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Antimitogenic Actions of Organic Nitrates Are Potentiated by Sildenafil and Mediated Via Activation of Protein Kinase A

MICHAEL T. OSINSKI, BERNHARD H. RAUCH, and KARSTEN SCHRÖR

Institut für Pharmakologie und Klinische Pharmakologie, Heinrich-Heine-Universität Düsseldorf, Germany

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ABSTRACT

Migration and proliferation of vascular smooth muscle cells (SMC) in response to platelet-derived growth factor (PDGF) and other mitogens play an important role in restenosis after coronary angioplasty. Elevation of both cAMP and cGMP has been shown to inhibit SMC mitogenesis. The aim of this study was to examine the antimitogenic actions of organic nitrates and sildenafil and to clarify the role of cyclic nucleotide-dependent protein kinases (PKA, PKG) in this action. Organic nitrates [glycerol trinitrate (GTN), isosorbide 5′-mononitrate (ISMN), pentaerythrityl-tetranitrate (PETN)] and the PDE5 inhibitor sildenafil reduced PDGF-induced DNA synthesis, measured by (^{3}H]thymidine incorporation. GTN, ISMN, and PETN acted synergistically with sildenafil (1 μM) on inhibition of PDGF-induced DNA synthesis, increase of intracellular cyclic nucleotides, and vasodilator-stimulated phosphoprotein phosphorylation. The highly selective PKA inhibitor PKI abolished these actions of sildenafil and organic nitrates, whereas the PKG inhibitors KT5823 and (*R*p)-8-pCPT-cGMPS had no effect. In addition, selective activation of PKG without inhibition of PDE3 by the cGMP analog 8-pCPT-cGMP (100 μM) had no antimitogenic effect. The data suggest that 1) organic nitrates and sildenafil exert antimitogenic actions by activation of PKA via inhibition of PDE3, but not by activation of PKG and 2) that antimitogenic effects of organic nitrates are potentiated by sildenafil at therapeutic plasma levels.

Proliferation of vascular smooth muscle cells (SMC) is a typical event in the pathogenesis and progression of atherosclerosis (Reines and Ross, 1993). SMC proliferation also occurs after percutaneous coronary interventions subsequent to endothelial injury (Ross and Fuster, 1996) and is mediated by growth factors. One of these growth factors is platelet-derived growth factor (PDGF), which initiates mitogenic signaling by activation of the MAP kinase pathway (Davis et al., 1993; Claesson-Welsh, 1994).

NO, formed endogenously by the endothelium or pharmacologically by release from organic nitrates, increases the intracellular cGMP concentration in SMC by stimulation of the soluble guanylate cyclase. The subsequent elevation of cellular cGMP levels has been associated with an antimitogenic effect in SMC (Cornwell et al., 1994; Yu et al., 1997). It has been also demonstrated that NO donors and structural analogs of cGMP inhibit SMC proliferation (Yu et al., 1997; Cornwell et al., 1994). Finally, administration of NO donors or L-arginine, the substrate of NO synthase, has been shown to reduce intimal thickening after coronary angioplasty in clinical trials in men (Lablanche et al., 1997). Despite these data demonstrating antimitogenic effects of locally enhanced NO levels, no consistent antimitogenic action has been reported for organic nitrates (Bult et al., 2000). Therefore, a more detailed identification of mechanisms involved in the antimitogenic action of cGMP is necessary to understand the mechanism of cell proliferation associated with bioactive NO generation subsequent to endothelial injury.

Cyclic nucleotides bind to and activate specific cyclic nucleotide-dependent protein kinases (PKA, PKG). Several studies have demonstrated that agents that stimulate PKA inhibit mitogen-induced phosphorylation of the p42/p44 MAP kinases (ERK-1/2) and DNA synthesis in SMC (Bönisch et al., 1998; Zucker et al., 1998). One possibility to stimulate PKA activity is accumulation of cAMP by inhibition of its breakdown by phosphodiesterases (PDEs). Selective inhibitors of the cGMP-inhibited PDE3 have been shown to prevent mitogenesis of SMC both, in in-vitro and in clinical studies (Tsuchikane et al., 1999; Osinski and Schrör, 2000). Thus, antiproliferative effects related to an increase in intracellular cGMP might also involve PKA activation (Cornwell et al.,

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ABBREVIATIONS: SMC, smooth muscle cells; PDGF, platelet-derived growth factor, isoform BB; MAP mitogen-activated protein; PKA, protein kinase A; PKG, protein kinase G; PDE, phosphodiesterase; GTN, glycerol trinitrate; ISMN, isosorbide 5′-mononitrate; PETN, pentaerythrityl tetranitrate; PKI, myristoylated protein kinase A inhibitor peptide; 8-pCPT-cGMP, 8-(*p*-chlorophenylthio)-cGMP; (*R*p)-8-pCPT-cGMPS, 8-(4-chlorophenylthio)guanosine-3′,5′-cyclic monophosphorothioate, *R*p isomer; VASP, vasodilator-stimulated phosphoprotein; IBMX, 3-isobutyl-1-methylxanthine.

