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Inhibition of cyclic GMP-dependent protein kinase-mediated effects by (Rp)-8-bromo-PET-cyclic GMPS

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1 The modulation of the guanosine 3':5'-cyclic monophosphate (cyclic GMP)- and adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase activities by the diastereomers of 8-bromo-βphenyl-1,N^2-ethenoguanosine 3',5'-cyclic monophosphorothioate, ((**Rp**)- and (Sp)-8-bromo-PET-cyclic GMPS) was investigated by use of purified protein kinases. In addition, the effects of (**Rp**)-8-bromo-PET-cyclic GMPS on protein phosphorylation in intact human platelets and on [^{3}H]-noradrenaline release and neurogenic vasoconstriction in electrical field stimulated rat tail arteries were also studied.

2 Kinetic analysis with purified cyclic GMP-dependent protein kinase (PKG) type Iα and Iβ, which are expressed in the rat tail artery, revealed that (**Rp**)-8-bromo-PET-cyclic GMPS is a competitive inhibitor with an apparent K_i of 0.03 μM. The activation of purified cyclic AMP-dependent protein kinase (PKA) type II was antagonized with an apparent K_i of 10 μM.

3 In human platelets, (**Rp**)-8-bromo-PET-cyclic GMPS (0.1 mM) antagonized the activation of the PKG by the selective activator 8-(4-chlorophenylthio)-guanosine 3':5'-cyclic monophosphate (8-pCPT-cyclic GMP; 0.2 mM) without affecting the activation of PKA by (Sp)-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3':5'-cyclic monophosphorothioate ((Sp)-5,6-DCl-cyclic BiMPS; 0.1 mM).

4 (**Rp**)-8-bromo-PET-cyclic GMPS was not hydrolysed by the cylcic GMP specific phosphodiesterase (PDE) type V from bovine aorta but potently inhibited this PDE.

5 The corresponding sulphur free cyclic nucleotide of the two studied phosphorothioate derivatives, 8-bromo-β-phenyl-1,N^2-ethenoguanosine-3':5'-cyclic monophosphate (8-bromo-PET-cyclic GMP), had no effect on electrically-induced [^{3}H]-noradrenaline release but concentration-dependently decreased the stimulation-induced vasoconstriction. (**Rp**)-8-bromo-PET-cyclic GMPS (3 μM) shifted the vasoconstriction response to the right without affecting stimulation evoked tritium overflow.

6 The NO donor, 3-morpholinosydnonimine (SIN-1) relaxed rat tail arteries precontracted with phenylephrine (1 μM). The SIN-1 concentration-relaxation curve was shifted in a parallel manner to the right by (**Rp**)-8-bromo-PET-cyclic GMPS, suggesting that the relaxation was mediated by a cyclic GMP/PKG-dependent mechanism.

7 The [^{3}H]-noradrenaline release-enhancing effect and stimulation-induced decrease in vasoconstriction of forskolin were unaffected by (**Rp**)-8-bromo-PET-cyclic GMPS. Moreover, the forskolin concentration-relaxation curve was not changed in the presence of the PKG inhibitor, suggesting a high selectivity in intact cells for PKG- over PKA-mediated effects.

8 The results obtained indicate that (**Rp**)-8-bromo-PET-cyclic GMPS presently is the most potent and selective inhibitor of PKG and is helpful in distinguishing between cyclic GMP and cyclic AMP messenger pathways activation. Therefore, this phosphorothioate stereomer may be a useful tool for studying the role of cyclic GMP *in vitro*.

Keywords: Cyclic GMP; cyclic nucleotide-dependent protein kinases; protein phosphorylation; neurogenic vasoconstriction; noradrenaline release; human platelets; rat tail artery

Introduction

Although protein kinase G (PKG) is considered to be one of the major targets for guanosine 3':5'-cyclic monophosphate (cyclic GMP), this cyclic nucleotide also regulates other intracellular proteins such as cyclic GMP-binding cyclic nucleotide phosphodiesterases and ion channels (Walter, 1989; Lincoln & Cornwell, 1993; Butt *et al.*, 1993). Cell-membrane-permeant analogues of cyclic GMP have been widely used to elucidate the functional role of PKG in the regulation of biological processes (Sekhar *et al.*, 1992; Butt *et al.*, 1993). The development of analogues in which one of the exocyclic oxygen atom of the phosphate group was substituted with a sulphur atom resulted in phosphorothioate analogues composed of two diasteromers which were shown to have different properties (Rothermel *et al.*, 1983; Butt *et al.*, 1990; Dostmann *et al.*, 1990; Butt *et al.*, 1994b). **Rp** analogues (with the sulphur in the equatorial position with respect to the sugar ring) are antagonists of the protein kinase whereas the Sp analogues, (with the sulphur in the axial position) are activators. The first synthesized **Rp** analogue of cyclic GMP, (**Rp**)-cyclic GMPS, was of limited use due to its weak antagonistic activity, non-specificity and very low lipophilicity (Butt *et al.*, 1990). In the search for useful inhibitors of PKG, a newly developed **Rp** analogue, 8-(4-chlorophenylthio)-guanosine 3':5'-cyclic monophosphorothioate [(**Rp**)-8-pCPT-cyclic GMPS], with increased lipophilicity, was recently shown to be a useful tool for studying the role of PKG in intact tissues (Butt *et al.*, 1994b).

