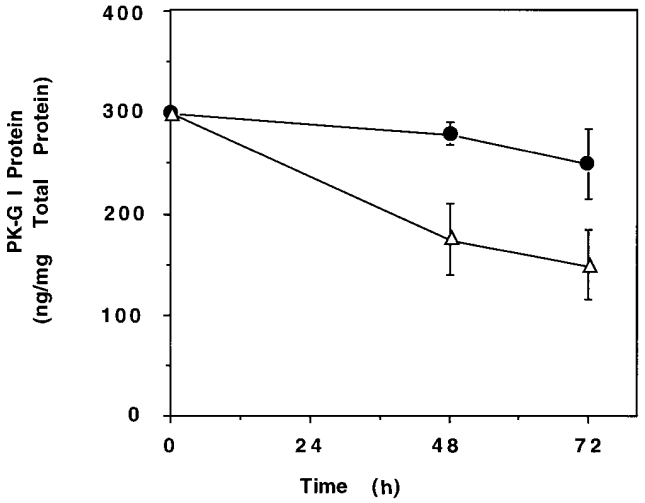

Figure 3. PK-G I antigen is suppressed by exposure to 8-CPT-cGMP. Primary cultures of rat aortic SMC were exposed to 10 (●) or 100 (△) μM 8-CPT-cGMP for up to 72 h. PK-G I was measured by slot-blot analysis, and normalized to total cellular protein. The data are the mean±SEM of three independent experiments ($P < 0.05$ for 100 μM 8-CPT-cGMP; data not significant for 10 μM 8-CPT-cGMP).

cloning of the PCR products, the cDNAs of the predicted size and sequence were obtained, yielding the isoform-specific cDNA, and confirming that the isoform-specific domains are in a single exon. The isoform-specific cDNA fragments were used for subsequent Northern blot analysis. Four of the Northern blots that had shown suppression of PK-G I by SNAP or 8-Br-cGMP were reprobed with the isoform-specific cDNA fragments. In all cases, the results using the Iα-specific probe showed the predicted 7-kb mRNA present in baseline conditions and a pattern of suppression indistinguishable from the total PK-G I (Fig. 1, and Fig. 2, *A* and *B*), whereas the Iβ-specific probe failed to yield a detectable signal (data not shown). As noted above, the common domain between Iα and Iβ represents > 95% of each mRNA, and the cDNA of the common coding region was used to detect total PK-G I mRNA. As a positive control of hybridization of the Iβ probe, hybridization to genomic DNA and rat lung RNA was performed. Tamura et al. have observed independently that in vascular SMC, only the PK-G Iα isoform is detected at the mRNA level (14).

Evaluation of PK-G I mRNA stability and transcription. To determine whether nitrovasodilators alter PK-G I mRNA half-life, early passage postconfluent bovine SMC were treated with 10 μg/ml actinomycin D, or 500 μM SNAP and actinomycin D. Actinomycin D, which suppresses mRNA transcription, yielded a half-life of ~ 5 h for PK-G I mRNA (Fig. 4 *A*). Addition of SNAP to the actinomycin D treatment yielded a comparable mRNA half-life, indicating no contribution of SNAP to the rate of PK-G I mRNA decay. These data are consistent with SNAP being an inhibitor of PK-G I gene transcription.

Nuclear runoff analysis was performed to confirm the transcriptional basis of the nitrovasodilator-mediated decrease in PK-G I mRNA levels (26, 27). Nuclei were harvested from control SMC and SMC treated with 500 μM SNAP for 24 h. As illustrated in Fig. 4 *B*, the PK-G I mRNA was reduced to 47% of control by SNAP treatment, while the levels of α-actin and GAPDH were not changed significantly. These data indicate that SNAP suppresses PK-G I transcription in vascular SMC.