1994). Short-acting nitrates (e.g., GTN) might not elevate cGMP levels long enough to allow sufficient cAMP elevation. In addition, GTN might have a promitogenic effect by generation of oxygen radicals (Bult et al., 2000).

The aim of this study was to elucidate in more detail the mechanism of cGMP-dependent antimitogenic action in SMC. Specially, we wanted to know whether elevation of cGMP exerts an antimitogenic effect by itself or whether any antimitogenic action is indirect in nature (e.g., by primary inhibition of PDE3 and subsequent elevation of cAMP). Three different organic nitrates were used as NO donors: GTN, ISMN, and PETN. Sildenafil was included as a selective inhibitor of PDE5 (Boolell et al., 1996), which inhibits cGMP breakdown, eventually resulting in a more prolonged action of organic nitrates. Whether or not a highly selective PDE5 inhibitor, such as sildenafil, suppresses PDGF-induced mitogenesis of SMC by itself has not been explored.

Materials and Methods

Cell Culture. Bovine coronary artery SMC were isolated as described previously (Zucker et al., 1998). The cells were cultivated in 80% Ham's F-12/20% Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were grown in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cell culture material was purchased from Life Technologies (Eggenstein, Germany) or Becton-Dickinson (Heidelberg, Germany).

Determination of DNA Synthesis. SMCs were seeded in 24-well plates with serum-free medium for 3 days. PDE inhibitors (Biomol, Hamburg, Germany) were preincubated for 10 min and the cells then stimulated with 10 ng/ml PDGF, isoform BB (Sigma, Deisenhofen, Germany). When the protein kinase inhibitors KT5823 (Sigma), (*R*p)-8-pCPT-cGMPS, and myristoylated protein kinase inhibitor peptide (PKI; Promega, Mannheim, Germany) were used, they were added for another 10 min before addition of GTN, ISMN, PETN (ISIS Chemie, Zwickau, Germany), sildenafil (extracted from Viagra; Pfizer, Karlsruhe, Germany) and the PKG activator 8-pCPT-cGMP (Biolog, Bremen, Germany). After 20 h, [^{3}H]thymidine (0.5 μCi/well; DuPont, Bad Homburg, Germany) was added. At the end of the total incubation period of 24 h, the media were removed and the cells were washed twice with 1 ml ice-cold PBS, 0.3 ml of ice-cold perchloric acid (0.3 M), and again with cold PBS. The cells were solubilized with 0.3 ml NaOH (0.1 M) for 30 min at 37°C. [^{3}H]Thymidine incorporation was measured by liquid scintillation counting. All measurements were done in triplicate.

p42/44 MAP Kinase Phosphorylation. SMCs, kept in serum-free medium as described above, were stimulated with PDGF (10 ng/ml) for 10 min. If indicated, the organic nitrates and sildenafil were added 10 min before the mitogen. The p42/p44 MAP kinase phosphorylation was detected by Western blotting using phospho-specific antibodies (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. Briefly, proteins were harvested into SDS sample buffer [6.25 mM Tris-HCl, pH 6.8, 2% SDS (w/v), 10% glycerol, 50 mM dithiothreitol], separated by SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). For immunodetection, membranes were probed with phospho-specific MAP kinase antibodies (1:1000; New England Biolabs) followed by incubation with peroxidase-conjugated secondary antibodies (1:3000; Dianova, Hamburg, Germany). Blots were mounted with amidoblack to control protein loading of the lanes.

VASP Phosphorylation. SMC were harvested after a stimulation period of 10 min with the compound to be studied. In experiments with inhibitors of PKA and PKG, the inhibitors were added 10 min before sildenafil and the organic nitrates. SDS-polyacrylamide gel electrophoresis and the following steps were carried out as described above, the vasodilator-stimulated phosphoprotein (VASP)-antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Both, dephospho-VASP (p46) and phospho-VASP (p50), were detected by the antibody. Blots were mounted with amidoblack (BioRad, München, Germany) to control protein loading of the lanes.

PKA Activity. PKA activity in SMC was determined using a nonradioactive protein kinase assay kit (Calbiochem, San Diego, CA). Subconfluent SMC were fasted for 48 h in serum-free medium and stimulated with forskolin (100 nM, Sigma, Deisenhofen, Germany) for 10 min. When combined with sildenafil (30 μM), the PDE3-inhibitor trequinsin (30 nM; Biomol) or the nonspecific PDE-inhibitor IBMX (1 mM; Sigma), forskolin was added after preincubation for 10 min with these compounds. Cells were lysed in 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 0.3% β-mercaptoethanol, 1% phenylmethylsulfonyl fluoride of a saturated solution in isopropanol and 10 mM Benzamidine, then briefly sonicated and centrifuged at 12,000*g* for 3 min at 4°C. Supernatants were assayed for PKA activity by enzyme-linked immunosorbent assay according to the manufacturer's instructions. PKA activity was referred to that sensitive to 1.0 μM specific PKA inhibitor (PKI; Sigma). PKA activity ratio was calculated as described by Torphy et al. (1982).