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Here we describe the development and some properties of a more active PKG inhibitor, 8-bromo-β-phenyl-1,N^2-ethenoguanosine 3',5'-cyclic monophosphorothioate [(**Rp**)-8-bromo-PET-cyclic GMPS], which was designed in analogy to the potent PKG activator 8-bromo-β-phenyl-1,N^2-ethenoguanosine-3':5'-cyclic monophosphate (8-bromo-PET-cyclic GMP; Sekhar *et al.*, 1992). The present study describes the effects of (**Rp**)-8-bromo-PET-cyclic GMPS on cyclic GMP-dependent protein kinase-mediated protein phosphorylation in intact human platelets, on both neurogenic vasoconstriction and modulation of noradrenaline release, and on SIN-1-induced relaxation of phenylephrine-preconstricted rat tail arteries.

Methods

Biochemical studies

Protein kinase assay The type II PKA and the soluble type Iα PKG were purified from bovine heart and lung, respectively, as described earlier (Kaczmarek *et al.*, 1980; Walter *et al.*, 1980). Human type Iβ PKG was expressed in Sf-9 cells and purified by affinity chromatography (Pöhler *et al.*, 1994). The activity of the purified kinases was measured by the phosphocellulose method (Roskoski, 1983) using Kemptide as substrate with minor modifications (Butt *et al.*, 1990). Briefly, protein kinase activity was assayed at 30°C in a total volume of 100 μl containing 20 mM Tris/HCl buffer (pH 7.4), 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.01% (w/v) bovine serum albumin, 10 μg Kemptide (130 μM), 75 ng protein kinase and cyclic nucleotide as indicated. The reaction was started by the addition of 50 μM [γ-^{32}P]-ATP (about 100 c.p.m. pmol^{-1}) and terminated by the addition of 0.1 M EDTA. PK activity is expressed as amount of phosphate transferred min^{-1} mg^{-1} protein kinase.

Phosphodiesterase assays The type V PDE was purified from bovine aorta by use of DEAE-trisacryl column and the activity of the purified enzyme was measured at 1 μM substrate concentration as previously described (Lugnier *et al.*, 1986). Both incubation time and enzyme concentration were adjusted so that no more than 15% of the substrate was hydrolysed under the assay conditions. The IC_{50} value (concentration of the drug which inhibited 50% of the enzymatic activity) was calculated by linear regression, and the IC_{50} values was converted to the apparent K_i value according to the equation of Cheng and Prusoff (1973).

High performance liquid chromatography (h.p.l.c.) Metabolic stability towards PDE V was monitored by h.p.l.c. (**Rp**)-8-bromo-PET-cyclic GMPS was incubated with phosphodiesterase as described for inhibition experiments above and analysed repeatedly (2 h) for potential degradation to polar metabolites. Isocratic reversed phase chromatography with u.v. detection (254 nm) was performed by use of a RP-18 column (250 × 4 mm, i.d., 10 μm), 22.5% acetonitrile and 50 mM phosphate buffer at a flow rate of 1.5 ml min^{-1}. Moreover, the lipophilicity (expressed as log K_W) was determined by h.p.l.c. (Braumann & Jastorff, 1985).

Phosphorylation experiments with human intact platelets Freshly donated blood was mixed 5:1 (v/v) with 100 mM sodium citrate, 7 mM citric acid, 140 mM glucose (pH 6.5) as anticoagulant and centrifuged 10 min at 300 *g* to yield platelet-rich plasma (PRP). PRP was centrifuged 20 min at 500 *g* and the platelet pellet was resuspended in an isotonic buffer containing 10 mM HEPES buffer (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 5.5 mM glucose and 1 mM EDTA at a density of 10^9 cells ml^{-1}. Aliquots were incubated at 37°C with reagents for the times indicated. At various time points, platelet aliquots were removed, mixed with a sodium dodecyl sulphate-containing stop-solution and boiled. Phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) was then measured by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blot analysis as described previously (Halbrügge *et al.*, 1990).

Reverse transcription-polymerase chain reaction (RT-PCR) Tail arteries were dissected under sterile conditions and frozen in liquid nitrogen. Bovine lung and aorta were obtained fresh from a local abattoir, frozen in liquid nitrogen and ground using Spex 6700 Freezer/mill. Total RNA was extracted by use of RNA Now (Biogentex) and RT-PCR performed with Perkin-Elmer reagents and standard conditions with the following cycle times and temperatures: RT was performed at 42°C for 30 min, followed by denaturation at 99°C for 5 min and then 5°C for 5 min; PCR was performed with 30 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min. PCR products were analysed on ethidium bromide-stained agarose gels. Primers were designed to amplify the 5' coding regions of G-kinase Iα and Iβ (Wernet *et al.*, 1989) that are different between the two enzyme isotypes. Hence, the primers for Iα were 5'-TCTAGAGGATCCATGAGCGAGCTGGAGGAAGACTTTG (upstream) and 5'-GATATCGAATTCTTTCGGATTTGGTGAACTTCCGGA (downstream) and for Iβ were 5'-GCTGAATTCATGGGCACCTTGCGGGATTTACAGT (upstream) and 5'-AGCGGATCCTGGGGTAGAAGGGCAGGGTCACATG (downstream). Downstream primers were used during reverse transcription.