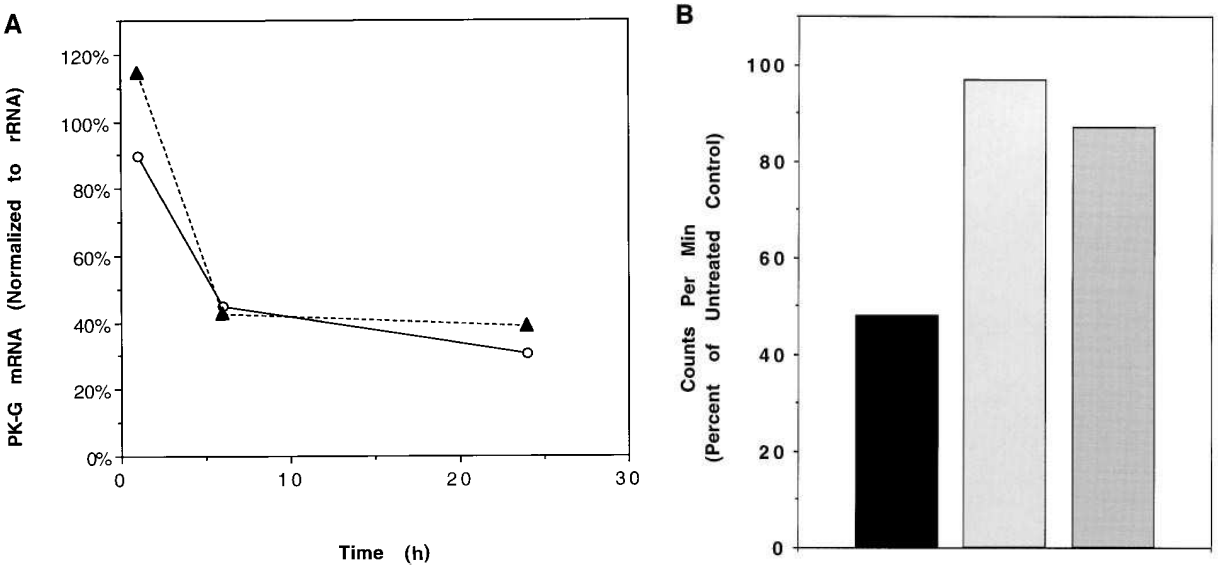

Figure 4. (*A*) PK-G I mRNA half-life was not affected by SNAP. Bovine SMC were treated with the transcription inhibitor actinomycin D (10 μg/ml). PK-G I mRNA (normalized to 18 S rRNA) was suppressed to 45% of baseline at 6 h and 31% at 24 h by actinomycin D alone (○). Treatment with both actinomycin D and SNAP (500 μM) together (▲) showed comparable PK-G I mRNA half-life, indicating no effect of SNAP on PK-G mRNA half-life. Treatment with SNAP alone also suppressed PK-G mRNA, consistent with previous experiments (not shown). The data represent the mean of two independent experiments. (*B*) Nuclear runoff experiment with bovine SMC demonstrates suppression of PK-G I transcription by SNAP treatment. When normalized to untreated cells, 24 h of treatment with 500 μM SNAP resulted in a 52% suppression of PK-G I mRNA (*black bar*). In comparison, the control mRNA species, GAPDH (*dark gray bar*) and actin (*light gray bar*), were not changed significantly by SNAP treatment.

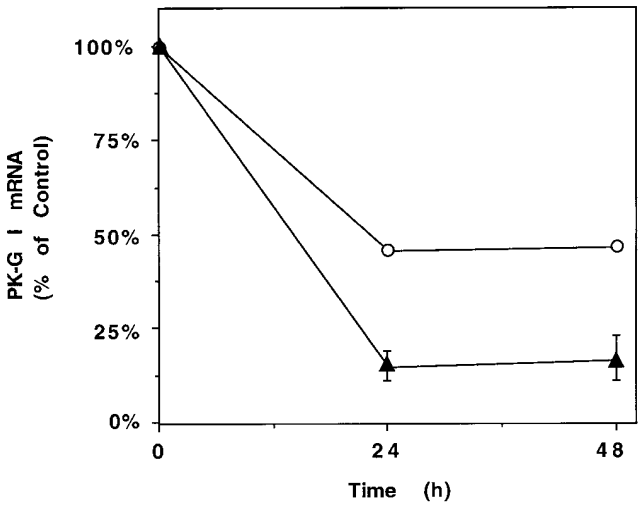

Figure 5. PK-G I mRNA is suppressed by DB-cAMP and theophylline. Exposure of early passage postconfluent bovine SMC to 500 μM DB-cAMP (▲) suppressed PK-G mRNA to 10% of baseline values by 24 h of treatment. The data are the mean±SEM of three experiments ($P < 0.01$). In a single experiment, 500 μM of theophylline treatment (○) also suppressed PK-G I mRNA.