Intracellular Concentration of Cyclic Nucleotides. Cells were seeded in 6-well plates in serum-free medium for 72 h. Then, the cells were washed twice with 2 ml of a balanced salt solution containing 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5.5 mM glucose and 20 mM HEPES, pH 7.3. Organic nitrates and sildenafil were added for 10 min. When forskolin was combined with sildenafil and/or organic nitrates, these were incubated for 10 min before forskolin was added for another 10 min. The reaction was stopped by removing the buffer and by addition of ice-cold ethanol (96%). After the ethanol was evaporated, intracellular cAMP and cGMP levels were determined by radioimmunoassay as described previously (Schröder and Schrör, 1993). All experiments were carried out in duplicate.

Statistics. Data are mean ± SE of n independent experiments, performed in duplicate or triplicate as indicated. Statistical significance was determined using Tukey's test of means. $P < 0.05$ was considered significant.

Results

Effects of Organic Nitrates and Sildenafil on PDGF-Induced DNA Synthesis. Cell proliferation is preceded by DNA synthesis, taken as a measure to determine cell proliferation. Addition of PDGF-BB (10 ng/ml) stimulated DNA synthesis in SMC 7 ± 1-fold above control (n = 24, data not shown). This effect was concentration-dependently inhibited by sildenafil at 1 to 30 μM (Fig. 1). The basal DNA synthesis under resting conditions was not affected (data not shown). PETN, ISMN, and GTN inhibited mitogenesis by 25, 40, and 10%, respectively, at the highest concentrations studied. However, in combination with 1 μM sildenafil, which by itself did not inhibit mitogenesis, there was a clear potentiation of the antimitogenic response. Interestingly, there was an almost complete inhibition of mitogenesis by the combined administration of ISMN and PETN with sildenafil (1 μM) but not by combined administration with the shorter-acting GTN (Figs. 2 to 4).

Effects of Sildenafil and Organic Nitrates on Intracellular Cyclic Nucleotide Accumulation. Sildenafil and NO donors have been reported to increase intracellular cGMP levels in SMC (Jeremy et al., 1997; Turko et al., 1999). We confirmed these findings in bovine coronary artery SMC with sildenafil (1 μM), ISMN (10 μM), and PETN (3 μM).

ISMN and sildenafil increased intracellular cGMP concentration synergistically. Forskolin (100 nM), a direct adenylate cyclase activator, had no effect on cGMP levels (Table 1).

In the same samples, cAMP levels were measured (Table 1). In the absence of forskolin, no marked effect of organic nitrates and sildenafil on cAMP concentration was seen. Only the combination of ISMN or PETN and sildenafil caused a light elevation of cAMP level in SMC. In contrast, the combination of 100 nM forskolin with sildenafil and ISMN increased the intracellular cAMP concentration 3-fold. The combination of both agents elevated cAMP 7-fold (Table 1). With PETN (3 μM), similar results where obtained (Table 1): PETN together with forskolin elevated cAMP levels 2-fold; in combination with sildenafil, a 5-fold increase of cellular cAMP concentration was observed.

Effects of Sildenafil and PDE3-Inhibition on PKA Activity. To investigate the effect of sildenafil on PKA, we measured PKA activity by enzyme-linked immunosorbent assay and calculated the PKA activity ratio. The PKI (1.0 μM) inhibitable PKA activity was taken as reference. Forskolin was used to stimulate intracellular cAMP levels. Both sildenafil and the selective PDE3-inhibitor trequinsin, used to compare the efficacy of sildenafil-induced PDE3-inhibition, were combined with forskolin. We found that combina-

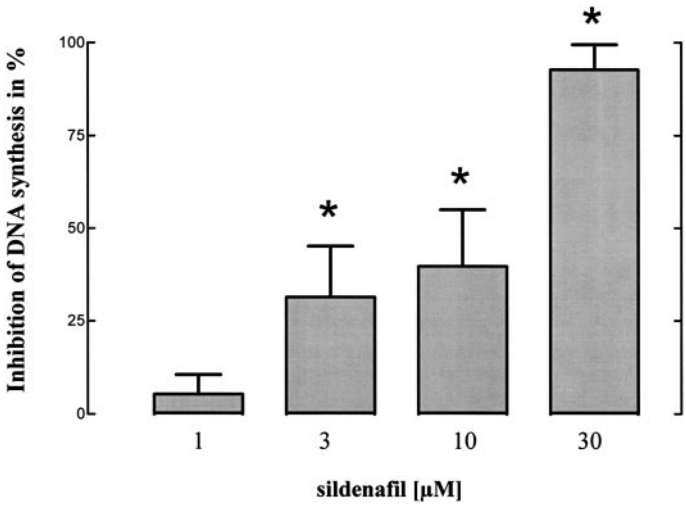

Fig. 1. Inhibition of PDGF-BB (10 ng/ml)-induced DNA synthesis in SMC by sildenafil (1–30 μM). Sildenafil was preincubated 10 min before stimulation with PDGF. Data are mean ± SE of three independent experiments performed in triplicate. *$p < 0.05$ versus PDGF.