Functional studies

Electrically-evoked [^{3}H]-noradrenaline release and vasoconstriction Experiments were carried out as described previously (Bucher *et al.*, 1992). Briefly, a segment of about 2–2.5 cm of the proximal part of the ventral rat tail artery was dissected out and kept in oxygenated (95% O_2/5% CO_2) medium of the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 0.9, $NaHCO_3$ 25, glucose 11, ascorbic acid 0.3 and disodium EDTA 0.03. The arteries were cannulated at one end and preincubated for 1 h in 1.5 ml of medium containing in addition 2.2 μM (−)-[^{3}H]-noradrenaline. They were then suspended vertically, distal end uppermost, between two platinum wire electrodes and perfused with physiological saline solution containing 10 μM cocaine. The intraluminal perfusion pressure was determined with a pressure transducer and recorded on a pen recorder. Changes in perfusion pressure reflected changes in the resistance to flow, i.e. the degree of vasoconstriction. Each artery was subjected to 4 stimulation periods (24 pulses at 0.4 Hz; 0.3 ms; 200 mA; intervals between stimulation periods 16 min). The compounds were infused into the perfusion stream for 8 min before S_3 until the end of the experiment when the arteries were solubilized in 1 ml Soluene 100 (Packard Instrument). The fractional rate of tritium outflow and stimulation-evoked tritium overflow were expressed as a percentage of the tissue tritium content at the onset of the respective collection period. The effects of drugs added after S_2 were evaluated by calculating the ratio S_4/S_2 of the tritium overflow or vasoconstriction evoked during the respective stimulation periods.

Relaxation of phenylephrine-preconstricted arteries Experiments were carried out as described previously (Bucher *et al.*, 1992). Cumulative concentrations of SIN-1 or forskolin were applied to the arteries precontracted with 1 μM phenylephrine. After an interval of 2 h a second concentration-response curve of SIN-1 or forskolin alone or in the presence of (**Rp**)-8-bromo-PET-cyclic GMPS was constructed. The effect of cumulatively added SIN-1 or forskolin concentrations were expressed as % reduction of precontractions induced by phenylephrine, and these values were calculated in each individual preparation. The relative potency of SIN-1 or forskolin was determined from the comparison of the EC_{50} value: i.e. the concentration that produced 50% of the maximal effect, calculated by logit/log regression analysis.

Data and statistical analysis

Results are given as mean ± s.e.mean. n is the number of experiments. Comparisons were made by the Mann-Whitney U test if Kruskall-Wallis analysis indicated a significant difference between multiple groups. A probability level of 0.05 or less was considered significant. For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons (Wallenstein *et al.*, 1980).

Drugs

(−)-Noradenaline hydrochloride, (−)-phenylephrine hydrochloride, forskolin, adenosine 3':5'-cyclic monophosphate (sodium salt; cyclic AMP) and guanosine 3':5'-cyclic monophosphate (sodium salt; cyclic GMP) were purchased from Sigma. Kemptide was purchased from Peninsula. 8-Bromo-β-phenyl-1,N^2-ethenoguanosine-3':5'-cyclic monophosphate (sodium salt; 8-bromo-PET-cyclic GMP), 8-(4-chlorophenylthio)-guanosine 3':5'-cyclic monophosphate (sodium salt; 8-pCPT-cyclic GMP); (**Rp**)- and (**Sp**)-8-bromo-β-phenyl-1,N^2-etheno guanosine 3':5'-cyclic monophosphorothioate (sodium salt; (**Rp**)- and (**Sp**)-8-bromo-PET-cyclic GMPS) and (**Sp**)-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3':5'-cyclic monophosphorothioate (sodium salt; (**Sp**)-5,6-DCl-cyclic BiMPS) were obtained from Biolog (Bremen, Germany). 3-Morpholinosydononimine (SIN-1) was kindly donated by Hoechst, France. Stock solutions of all substances were prepared with Milli-Q water (Millipore) and diluted as required with the exception of SIN-1 which was initially dissolved in 5% glucose as a 4 mM solution and forskolin which was initially dissolved in ethanol 100% as a 8 mM solution. Both compounds were further diluted in water as required. (−)-[Ring-2,5,6-^{3}H]-noradrenaline, specific activity 43.7–56.9 Ci mmol^{-1} (New England Nuclear, Dreieich, Germany) was diluted with unlabelled (−)-noradrenaline hydrochloride in order to obtain a specific activity of 4.4 Ci mmol^{-1}. [γ^{32}P]-ATP and [^{125}I]-protein A were obtained from Amersham (Braunschweig, Germany). All other chemicals were from commercial sources.

Results

Biochemical studies

Analogues modified at the 1,N^2- and 8-position of cyclic GMP are potent PKG activators (Sekhar *et al.*, 1992). Based on this information we synthesized new (**Rp**)- and (**Sp**)-configured phosphorothioate derivatives of cyclic GMP, (**Rp**) and (**Sp**)-8-bromo-PET-cyclic GMPS. The structure of (**Rp**)-8-bromo-PET-cyclic GMPS is shown in Figure 1.