Evaluation of cAMP analogues and agonists. Several genes have been shown to be responsive to cAMP-mediated gene regulation, including some in SMC (i.e., 23, 26, 31). In addition, cAMP has been shown to activate PK-G Iα and Iβ in vitro (7) and in the intact cell (13, 32). For these reasons, the effects of the cAMP analogue dibutyryl cAMP (DB-cAMP) on PK-G I expression in cultured SMC were evaluated. As shown in Fig. 5, 500 μM DB-cAMP resulted in a marked suppression of PK-G I mRNA levels in early passage, postconfluent bovine SMC. By 24 h of treatment, the PK-G I mRNA levels were 15% of baseline, and this effect was sustained at the 48-h time point. These data indicate that both cyclic nucleotides, cAMP and cGMP, suppress PK-G I mRNA levels in vascular SMC. The phosphodiesterase inhibitor theophylline also suppressed PK-G, to 46% of baseline at 24 h of treatment.

Table I. Activation of PK-A in Bovine Aortic SMC

Treatment	PK-A activity ratio (−cAMP/+cAMP)
None	0.33±0.04
Forskolin (5 μM)	0.93±0.02
SNAP (100 μM)	0.91±0.05
SNP (100 μM)	0.96±0.06

Treatment of bovine SMC with the nitrovasodilators SNAP and SNP resulted in marked activation of PK-A, to > 90% of maximal activity, despite the fact that SNAP and SNP treatment were not associated with increases in cAMP levels. The adenylyl cyclase activator forskolin served as a positive control. An activity ratio of 1.0 represents completely activated PK-A. The untreated SMC had a PK-A activity ratio of 0.33, indicating 33% of maximal activation in control conditions. The results are given as the mean±SEM for $n = 3$.

Evaluation of activation of PKA by nitrovasodilators. To determine whether or not nitrovasodilators activated PK-A by cGMP-mediated cross-activation of PK-A (30, 32), the activity ratio of PK-A was determined after exposure of bovine aortic SMC to SNAP and SNP. As shown in Table I, a 5-min exposure to either NO-donor drug activated PK-A in the intact cell to a similar extent as did the adenylyl cyclase activator, forskolin. This activation of PK-A by SNAP and SNP occurred despite the fact that neither drug elevated cAMP levels (not shown). These data suggested that cGMP cross-activated PK-A in bovine aortic SMC similar to previous reports of cGMP activation of PKA in rat aortic SMC (30).

In vivo effects of continuous exposure of rats to the nitrovasodilator isosorbide dinitrate. It is well-established that prior exposure to nitrovasodilators results in resistance to these drugs over time. To determine whether continuous exposure of rats to the nitrovasodilator isosorbide dinitrate could sup-