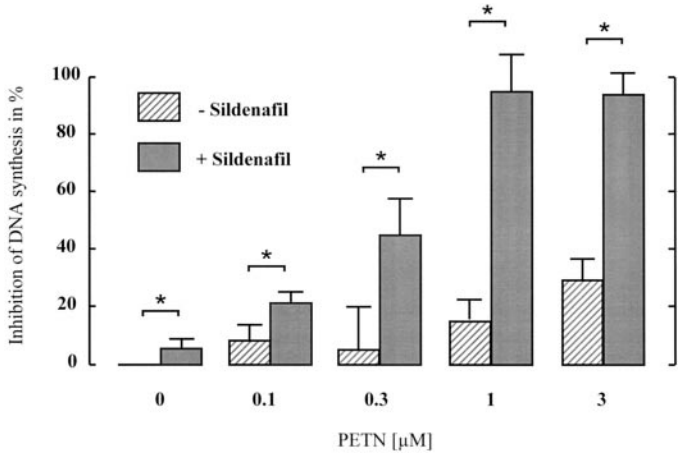

Fig. 2. Inhibition of PDGF-BB (10 ng/ml)-induced DNA synthesis in SMC by PETN (0.1–3 μM) in the absence and presence of sildenafil (1 μM). Sildenafil was preincubated 10 min before PETN and 20 min before PDGF. Data are mean ± SE of three independent experiments performed in triplicate. *$p < 0.05$ PETN versus PETN + sildenafil.

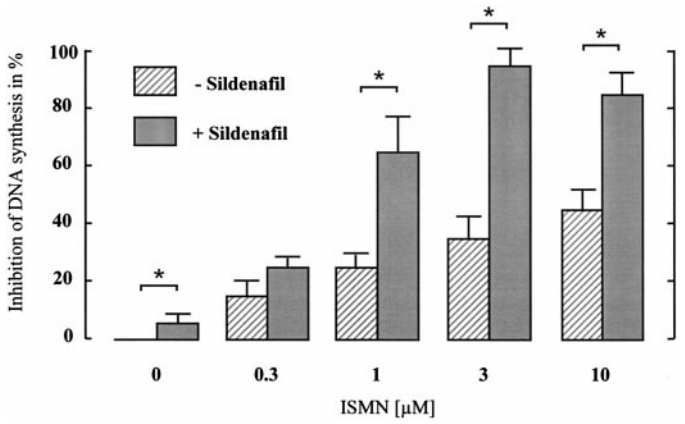

Fig. 3. Inhibition of PDGF-BB (10 ng/ml)-induced DNA synthesis in SMC by ISMN (0.3–10 μM) in the absence and presence of sildenafil (1 μM). Sildenafil was preincubated 10 min before ISMN and 20 min before PDGF. Data are mean ± SE of three independent experiments performed in triplicate. *$p < 0.05$ ISMN versus ISMN + sildenafil.

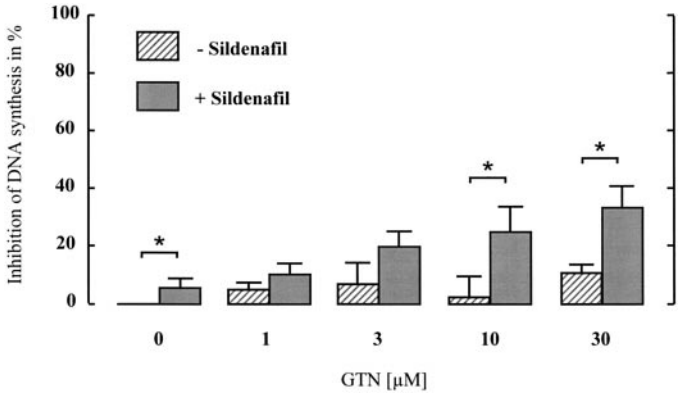

Fig. 4. Inhibition of PDGF-BB (10 ng/ml)-induced DNA synthesis by GTN (1–30 μM) in the absence and presence of sildenafil (1 μM). In these concentrations, neither GTN nor sildenafil had any significant effect on DNA synthesis. Sildenafil was preincubated 10 min before GTN and 20 min before PDGF. Data are mean ± SE of three independent experiments performed in triplicate. *$p < 0.05$ GTN versus GTN + sildenafil.

TABLE 1

Effects of sildenafil and organic nitrates on intracellular cyclic nucleotide concentration.

Data are mean ± SE of three independent experiments performed in duplicate.