Protein kinase assay It has been shown that bovine aortic and tracheal smooth muscle contain the two known isozyme forms of PKG I (Keilbach *et al.*, 1992), designated Iα and Iβ. Therefore, we investigated the inhibitory effects of (**Rp**)-8-bromo-PET-cyclic GMPS in *in vitro* studies with purified bovine PKG Iα and recombinant human PKG Iβ. Kinetic analysis by double reciprocal plots revealed that (**Rp**)-8-bromo-PET-cyclic GMPS is a competitive inhibitor of both protein kinases with an identical apparent K_i of 0.03 μM (Table 1). In contrast, the (**Sp**)-diastereomer with an axial exocyclic sulphur atom, (**Sp**)-8-bromo-PET-cyclic GMPS, acts as an activator (Table 1). For complete analysis, we also present the data for the cyclic AMP-dependent protein kinase type II (apparent K_i of 10 μM; Table 1).

Phosphodiesterase assays The effects of the new analogues on the cyclic GMP-specific PDE type V purified from bovine aorta were studied. Both phosphorothioates, (**Rp**)- and (**Sp**)-8-bromo-PET-cyclic GMPS, as well as the corresponding cyclic nucleotide 8-bromo-PET-cyclic GMP were resistant to hydrolysis by PDE V. However, all three derivatives inhibited the cyclic GMP-specific PDE V with an apparent K_i of 12.6 ± 1.4 μM (n=4), 6.5 ± 0.3 μM (n=4) and 8.0 ± 1.8 μM (n=4) for respectively (**Rp**)- (**Sp**)-8-bromo-PET-cyclic GMPS and 8-bromo-PET-cyclic GMP. These compounds were nevertheless 20 to 40 times less potent than zaprinast (apparent K_i of 0.32 ± 0.02 μM; n=4; P<0.05), a specific PDE V inhibitor.

Since the potency of an inhibitor in intact cells is also dependent on its permeability, the lipophilicity (expressed as log K_W) was determined by h.p.l.c. With a log K_W of 2.83, (**Rp**)-8-bromo-PET-cyclic GMPS is about 90 times more lipophilic than (**Rp**)-cyclic GMPS (log K_W 0.89) and 1.7 times more lipophilic than (**Rp**)-8-pCPT-cyclic GMPS (Butt *et al.*, 1994b).

Intact cell studies To investigate whether extracellularly applied (**Rp**)-8-bromo-PET-cyclic GMPS is capable of selectively inhibiting intracellular PKG, we used human platelets since these cells contain high levels of both cyclic AMP- and cyclic GMP-dependent protein kinases (Eigenthaler *et al.*, 1992). One major substrate of both protein kinases is the 46/50 kDa vasodilator-stimulated phosphoprotein (VASP). Phosphorylation of VASP at Ser 157 results in a shift of its apparent

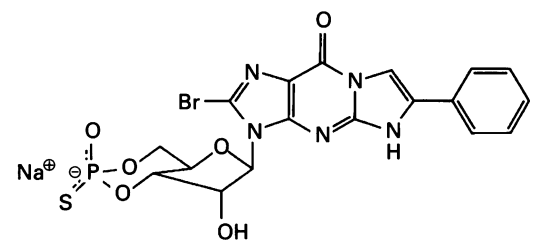

Figure 1 Chemical structure of (**Rp**)-8-bromo-β-phenyl-1,N^2-ethenoguanosine 3',5'-cyclic monophosphorothioate.

Table 1 Apparent activation constants K_a and inhibition constants K_i of bovine lung cyclic GMP-dependent protein kinase type Iα (PKG Ia), human placenta cyclic GMP-dependent protein kinase type Iβ (PKG Iβ) and bovine heart cyclic AMP-dependent protein kinase II (PKA II)

	PKG Iα		*PKG Iβ*		*PKA II*	
Compound	K_i (μM)	K_a (μM)	K_i (μM)	K_a (μM)	K_i (μM)	K_a (μM)
(**Rp**)-8-bromo-PET-cyclic GMPS	0.035 ± 0.005 (2)		0.030 ± 0.002 (3)		11 ± 1 (4)	
(**Sp**)-8-bromo-PET-cyclic GMPS		2.6 ± 0.5 (3)		2.5 ± 0.4 (3)		> 1000
8-Bromo-PET-cyclic GMP		0.013*		0.009*		

The data represent the mean ± s.e.mean from triplicate determinations from 2–4 separate kinase assays.
*The data have been published previously (Sekhar *et al.*, 1992).

molecular mass in SDS–PAGE from 46 to 50 kDa (Butt *et al.*, 1994a). A specific antibody developed against both forms of the VASP protein enabled us to study the protein phosphorylation in intact cells by Western blot technique. Washed human platelets were preincubated with or without 0.1 mM (**Rp**)-8-bromo-PET-cyclic GMPS and subsequently incubated with 0.2 mM of the selective cell membrane permeant PKG activator 8-pCPT-cyclic GMP (Butt *et al.*, 1992). In control cells, a conversion of VASP to the 50 kDa protein was visible within 30 min whereas only 3–5% phosphorylation was observed in cells preincubated with (**Rp**)-8-bromo-PET-cyclic GMPS (Figure 2a). The phosphorylation is totally blocked in platelets preincubated with 0.2 mM (**Rp**)-8-bromo-PET-cyclic GMPS before activation (data not shown) suggesting an inhibition of the cyclic GMP-dependent protein kinase in intact human platelets. In contrast, stimulation of platelets with 0.1 mM (**Sp**)-5,6-DCl-cyclic BiMPS, a selective activator of PKA (Sandberg *et al.*, 1991) converted up to 50% of the VASP protein to the 50 kDa form within 10 min in platelets with or without (**Rp**)-8-bromo-PET-cyclic GMPS pretreatment (Figure 2b) indicating that the cyclic AMP-dependent protein kinase is not inhibited by this cyclic GMP analogue under these conditions. At a higher concentration (0.5 mM (**Rp**)-8-bromo-PET-cyclic GMPS), partial inhibition of the PKA was observed.