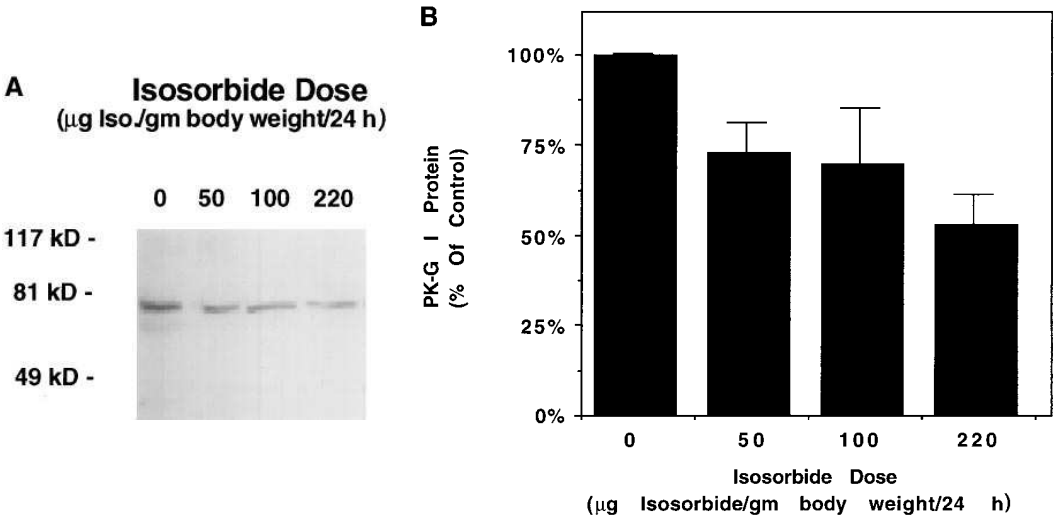

Figure 6. (*A*) Western blot analysis of PK-G I in extracts of isosorbide dinitrate–treated rat aorta. Rats were treated with various doses of isosorbide dinitrate for 4 d. Extracts of aortic tissue were analyzed for PK-G I antigen by Western blot. Equal amounts of extract protein (75 μg) were assayed, and immunoreactivity was visualized by enhanced chemiluminescence. A representative blot from one of three groups of rats is shown, illustrating decreased PK-G I antigen with increasing dose of isosorbide dinitrate ingested. (*B*) Suppression of PK-G I antigen in rat aorta. Cohorts of 400-g rats were given isosorbide dinitrate in the drinking water for 4 d and PK-G I protein was measured by Western blot analysis. Bands on autoradiograms after enhanced chemiluminescence were quantified by densitometry. Values are expressed as percentage of control animal PK-G I levels. Data are presented as the mean±SEM of three animals per group. A significant, dose-dependent suppression of PK-G I protein was observed ($P < 0.05$ for 50- and 100-μg doses, $P < 0.01$ for 220-μg dose).

press PK-G I levels, cohorts of 400-g rats were given water supplemented with isosorbide dinitrate at various concentrations. The actual dose received per animal was calculated by the volume of water consumed per 24 h and the concentration of drug in the water. No gross evidence of ill effects was seen in the animals. On the fourth day of treatment, the rats were killed, and PK-G I protein in the aorta was quantified by Western blot analysis. As illustrated in Fig. 6, *A* and *B*, the PK-G I levels were suppressed in a dose-dependent manner. By 4 d of treatment, the PK-G I protein levels were reduced by 50% in response to 220 μg isosorbide dinitrate/g body wt. The lower doses yielded a significant but more limited suppression.

As PK-G I is expressed in lung tissue (12, 14), the amount of PK-G I in lung tissue was assayed by Western blot analysis in the rats that had received isosorbide dinitrate or theophylline. As shown in Fig. 7, the levels of PK-G I protein were also suppressed to ~ 60% of baseline values in the lung in response to the 220-μg dose of isosorbide, and suppressed to 50% of baseline in response to 107 μg theophylline/g body wt. Doses of 100 μg or less isosorbide per day did not result in significant PK-G I suppression.

Discussion

We reported recently that the state of confluence affects the expression of PK-G I in SMC in culture (18). This study elaborates further on the mechanisms involved in regulating the expression of PK-G. The salient observation in this study is that PK-G I mRNA and protein are suppressed in SMC after continuous exposure to nitrovasodilators or analogues of cGMP or cAMP. Several models were used to demonstrate this effect independently. In tissue culture, PK-G I mRNA and protein were shown to be suppressed in bovine and rat SMC, respectively. Furthermore, three nitrovasodilators were shown to suppress PK-G I; SNAP and SNP were evaluated in vitro, and isosorbide dinitrate in vivo.

Our studies with PK-G I isoform–specific cDNA probes indicated that the predominant mRNA in vascular SMC was the Iα isoform, and that the level of Iβ isoform was below our ability to detect by Northern blot. This is similar to the recent report of Tamura et al. (14). The parallel changes in total PK-G I mRNA and Iα isoform support the conclusion that the cyclic nucleotide–mediated suppression of PK-G I was not due to a change in the isoform being expressed.

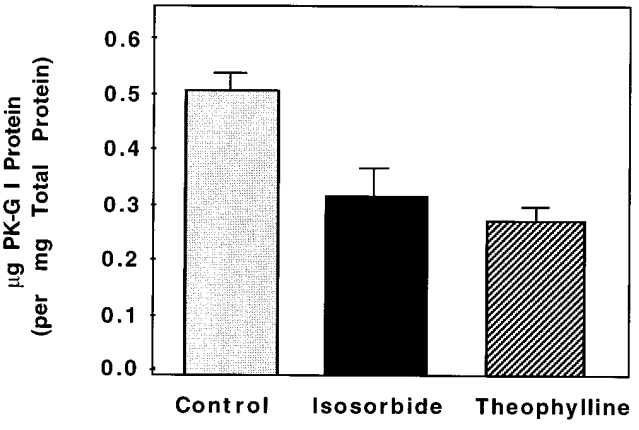

Figure 7. Suppression of PK-G I protein in rat lung tissue by exposure to isosorbide or theophylline. A significant, dose-dependent suppression of PK-G I protein in the rat lung was observed by 4 d of exposure to 220 μg/g/d of isosorbide ($P < 0.05$) and 107 μg/g/d theophylline ($P < 0.01$). Data are presented as mean±SEM of three animals per group.