Compounds	cAMP	cGMP
	pmol/mg/protein	
− Forskolin (100 nM)		
Control	1.6 ± 0.4	0.3 ± 0.1
Sildenafil (1 μM)	1.6 ± 0.3	0.9 ± 0.3*
ISMN (10 μM)	1.8 ± 0.0	0.9 ± 0.3*
Sildenafil (1 μM) + ISMN (10 μM)	2.8 ± 0.2*	2.4 ± 0.4*
PETN (3 μM)	1.4 ± 0.1	0.9 ± 0.1*
PETN (3 μM) + Sildenafil (1 μM)	2.2 ± 0.4	2.4 ± 0.1*
+ Forskolin (100 nM)		
Control	2.2 ± 0.4	0.4 ± 0.1
Sildenafil (1 μM)	2.6 ± 0.3	0.7 ± 0.3
ISMN (10 μM)	2.5 ± 0.2	0.7 ± 0.1*
Sildenafil (1 μM) + ISMN (10 μM)	12.3 ± 1.2*	2.3 ± 0.2*
PETN (3 μM)	4.4 ± 0.3*	0.8 ± 0.3*
PETN (3 μM) + Sildenafil (1 μM)	10.2 ± 1.2*	2.3 ± 0.1*

* $p < 0.05$ versus control.

tion of forskolin with sildenafil or trequinsin caused a comparable activation of PKA. The combination of forskolin with the nonselective PDE-inhibitor IBMX strongly stimulated PKA activity (Fig. 5).

Role of PKG on PDGF-Induced DNA Synthesis. To elucidate the significance of PKG-stimulation for antimitogenic effects of organic nitrates and sildenafil, the cGMP analog 8-pCPT-cGMP was used. This selective PKG activator (Geiger et al., 1992) did not inhibit SMC mitogenesis (data not shown). In the same concentrations (10 μM and 100 μM), 8-pCPT-cGMP has no effects on PKA activity and also does not inhibit PDE3 (Geiger et al., 1992). To determine whether 8-pCPT-cGMP actually increased intracellular PKG activity, the 8-pCPT-cGMP–induced VASP phosphorylation was determined. 8-pCPT-cGMP (10 μM and 100 μM) caused a phosphorylation-dependent shift from 46 to 50 kDa, indicating intracellular PKG activation by 8-pCPT-cGMP (Fig. 6).

The PKG inhibitors KT5823 and (*R*p)-8-pCPT-cGMPS (Butt et al., 1994; Francis and Corbin, 1994) were also used to study the possible involvement of PKG in the antimitogenic actions of sildenafil and organic nitrates. As shown in Fig. 7, no significant influence of the PKG inhibitor on the antimitogenic effects of sildenafil was detected. Similar negative results were obtained with KT5823 (data not shown). In addition, the antimitogenic effect of the combined administration of organic nitrates with sildenafil (1 μM) was not significantly influenced by the PKG inhibitors (data not shown).

Role of PKA on PDGF-Induced DNA Synthesis. The alternative possibility to explain the antimitogenic actions is activation of PKA. In contrast to the PKG inhibitors, the highly selective PKA inhibitor PKI_{myr} abolished the antimitogenic action of sildenafil nearly completely (Table 2). In experiments with ISMN and PETN together with sildenafil (1 μM), a comparable inhibition of the antimitogenic effect by PKI_{myr} was observed. PKI_{myr} itself did not exhibit any effect on either stimulation of DNA synthesis by PDGF on untreated cells (data not shown).

Effects of Sildenafil and Organic Nitrates on PDGF-Induced p42/p44 MAP Kinase Phosphorylation. One of the central mitogenic signaling pathways of PDGF and other mitogens is the MAP kinase cascade (Davis et al., 1993). After stimulation with PDGF, maximal phosphorylation of the p42/p44 MAP kinase (ERK1/2) was seen after 10 min (data not shown). Therefore, this time point was chosen for the experiments with sildenafil and organic nitrates on the PDGF-induced phosphorylation of MAP kinase. Preincubation with sildenafil inhibited the phosphorylation of p42/p44 MAP kinase in a concentration-dependent manner (Fig. 8). After preincubation with sildenafil (1 μM), PETN and ISMN inhibited PDGF-induced p42/p44 MAP kinase phosphorylation. The selective PKA inhibitor PKI_{myr} reversed this effect of sildenafil and organic nitrates (Fig. 8).

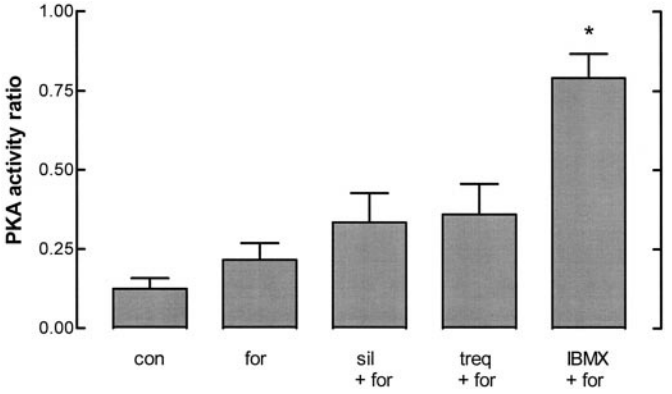

Fig. 5. Effect of sildenafil (30 μM) and the specific PDE3 inhibitor trequinsin (30 nM) on PKA activity, measured by enzyme-linked immunosorbent assay. Cells were preincubated with these compounds, then stimulated with forskolin (100 nM) for another 10 min. PKA activity ratio was calculated as described by Torphy et al. (1982). Combination of forskolin with the nonselective PDE inhibitor IBMX (1 mM) markedly activated PKA in the cells. A smaller but comparable activation was seen with trequinsin and sildenafil, respectively. Data are mean ± SE of five independent experiments performed in duplicate or triplicate.