RT-PCR studies Our recent studies with cyclic nucleotide analogues suggest that the postjunctional action of cyclic GMP in rat tail artery involves the activation of PKG Iα and probably Iβ (Ouedraogo *et al.*, 1994b). Therefore, we investigated the presence of PKG mRNA in this tissue. After 30 cycles of PCR, a clear product was observed using primers specific to the 5′ regions of Iα and Iβ PKG isozymes (Figure 3). The primers used are designed from the sequences of bovine G-kinase cDNA (Wernet *et al.*, 1989), which is known to be identical with human PKG Iβ sequence (Sandberg *et al.*, 1989) in this region. The gel bands showed the sizes expected for the PKG cDNA fragments, i.e. 265 bp (289 bp including PCR primers) for Iα (Figure 3a) and 301 bp (318 bp including PCR primers) for Iβ (Figure 3b). The identity of the cDNA was verified by sequencing and the gel products could also be identified in Southern blots by use of appropriate radiolabelled riboprobes (data not shown). The presence of PKG mRNAs was not due to contamination, as confirmed by the absence of PCR product in the control. It is therefore clear that mRNA encoding both PKG type I isotypes is transcribed in rat tail arteries.

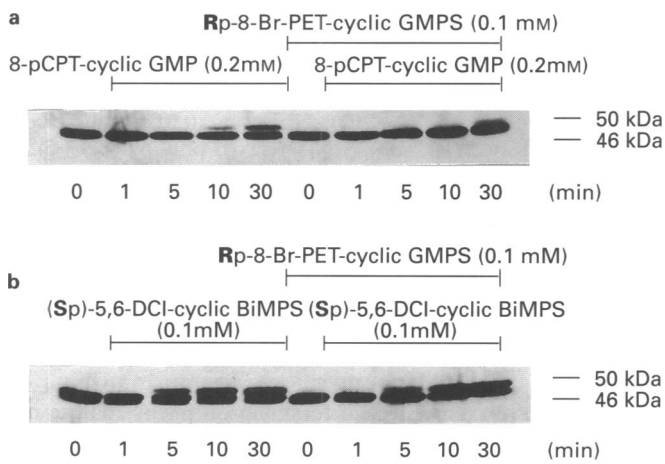

Figure 2 Western blot showing the inhibition of cyclic GMP-dependent protein kinase-mediated vasodilator-stimulated phosphoprotein (VASP) phosphorylation by (**Rp**)-8-bromo-PET-cyclic GMPS in intact human platelets. Washed human platelets were preincubated with or without 0.1 mM (**Rp**)-8-bromo-PET-cyclic GMPs for 30 min at 30°C followed by a time-dependent stimulation with 0.2 mM 8-pCPT-cyclic GMP (a) or 0.1 mM (**Sp**)-5,6-DCl-cyclic BiMPS (b) as indicated.

Functional studies

Electrically-evoked [^{3}H]-noradrenaline release and vasoconstriction In control as well as in treated arteries, tritium overflow and peak vasoconstriction evoked by the stimulation period S_2 amounted respectively to 0.253±0.007% of tissue tritium and 113.8±4.3 mmHg (n=73; all appropriate experiments pooled). Under control conditions, stimulation-evoked overflow of tritium and vasoconstriction did not change upon subsequent stimulations, resulting thus in S_4/S_2 ratio which were not different from unity (Figures 4 and 5) but the fractional rate of basal tritium outflow declined with time (not shown).

8-Bromo-PET-cyclic GMP (0.1–10 μM) had no significant effect on electrically-induced [^{3}H]-noradrenaline release (Figure 4a). However, 8-bromo-PET-cyclic GMP produced a concentration-dependent decrease in the stimulation-elicited vasoconstriction (Figure 4b). At the concentration tested (3 μM), the membrane permeant inhibitor of PKG (**Rp**)-8-bromo-PET-cyclic GMPS alone did not modify the tritium overflow or the vasoconstrictor response induced by perivascular nerve stimulation. However, it shifted the dose-response curve of the 8-bromo-PET-cyclic GMP effect to the right without affecting the stimulation-evoked [^{3}H]-noradrenaline overflow (Figure 4).

At an equimolar concentration (3 μM), (**Rp**)-8-bromo-PET-cyclic GMPS did not modify the inhibitory effect of forskolin (3 μM) on stimulation-induced vasoconstriction, nor did it change the facilitatory effect of this adenylyl cyclase stimulating drug on evoked tritium overflow (Figure 5). Therefore,