Analysis of PK-G I mRNA half-life using the transcription inhibitor actinomycin D indicated no significant effect of SNAP on mRNA half-life. Furthermore, nuclear runoff studies demonstrated that SNAP suppresses PK-G I transcription in SMC. These data indicate that the nitrovasodilators suppress PK-G I levels in vascular SMC by suppressing transcription of the PK-G I gene. The mechanism of transcriptional regulation of the PK-G I gene is still unknown.

The comparable suppression of PK-G I by nitrovasodilators and cGMP analogues supports the model that the effect may be mediated through cGMP. However, it is not known if the effect is mediated through activation of PK-G I itself, or through other cGMP-dependent mechanisms. The selective cAMP analogue DB-cAMP mimicked the suppressive effect of cGMP analogues on PK-G I expression and appeared to be more potent than 8-Br-cGMP in suppressing PK-G I mRNA levels. It is also known that cGMP is capable of activating PK-A in vitro and in intact SMC, and may be the major mechanism by which NO and cGMP inhibit rat aortic SMC proliferation (30). The data reported here demonstrate a similar ability of nitrovasodilators to activate PK-A in bovine aortic SMC, and, therefore, are consistent with the hypothesis that the suppression of PK-G Iα may be mediated by continuous activation of PK-A. These findings may also provide an explanation for the suppression of PK-G I mRNA expression observed with repetitive passaging of SMC (18). Lermioglu et al. (33) observed that subconfluent cultures of rat aortic SMC have up to 100-fold higher concentrations of intracellular cGMP than confluent cultures of SMC. Repetitive passaging, which would subject cells to low density growth and high intracellular cGMP levels, could conceivably suppress PK-G I expression via activation of PK-A.

The results described in this study may have important implications for vascular diseases. A long-standing clinical problem has been the acquired resistance to nitroglycerin and other nitrovasodilators after continued exposure to the agents without an ~ 10–12-h nitrate-free period (34–37). Several mechanisms have been described as possibly contributing to nitrovasodilator resistance or tolerance. One of the more widely studied is that depletion of thiol moieties may be responsible for nitrovasodilator tolerance (i.e., 38–42). Formation of low molecular weight *S*-nitroso-thiols, such as *N*-acetylcysteine, facilitates NO entrance to the SMC of the vascular media (43). In several reports, administration of *N*-acetylcysteine may reverse nitrovasodilator resistance (38–42). However, other studies failed to demonstrate benefit of sulfhydryl repletion (44–46), and when tissue thiol levels have been evaluated directly, no deficiency was observed (47). The clinically beneficial effect of thiol administration may represent a compensation, not a correction, of the underlying defect. In other studies, administration of nitrovasodilators was associated with marked reduction in the levels of cGMP in platelets (48, 49). This supports the hypothesis that reduced platelet cGMP levels are a marker of nitrovasodilator tolerance, and possibly contribute to the phenomenon. A limitation to this observation is the retrospective, nonrandomized nature of the study, as well as the observation that platelet activation per se is asso-

ciated with reduced platelet cytoplasmic levels of cGMP (50). Other mechanisms that have been postulated to contribute to nitroglycerin tolerance include neurohormonal compensation (51). However, these changes are detected inconsistently, and are not causally associated with resistance to the hypotensive effect.

This report reveals an additional possibility for the mechanism of nitrovasodilator tolerance. Continuous exposure of SMC to nitrovasodilators suppresses PK-G I, the primary mediator of the vasorelaxant effect of NO. While additional experiments are required to define the extent to which PK-G I suppression contributes to loss of vasorelaxant response to subsequent doses of nitrovasodilators, it is plausible that a significant reduction of PK-G I will make the SMC less responsive to activation of the system.

Acknowledgments

This work was supported by grants-in-aid from the American Heart Association of Metropolitan Chicago and the Feinberg Institute for Cardiovascular Research (G.A. Soff), and by National Institutes of Health grants HL-34646 and HL-53426 (T.M. Lincoln).