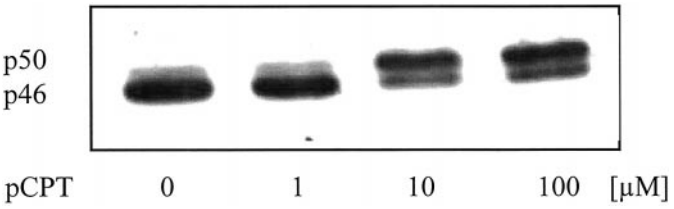

Fig. 6. Effect of the PKG agonist 8-pCPT-cGMP (pCPT) on VASP phosphorylation. The cGMP analog was added for 10 min. Dephospho-VASP (p46) and phospho-VASP (p50) were detected by Western blotting with specific VASP antibodies. The figure shows a representative experiment of three with similar results.

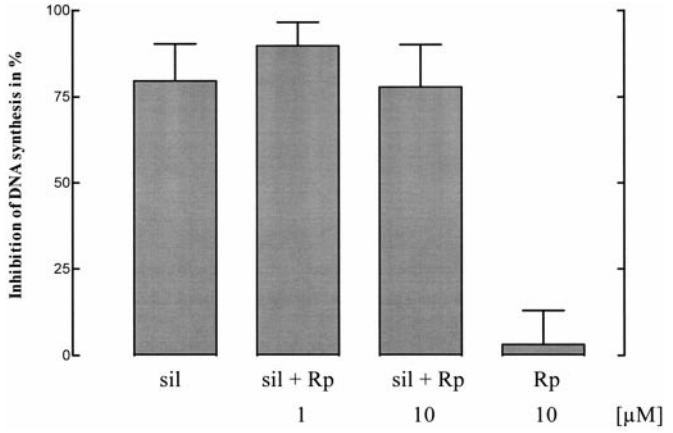

Fig. 7. Inhibition of PDGF-BB (10 ng/ml)-induced DNA synthesis by sildenafil (sil, 30 μM) and the influence of the selective PKG inhibitor (*R*p)-8-pCPT-cGMPS (Rp, 1 and 10 μM). (*R*p)-8-pCPT-cGMPS was added for 10 min before sildenafil and 20 min before PDGF. Data are mean ± SE of three independent experiments performed in triplicate.

TABLE 2

Effects of sildenafil, organic nitrates, and the PKA inhibitor PKI_{myr} on PDGF (10 ng/ml)-induced DNA synthesis in SMC.

Data are mean ± SE of three independent experiments performed in triplicate.

Compounds	Inhibition of PDGF-Induced DNA Synthesis
	%
sildenafil (1 μM)	5 ± 12
sildenafil (30 μM)	93 ± 15*
sildenafil (30 μM) + PKI_{myr} (1 μM)	25 ± 9**
ISMN (10 μM)	42 ± 7*
ISMN (10 μM) + sildenafil (1 μM)	92 ± 8*
ISMN (10 μM) + sildenafil (1 μM) + PKI_{myr} (1 μM)	12 ± 8**
PETN (3 μM)	30 ± 6*
PETN (3 μM) + sildenafil (1 μM)	87 ± 8*
PETN (3 μM) + sildenafil (1 μM) + PKI_{myr} (1 μM)	26 ± 7**

* $p < 0.05$ versus control.
** $p < 0.05$ versus sildenafil + organic nitrate.

PKA and/or PKG-Dependent Phosphorylation of VASP. VASP is phosphorylated by PKA and/or PKG (Halbrügge et al., 1990; Makert et al., 1996). Therefore, VASP phosphorylation was taken as an indicator for target protein phosphorylation by cyclic nucleotide-dependent protein kinases. The nitric oxide donors GTN (10 μM), ISMN (10 μM), and PETN (3 μM), as well as sildenafil, caused a phosphorylation of VASP in SMC. In Western blot analysis, the phosphorylation resulted in a band-shift from 46 to 50 kDa. Figure 9 shows the phosphorylation of VASP induced by 1 μM and 30 μM sildenafil and 10 μM ISMN. When the selective PKG inhibitor (*R*p)-8-pCPT-cGMPS was added 10 min before sildenafil or ISMN, the phosphorylation-dependent shift was reduced. The same effect was seen when the PKA inhibitor PKI_{myr} was added before 30 μM sildenafil or before 10 μM ISMN. Only the combination of PKI_{myr} and (*R*p)-8-pCPT-cGMPS resulted in a complete inhibition of VASP phosphorylation induced by sildenafil and ISMN.

Discussion

This study demonstrates that sildenafil causes a significant antimitogenic effect in SMC. Similar yet somehow smaller responses were seen with PETN and ISMN. There was a clear potentiation of these reactions by the combined administration of sildenafil and organic nitrates; however, this reaction was mediated not by PKG but by PKA. This suggests that accumulation of cGMP results in primary inhibition of PDE3 with subsequent stimulation of PKA. A schematic presentation of this hypothesis, including the interaction of PDEs 3 and 5, cyclic nucleotides, and their corresponding protein kinases is shown on Fig. 10.