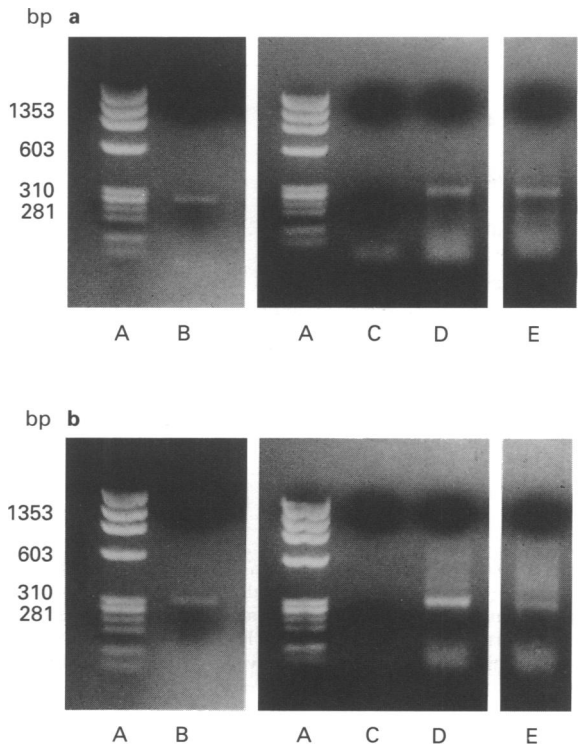

Figure 3 Ethidium-bromide-stained agarose gel showing products of RT-PCR using primers specific for protein kinase G (PKG) Iα (a) or Iβ (b). RNA was prepared from rat tail artery (lane B), bovine aorta (lane D) and bovine lung (lane E). Negative control, without RNA (lane C) and size standard ladders (lane A) are shown. bp: number of base pairs indicated on the left.

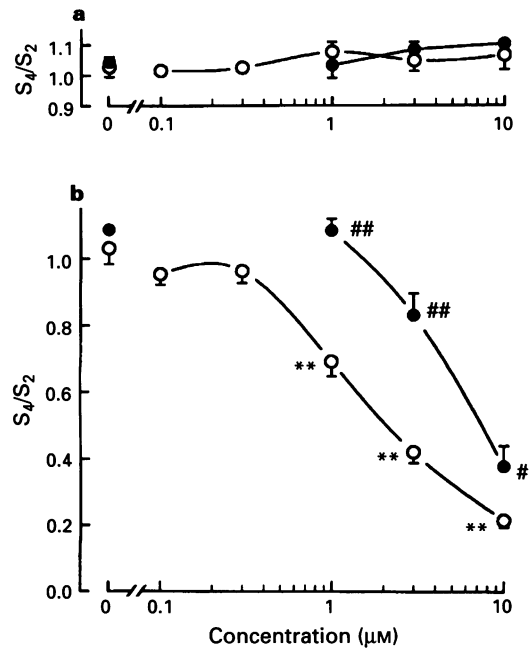

Figure 4 Effect of 8-bromo-β-phenyl-1,N^2-ethenoguanosine-3′:5′-cyclic monophosphate (8-bromo-PET-cyclic GMP) alone (○) or in combination with 3 μM (**Rp**)-8-bromo-PET-cyclic GMPS (●) on electrically-evoked overflow of tritium (a) and change in perfusion pressure (b) in rat tail arteries preincubated with [^{3}H]-noradrenaline. Four periods (S_1-S_4) of field stimulation were delivered. The effect of the drug is presented as the ratio of tritium overflow or change in perfusion pressure evoked by S_4 over that evoked by S_2. Each point represents the mean from 4–7 arteries; s.e.mean shown by vertical lines. $^{**}P<0.01$ vs control values; $\#P<0.05$, $\#\#P<0.01$ vs values in the absence of (**Rp**)-8-bromo-PET-cyclic GMPS.

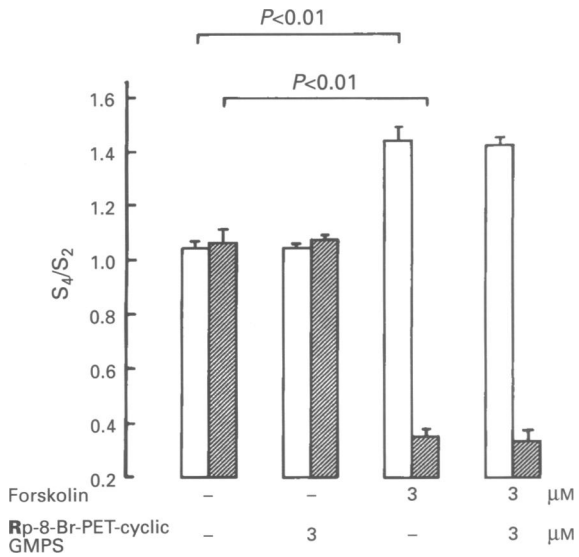

Figure 5 Effect of (**Rp**)-8-bromo-PET-cyclic GMPS and forskolin alone or in combination on electrically-evoked overflow of tritium (open columns) and change in perfusion pressure (hatched columns) in rat tail arteries preincubated with [^{3}H]-noradrenaline. Four periods (S_1-S_4) of field stimulation were delivered. The effect of the drug is presented as the ratio of tritium overflow or change in perfusion pressure evoked by S_4 over that evoked by S_2. Each column represents the mean ± s.e.mean from 6 arteries. Statistically significant differences are indicated.

these results indicate a good selectivity of (**Rp**)-8-bromo-PET-cyclic GMPS towards cyclic GMP/PKG-mediated effects on the vascular preparation.