References

1. Moncada, S., and A. Higgs. 1993. The L-arginine-nitric oxide pathway. *N. Engl. J. Med.* 329:2002–2012.

2. Lincoln, T.M., and T.L. Cornwell. 1993. Intracellular cyclic GMP receptor proteins. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 7:328–338.

3. Nathan, C., and Q.-W. Xie. 1994. Nitric oxide synthases: roles, tolls, and controls. *Cell.* 78:915–918.

4. Radomski, M.W., and S. Moncada. 1993. Regulation of vascular homeostasis by nitric oxide. *Thomb. Haemostasis.* 70:36–41.

5. Wolfe, L., S.H. Francis, and J.D. Corbin. 1989. Properties of a cGMP-dependent monomeric protein kinase from bovine aorta. *J. Biol. Chem.* 264: 4157–4162.

6. Mery, P.F., S.M. Lohmann, U. Walter, and R. Fischmeister. 1991. Ca^{2+} current is regulated by cyclic GMP-dependent protein kinase in mammalian cardiac myocytes. *Proc. Natl. Acad. Sci. USA.* 88:1197–1201.

7. Landgraf, W., P. Ruth, A. Keilbach, B. May, A. Welling, and F. Hofmann. 1992. Cyclic GMP-dependent protein kinase and smooth muscle relaxation. *J. Cardiovasc. Pharmacol.* 20(Suppl. 1):S18–S22.

8. Koga, T., Y. Yoshida, J.Q. Cai, M.O. Islam, and S. Imai. 1994. Purification and characterization of 240-kDa cGMP-dependent protein kinase substrate of vascular smooth muscle. Close resemblance to inositol 1,4,5-trisphosphate receptor. *J. Biol. Chem.* 269:11640–11647.

9. Komalavilas, P., and T.M. Lincoln. 1994. Phosphorylation of the inositol 1,4,5-trisphosphate receptor by cyclic GMP-dependent protein kinase. *J. Biol. Chem.* 269:8701–8707.

10. Archer, S.L., J.M. Huang, V. Hampl, D.P. Nelson, P.J. Shultz, and E.K. Weir. 1994. Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA.* 91:7583–7587.

11. Bolotina, V.M., S. Najibi, J.J. Palacino, P.J. Pagano, and R.A. Cohen. 1994. Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature.* 368:850–853.

12. Wernet, W., V. Flockerzi, and F. Hofmann. 1989. The cDNA of the two isoforms of bovine cGMP-dependent protein kinase. *FEBS (Fed. Eur. Biol. Soc.) Lett.* 251:191–196.

13. Francis, S.H., T.A. Woodford, L. Wolfe, and J.D. Corbin. 1988. Types I alpha and I beta isozymes of cGMP-dependent protein kinase: alternative mRNA splicing may produce different inhibitory domains. *Second Messengers Phosphoproteins.* 12:301–310.

14. Tamura, N., H. Itoh, Y. Ogawa, O. Nakagawa, M. Harada, T.-H. Chun, S. Suga, T. Yoshimasa, and K. Nakao. 1996. cDNA cloning and gene expression of human type Iα cGMP-dependent protein kinase. *Hypertension (Dallas).* 27: 552–557.

15. Gamm, D.M., S.H. Francis, T.P. Angelotti, J.D. Corbin, and M.D. Uhler. 1995. The type II isoform of cGMP-dependent protein kinase is dimeric and possesses regulatory and catalytic properties distinct from the type I isoforms. *J. Biol. Chem.* 270:27380–27388.

16. Markert, T., A.B. Vaandrager, S. Gambaryan, D. Pohler, C. Hausler, U. Walter, H.R. de Jonge, T. Jarchau, and S.M. Lohmann. 1995. Endogenous expression of type II cGMP-dependent protein kinase mRNA and protein in rat intestine. Implications for cystic fibrosis transmembrane conductance regulator. *J. Clin. Invest.* 96:822–830.

17. Jarchau, T., C. Hausler, T. Markert, D. Pohler, J. Vanderkerckhove, H.R. De Jonge, S.M. Lohmann, and U. Walter. 1994. Cloning, expression, and in situ localization of rat intestinal cGMP-dependent protein kinase II. *Proc. Natl. Acad. Sci. USA.* 91:9426–9430.