Although the inhibition of SMC proliferation by both cyclic nucleotides, cAMP and cGMP, has been reported before (Cornwell et al., 1994; Grosser et al., 1995), the antimitogenic signaling pathway of cGMP is a matter of debate. The highly selective PKA inhibitor PKI almost completely abolished the antimitogenic effects of sildenafil and NO donors. Unfortunately, selective inhibition of PKG is much more difficult to obtain than inhibition of PKA. In this study, the PKG inhibitors KT5823 and the cGMP analog (*R*p)-8-pCPT-cGMPS were used. It has been reported that KT5823 inhibits not only PKG, but also PKA, and might have additional effects on other enzymes (Wyatt et al., 1991; Murad et al., 1993). To determine whether both PKG inhibitors permeate the cell membrane and actually inhibit PKG, their influence on VASP phosphorylation was measured by Western blotting. Although (*R*p)-8-pCPT-cGMPS had no effect on basal VASP phosphorylation, it inhibited the phosphorylation of VASP by sildenafil and organic nitrates, and KT5823 stimulated basal VASP phosphorylation (data not shown). This might account for its poor selectivity for PKG activation. With the two structurally different PKG inhibitors KT5823 and (*R*p)-8-pCPT-cGMPS, no significant inhibition of DNA synthesis and MAP kinase phosphorylation by sildenafil and organic nitrates was seen (Figs. 7 and 8). This suggests that the antimitogenic effect of organic nitrates and sildenafil is PKA-mediated. This conclusion was supported in experiments using the selective PKG agonist 8-pCPT-cGMP. This cGMP analog phosphorylated VASP at concentrations $\geq$ 10 μM (Fig. 6), but it did not influence PDGF-induced DNA synthesis, even at 100 μM.

The results of this study are consistent with the findings of

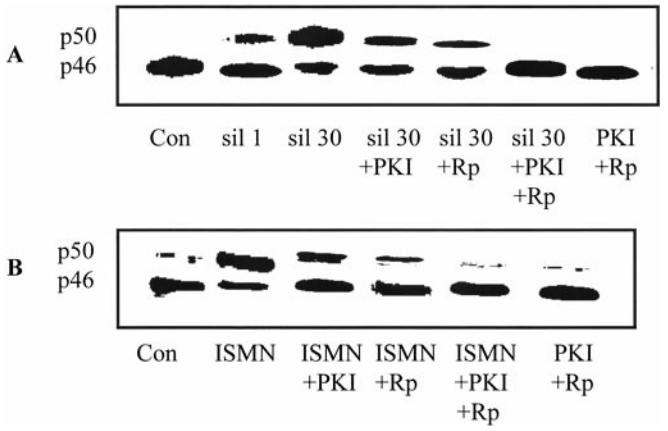

Fig. 9. A, VASP phosphorylation induced by sildenafil (sil, 1 and 30 μM) and inhibition of sildenafil-induced VASP phosphorylation by the PKA inhibitor PKI_{myr} (PKI, 1 μM) and the PKG inhibitor (*R*p)-8-pCPT-cGMPS (Rp, 10 μM). B, VASP phosphorylation induced by ISMN (10 μM) and inhibition of sildenafil-induced VASP phosphorylation by the PKA inhibitor PKI_{myr} (PKI) and the PKG inhibitor (*R*p)-8-pCPT-cGMPS (Rp, 10 μM). Cells were treated for 10 min with ISMN or sildenafil. If indicated, protein kinase inhibitors were added for 10 min. Dephospho-VASP (p46) and phospho-VASP (p50) were detected by Western blotting with specific VASP antibodies. The figure shows representative experiments of three with similar results.

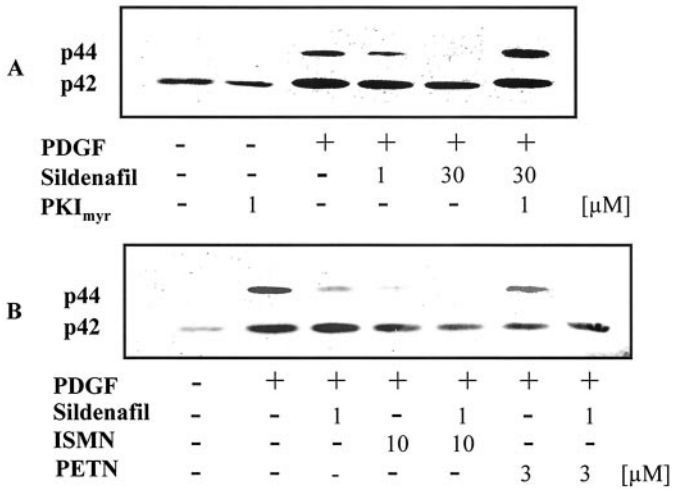

Fig. 8. A, influence of the selective PKA inhibitor PKI (1 μM) on the effect of sildenafil (30 μM) on PDGF (10 ng/ml)-induced p42/p44 MAP kinase phosphorylation. B, action of sildenafil (1 μM), PETN (3 μM), and ISMN (10 μM) on PDGF (10 ng/ml)-induced p42/p44 MAP kinase phosphorylation. The figure shows representative experiments of three with similar results.