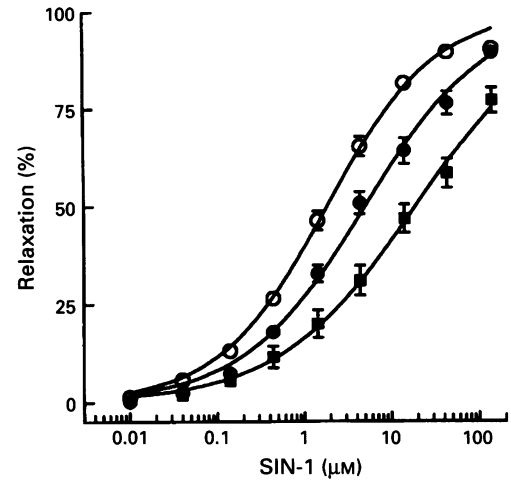

Figure 6 Concentration-dependent vasodilator effects of 3-morpholinosydnonimine (SIN-1) alone (○) or in the presence of (**Rp**)-8-bromo-PET-cyclic GMPS 10 μM (●) and 30 μM (■) on the change in perfusion pressure in rat tail arteries precontracted with exogenously-applied phenylephrine. Cumulative concentrations of SIN-1 were added after persistent vasoconstriction evoked by 1 μM phenylephrine. After two hours during which time the medium was replaced every 10 min, a second concentration-response curve to SIN-1 was constructed on the same arteries in the presence of the protein kinase G (PKG) antagonist. Each point represents the mean ± s.e.mean (vertical lines) from 6–15 arteries. The EC_{50} values in the presence of (**Rp**)-8-bromo-PET-cyclic GMPS differ significantly ($P<0.01$) from that in the absence of the PKG inhibitor.

Relaxation of phenylephrine-preconstricted arteries Figure 6 shows that cumulative concentrations of exogenously applied SIN-1 (0.01–100 μM) induced a concentration-dependent relaxation of rat tail arteries precontracted with 1 μM phenylephrine with a maximum relaxation (90.2 ± 1.1%, $n=15$) to slightly above the pre-phenylephrine tension and an EC_{50} of 1.85 ± 0.27 μM; $n=15$. A second concentration-dependent curve to SIN-1 constructed two hours after the first was not different from the former, indicating no time-dependent alteration in the relaxing response (EC_{50}: 1.66 ± 0.51 μM and 1.43 ± 0.40 μM; $n=7$; $P>0.05$; respectively for the first and second curve). Addition of (**Rp**)-8-bromo-PET-cyclic GMPS (10 μM) shifted, by about 4 fold, the SIN-1 concentration-response curve to the right (EC_{50}: 6.42 ± 1.50 μM; $n=9$; $P<0.01$) without affecting significantly the maximal effect of SIN-1 (Figure 6). In another series of experiments, in the presence of (**Rp**)-8-bromo-PET-cyclic GMPS (30 μM), the SIN-1 concentration-response curve was further shifted in a parellel manner (by approximately 12 fold) to the right (EC_{50}: 23.1 ± 4.5 μM; $n=6$; $P>0.01$). However, at the highest concentration tested (100 μM), SIN-1 relaxed only by 76.9 ± 3.2% the phenylephrine elicited constriction.

Forskolin (3–1000 nM) produced a concentration-dependent relaxation of rat tail arteries precontracted with 1 μM phenylephrine with a maximum relaxation close to the pre-phenylephrine tension level (97.8 ± 0.7%; $n=12$) and an EC_{50} of 64.4 ± 6.2 nM; $n=12$ (Figure 7). A second concentration-effect curve constructed 2 h after the first was not different (EC_{50}: 51.1 ± 3.9 nM; $n=6$; $P>0.05$). Figure 7 shows that the presence of (**Rp**)-8-bromo-PET-cyclic GMPS (10 μM) did not modify the relaxant effect of forskolin on phenylephrine precontracted rat tail arteries (EC_{50}: 56.7 ± 7.4 nM; $n=6$; $P>0.05$).

Discussion

Guanosine-3′,5′-cyclic monophosphate-dependent protein kinase (PKG) is one of several cyclic GMP-regulated proteins mediating the effects of intracellular cyclic GMP (Walter, 1989;

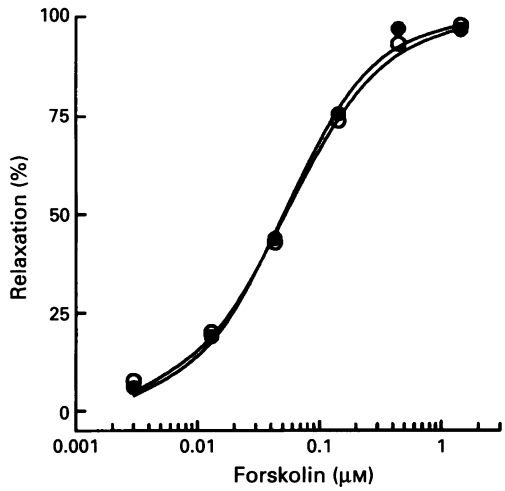

Figure 7 Concentration-dependent vasodilator effects of forskolin alone (○) or in the presence of (**Rp**)-8-bromo-PET-cyclic GMPS 10 μM (●) on the change in perfusion pressure in rat tail arteries precontracted with exogenously applied phenylephrine. Cumulative concentrations of forskolin were added after persistent vasoconstriction had been evoked by 1 μM phenylephrine. After two hours during which time the medium was replaced every 10 min, a second concentration-response curve to forskolin was constructed on the same arteries in the presence of the protein kinase G (PKG) antagonist. Each point represents the mean from 6–12 arteries; s.e.mean were smaller than the symbols.