18. Cornwell, T.L., G.A. Soff, A.E. Traynor, and T.M. Lincoln. 1994. Regulation of the expression of cyclic GMP-dependent protein kinase by cell density in vascular smooth muscle cells. *J. Vasc. Res.* 31:330–337.

19. Brown, K.E., R. Lawrence, and G.E. Sonenshein. 1991. Concerted modulation of alpha 1(XI) and alpha 2(V) collagen mRNAs in bovine vascular smooth muscle cells. *J. Biol. Chem.* 266:23268–23273.

20. Majors, A.K., and L.A. Ehrhart. 1992. Cell density and proliferation modulate collagen synthesis and procollagen mRNA levels in arterial smooth muscle cells. *Exp. Cell Res.* 200:168–174.

21. Rovner, A.S., R.A. Murphy, and G.K. Owens. 1986. Expression of smooth muscle and nonmuscle myosin heavy chains in cultured vascular smooth muscle cells. *J. Biol. Chem.* 261:14740–14745.

22. Shirinsky, V.P., K.G. Birukov, V.E. Kotelliansky, M.A. Glukhova, E. Spanidis, J.D. Rogers, J.H. Campbell, and G.R. Campbell. 1991. Density-related expression of caldesmon and vinculin in cultured rabbit aortic smooth muscle cells. *Exp. Cell Res.* 194:186–189.

23. Soff, G.A., R.W. Jackman, and R.D. Rosenberg. 1991. Expression of thrombomodulin by smooth muscle cells in culture: different effects of tumor necrosis factor and cyclic adenosine monophosphate on thrombomodulin expression by endothelial cells and smooth muscle cells in culture. *Blood.* 77:515–518.

24. Gunther, S., R.W. Alexander, W.J. Atkinson, and M.A Gimbrone, Jr. 1982. Functional angiotensin II receptors in cultured vascular smooth muscle cells. *J. Cell Biol.* 92:289–298.

25. Smith, J.B., and T.A. Brock. 1983. Analysis of angiotensin stimulated sodium transport in cultured smooth muscle cells from rat aorta. *J. Cell. Physiol.* 114:284–290.

26. Traynor, A.E., D.L. Cundiff, and G.A. Soff. 1995. cAMP influence on transcription of thombomodulin and myogenic proteins in smooth muscle is dependent on de novo synthesis of a protein intermediate. *J. Lab. Clin. Med.* 126: 316–323.

27. Nevins, J. 1987. Isolation and analysis of nuclear RNA. *In* Methods in Enzymology: Guide to Molecular Cloning Techniques. S. Berger and A. Kimmel, editors. Academic Press Inc., San Diego. 234–241.

28. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

29. Forte, L.R., P.K. Thorne, S.L. Eber, W.J. Krause, R.H. Freeman, S.H. Francis, and S.D. Corbin. 1992. Stimulation of intestinal Cl^- transport by heat-stable enterotoxin: activation of cAMP-dependent protein kinase by cGMP. *Am. J. Physiol.* 263:C607–C615.

30. Cornwell, T.L., E. Arnold, N.J. Boerth, and T.M. Lincoln. 1994. Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. *Am. J. Physiol.* 267:C1405–C1413.

31. Ohara, O., T. Nakano, H. Teraoka, and H. Arita. 1991. cAMP negatively regulates mRNA levels of actin and tropomyosin in rat cultured vascular SMC. *J. Biochem.* 109:834–839.

32. Jiang, H., J.B. Shabb, and J.D. Corbin. 1992. Cross-activation: overriding cAMP/cGMP selectivities of protein kinases in tissues. *Biochem. Cell Biol.* 70:1283–1289.

33. Lermioglu, F., J. Goyal, and A. Hassid. 1991. Cell density modulates the decrease of cytosolic free Ca^{2+} induced by atrial natriuretic hormone, *S*-nitroso-*N*-acetylpenicillamine and 8-bromo cyclic GMP in cultured rat mesangial cells. *Biochem. J.* 274:323–328.

34. Colditz, G.A., K.T. Halvorsen, and S.Z. Goldhaber. 1988. Randomized clinical trials of transdermal nitroglycerin systems for the treatment of angina: a meta-analysis. *Am. Heart J.* 116:174–180.

35. Parker, J.O. 1989. Intermittent transdermal nitroglycerin therapy in the treatment of chonic stable angina. *J. Am. Coll. Cardiol.* 13:794–795.