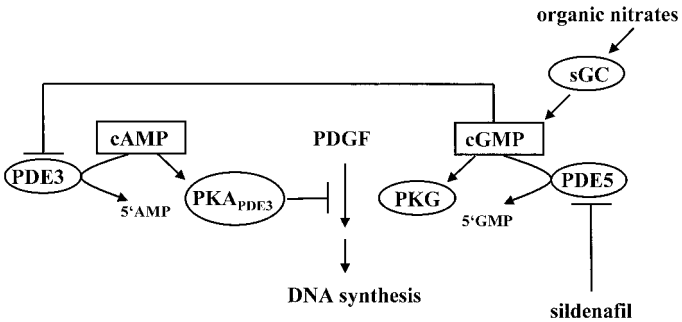

Fig. 10. Suppression of DNA synthesis via PDE3 inhibition by cGMP accumulation induced by sildenafil and/or organic nitrates.

other authors. Southgate and Newby (1990) reported that the PKA agonist 8-bromo-cAMP, but not the PKG agonist 8-bromo-cGMP, had antimitogenic effects in rabbit SMC. In another study, it was demonstrated that 8-bromo-cAMP inhibited SMC proliferation in vivo (Indolfi et al., 1997). Cornwell et al. (1994) demonstrated antimitogenic effects of elevated cGMP levels by NO donors in rat SMC. As in our study, pCPT failed to inhibit mitogenesis. Collectively, their results suggest that PKA activity is stimulated secondary to accumulation of cAMP after inhibition of its breakdown by PDE3.

To clarify the signal transduction of sildenafil and organic nitrates in SMC, cyclic nucleotide concentrations were determined. Elevation of cGMP levels by ISMN, PETN, and sildenafil has been reported previously (Jeremy et al., 1997; Turko et al., 1999) and was confirmed here. Interestingly, there was also an increase in cAMP concentration by sildenafil, PETN, and ISMN in the same magnitude as obtained with selective inhibitors of PDE3 (Osinski and Schrör, 2000). The stronger antimitogenic effects of PETN and ISMN compared with GTN might be explained by a longer-lasting NO delivery and subsequent higher cGMP synthesis.

There was a tendency toward increased forskolin-stimulated PKA activity by sildenafil to levels similar to those of the selective PDE3 inhibitor trequinsin (Fig. 5). Antiproliferative effects by inhibition of PDE3 in the same cell system have been shown previously and were also accompanied by only a small increase in cAMP (Osinski and Schrör, 2000) as well as antimitogenic effects of forskolin (Grosser et al., 1995) and cAMP (Assender et al., 1992). Cornwell et al. (1994) have also shown increases in cAMP and subsequent PKA stimulation by PKA-dependent antimitogenic agents. The reason for the very small increase in global cAMP levels may be an additional activity of PDE4, representing the second major cAMP metabolizing enzyme in SMC (Polson and Strada, 1996; Osinski and Schrör, 2000). Metabolization of cAMP by PDE4 might overcome the inhibition of PDE3 in terms of regulating the total cellular cAMP concentrations. However, for antimitogenic actions, the local cAMP concentration in the subcellular compartment regulated by PDE3 might be more important than the total intracellular cAMP level as shown by experiments using selective inhibitors of PDE3 and PDE4 (Chini et al., 1997; Osinski and Schrör, 2000).

In rat aortic SMC, somewhat different findings have been reported. As in our study, Yu et al. (1997) demonstrated antimitogenic actions of cGMP-elevating agents. However, suppression of the epidermal growth factor-induced SMC mitogenesis was not reversed by the PKA inhibitor 3′,5′-cyclic monophosphorothioate, *R*p isomer (10 μM). On the other hand, KT5823 (10 μM) inhibited the antimitogenic effects of the cGMP-elevating agents (Yu et al., 1997). These differences might be explained by different species, cell culture conditions, and mitogens used. In addition, 10 μM KT5823 might not be selective for PKG: IC_{50} value for PKA inhibition, 4 μM; IC_{50} for PKG inhibition, 0.2 μM (Kase et al., 1987).

In conclusion, this study demonstrates for the first time that sildenafil suppresses mitogenesis of SMC, that this effect is synergistic with that of organic nitrates and is mediated via activation of PKA but not PKG. One might speculate that a similar mechanism of activation might also be involved in relaxation of the corpus cavernosum (Stief et al., 2000). Sildenafil might also potentiate the actions of other cAMP-elevating agents, including sympathomimetics, eventually resulting in arrhythmias and a fall of blood pressure (Shah, 1998; Wallis et al., 1999). The clinical significance of these findings remains to be determined.

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Send reprint requests to: Dr. Karsten Schrör, Institut für Pharmakologie und Klinische Pharmakologie, Heinrich-Heine-Universität, Moorenstr. 5, 40225 Düsseldorf, Germany. E-mail: kschroer@uni-duesseldorf.de