Lincoln & Cornwell, 1993; Butt *et al.*, 1993). However, although the role of this cyclic nucleotide in cellular function and physiological processes, especially in smooth muscle cells and platelets is well known (Butt *et al.*, 1993; Lincoln & Cornwell, 1993), its mechanism of action is at this time not clearly defined. Therefore, the development of selective and potent activators and inhibitors of PKG is of considerable interest for understanding the role and mechanism of action of cyclic GMP. Studies utilizing (**Rp**)-diastereomers of cyclic nucleotides have indicated that these analogues act as inhibitors of PKA and PKG (Rothermel *et al.*, 1983; Butt *et al.*, 1990; Dostmann *et al.*, 1990; Butt *et al.*, 1994b). These derivatives bind to PKA or PKG, but apparently do not cause the conformational change of the enzyme required for its activation. However, (**Rp**)-cyclic GMPS is a weak antagonist for both PKG and PKA (Butt *et al.*, 1990), whereas (**Rp**)-8-Cl-cyclic GMPS (Butt *et al.*, 1990) and more recently (**Rp**)-8-pCPT-cyclic GMPS (Butt *et al.*, 1994b) are more potent and selective inhibitors of PKG. The newly developed PKG inhibitor, (**Rp**)-8-bromo-PET-cyclic GMPS, is about 15 fold more potent than (**Rp**)-8-pCPT-cyclic GMPS. Although it does not discriminate between the two isozyme forms of PKG I (Iα and Iβ), (**Rp**)-8-bromo-PET-cyclic GMPS is the most potent and selective PKG inhibitor amongst the phosphorothioate cyclic GMP analogues tested so far. High selectivity towards PKG is also shown for the (Sp)-derivative which activates PKG with a K_a 6 times lower than (Sp)-8-pCPT-cyclic GMPS (Butt *et al.*, 1994b), whereas the K_a for activating PKA is in the μM range. The selectivity towards PKG is also confirmed in the present functional studies. Activation of PKA has been implicated in the enhancing effect of cyclic AMP-elevating compounds on prejunctional release of noradrenaline (Ouedraogo *et al.*, 1994a) and in the present study, forskolin induced a concentration-dependent vasodilator effect on preconstricted arteries. Both effects which are assumed to operate via activation of PKA (reviewed by Beebe & Corbin, 1986) are unaffected by (**Rp**)-8-bromo-PET-cyclic GMPS at a concentration which produced an inhibition of effects involving the cyclic GMP/PKG-operated pathway. Therefore, the high degree of selectivity (**Rp**)-8-bromo-PET-cyclic GMPS has for PKG over PKA in biochemical experiments translates also to the intact tissue.

The potency of an inhibitor in intact cells is also dependent on its cell membrane permeability. (**Rp**)-8-pCPT-cyclic GMPS has been shown to be 50 times more lipophilic than (**Rp**)-cyclic GMPS (Butt *et al.*, 1994b). In comparison (**Rp**)-8-bromo-PET-cyclic GMPS is 90 times more lipophilic than (**Rp**)-cyclic GMPS and therefore more effective in penetrating the cell membrane and attaining an intracellular concentration sufficient to inhibit PKG. Moreover, the high lipophilicity exhibited by this antagonist makes it suitable for functional studies in intact tissues. In preliminary studies we also investigated the capacity of (**Rp**)-8-bromo-PET-cyclic GMPS to serve as a substrate for the cyclic GMP PDE type V. (**Rp**)-8-bromo-PET-cyclic GMPS was resistant to hydrolysis by the PDE type V. However, this phosphorothioate cyclic GMP analogue was also a potent inhibitor of the cyclic GMP-specific PDE type V.

Among the cyclic GMP analogues described in previous studies, 8-bromo-PET-cyclic GMP is one of the most potent smooth muscle relaxing agents, presumably acting via the activation of PKG (Sekhar *et al.*, 1992). This cyclic GMP analogue decreases the vasoconstrictor response in a concentration-dependent fashion without affecting nerve-induced neurotransmitter release, in agreement with our previous observations with other analogues (Ouedraogo *et al.*, 1994b). The relaxation induced by the NO donor SIN-1 which activates guanylyl cyclase (Böhme *et al.*, 1984) is thought to be mediated by the endogenous production of cyclic GMP which then activates PKG. In the present study, we now show that the two type I isoforms of PKG are expressed in the rat tail artery. In both experiments, with intact arteries, (**Rp**)-8-bromo-PET-cyclic GMPS attenuated the relaxing effect, providing further evidence that activation of PKG mediates cyclic GMP-induced relaxation of smooth muscle cells. However, the shift of the concentration-response curve for SIN-1 observed in the presence of 10 μM (**Rp**)-8-bromo-PET-cyclic GMPS is rather small compared to its high potency in inhibiting PKG in *in vitro* studies with purified kinase. It is possible that the intracellular concentration reached is not sufficient for a more potent inhibition of PKG. It is also possible that the inhibition of PDE V by (**Rp**)-8-bromo-PET-cyclic GMPS observed in the present study resulted in an increase in the endogenous cyclic GMP level which would in turn counteract the inhibition of PKG.

In conclusion, (**Rp**)-8-bromo-PET-cyclic GMPS is, at present, the most potent and selective (**Rp**)-configurated cyclic GMP-derived PKG inhibitor. The high lipophilicity and hydrolytic stability exhibited by this analogue makes it widely applicable for *in vitro* experiments and functional studies with intact cells.

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