36. Thadani, U., H.L. Fung, A.C. Darke, and J.O. Parker. 1982. Oral isosorbide dinitrate in angina pectoris: comparison of duration of action and dose-response relation during acute and sustained therapy. *Am. J. Cardiol.* 49: 411–419.

37. Bassan, M.M. 1990. The daylong pattern of the antianginal effect of long-term thee times daily administered isosorbide dinitrate. *J. Am. Coll. Cardiol.* 16:936–940.

38. May, D.C., J.J. Popma, W.H. Black, S. Schaefer, H. Lee, B.D. Levine, and L.D. Hillis. 1987. In vivo induction and reversal of nitroglycerin tolerance in human coronary arteries. *N. Engl. J. Med.* 317:805–809.

39. Horowitz, J.D., E.M. Antman, B.H. Lorell, W.H. Barry, and T.N. Smith. 1983. Potentiation of the cardiovascular effects of nitroglycerin by *N*-acetylcysteine. *Circulation.* 68:1247–1253.

40. Torresi, J., J.D. Horowitz, and G.J. Dusting. 1985. Prevention and reversal of tolerance to nitroglycerin with *N*-acetylcysteine. *J. Cardiovasc. Pharmacol.* 7:777–783.

41. Packer, M., W.H. Lee, P.D. Kessler, S.S. Gottlieb, N. Medina, and M.

Yushak. 1987. Prevention and reversal of nitrate tolerance in patients with congestive heart failure. *N. Engl. J. Med.* 17:799–804.

42. Levy, W.S., R.J. Katz, R.L. Ruffalo, R.H. Leiboff, and A.G. Wasserman. 1988. Potentiation of the hemodynamic effects of acutely administered nitroglycerin by methionine. *Circulation.* 78:640–645.

43. Scharfstein, J.S., J.F. Keaney, Jr., A. Slivka, G.N. Welch, J.A. Vita, J.S. Stamler, and J. Loscalzo. 1994. In vivo transfer of nitric oxide between a plasma protein–bound reservoir and low molecular weight thiols. *J. Clin. Invest.* 94: 1432–1439.

44. Parker, J.O., B. Farrell, K.A. Labey, and B.F. Rose. 1987. Nitrate tolerance: the lack of effect of *N*-acetylcysteine. *Circulation.* 76:572–576.

45. Munzel, T., J. Holtz, A. Mulsch, D.J. Stewart, and E. Bassenge. 1989. Nitrate tolerance in epicardial arteries or in the venous system is not reversed by *N*-acetylcysteine in vivo, but tolerance-independent interactions exist. *Circulation.* 79:188–197.

46. Dupuis, J., G. Lalonde, R. Lemieux, and J.L. Rouleau. 1990. Tolerance to intravenous nitroglycerin in patients with congestive heart failure. Role of increased volume, neurohumoral activation and lack of prevention with *N*-acetylcysteine. *J. Am. Coll. Cardiol.* 16:932–935.

47. Boesgaard, S., J. Aldershvile, H.E. Poulsen, S. Loft, M.E. Anderson, and A. Meister. 1994. Nitrate tolerance in vivo is not associated with depletion of arterial or venous thiol levels. *Circ. Res.* 74:115–120.

48. Watanabe, H., M. Kakihana, S. Ohtsuka, T. Enomoto, K. Yasui, and Y. Sugishita. 1993. Platelet cyclic GMP. A potentially useful indicator to evaluate the effects of nitroglycerin and nitrate tolerance. *Circulation.* 88:29–36.

49. Baines, C., R.S. Szwarc, and H.A. Ball. 1994. Parallel tolerance between platelet cyclic GMP and preload effects of nitroglycerin in anaesthetized minipigs. *Br. J. Pharmacol.* 113:334–335.

50. Wu, X.B., B. Brune, F. Von Appen, and V. Ullrich. 1993. Efflux of cyclic GMP from activated human platelets. *Mol. Pharmacol.* 43:564–568.

51. Bauer, J.A., H.L. Fung, W. Zheng, Y.W. Kwon, M. Gopalakrishnan, and D.J. Triggle. 1993. Continuous versus intermittent nitroglycerin administration in experimental heart failure: vascular relaxation and radioligand binding to adrenoceptors and ion channels. *J. Cardiovasc. Pharmacol.* 22:600–608.