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Effects of cyclic GMP elevation on isoprenaline-induced increase in cyclic AMP and relaxation in rat aortic smooth muscle: role of phosphodiesterase 3

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1 In rat aortic rings precontracted with phenylephrine, the β-adrenoceptor agonist isoprenaline (10 nM to 30 μM) produces greater relaxant effects in preparations with endothelium than in endothelium-denuded preparations. The aim of this study was to determine the mechanisms involved in this effect and in particular investigate the possibility of a synergistic action between adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and guanosine 3′:5′-cyclic monophosphate (cyclic GMP).

2 Isoprenaline-induced relaxation of rat aortic rings precontracted with phenylephrine was greatly reduced by the nitric oxide (NO) synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 300 μM) or the soluble guanylate cyclase inhibitors methylene blue (10 μM) or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM) but unaffected by indomethacin (10 μM), a cyclo-oxygenase inhibitor. Similarly, in intact rings, the concentration-response curve of forskolin (10 nM to 1 μM) was shifted to the right upon endothelium removal or treatment with methylene blue.

3 In endothelium-denuded rat aortic rings, isoprenaline-induced relaxation was potentiated by the guanylate cyclase activators atrial natriuretic factor (ANF, 1 to 10 nM) and sodium nitroprusside (SNP, 1 to 10 nM), and to a greater extent in the presence of the cyclic GMP-specific phosphodiesterase (PDE 5) inhibitor, 1,3dimethyl-6-(2-propoxy-5-methane sulphonylamidophenyl) pyrazolo [3,4-d] pyrimidin-4-(5H)-one (DMPPO, 30 nM). Relaxation induced by isoprenaline was also potentiated by the cyclic GMP-inhibited PDE (PDE 3) inhibitor cilostamide (100 nM).

4 Intracellular cyclic nucleotide levels were measured either in rat cultured aortic smooth muscle cells or in de-endothelialized aortic rings. In both types of preparation, isoprenaline (5 nM and 10 μM) increased cyclic AMP levels and this effect was potentiated by cilostamide (10 μM), by rolipram, a cyclic AMP-specific PDE (PDE 4) inhibitor (10 μM) and by cyclic GMP-elevating agents (50 nM ANF or 30 nM SNP plus 100 nM DMPPO). In isoprenaline-stimulated conditions, the increase in cyclic AMP induced by rolipram was further potentiated by cilostamide and by cyclic GMP-elevating agents. Cilostamide and cyclic GMP-elevating agents did not potentiate each other, suggesting a similar mechanism of action.

5 We conclude that in vascular smooth muscle (VSM) cells an increase in cyclic GMP levels may inhibit PDE 3 and, thereby, cyclic AMP catabolism. Under physiological conditions of constitutive NO release, and to a greater extent in the presence of the PDE 5 inhibitor DMPPO, cyclic GMP should act synergistically with adenylate cyclase activators to relax VSM.

Keywords: Isoprenaline; relaxation; cyclic AMP; cyclic GMP; phosphodiesterase (PDE) inhibitors; 1,3 dimethyl-6-(2-propoxy-5-methane sulphonylamidophenyl) pyrazolo [3,4-d] pyrimidin-4-(5H)-one (DMPPO); endothelium; smooth muscle cells

Introduction

An increase in the intracellular concentration of the cyclic nucleotides adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) produces relaxation of vascular smooth muscle (VSM), mainly by reducing concentrations of intracellular Ca^{2+} (Cornwell & Lincoln, 1989; Lincoln *et al.*, 1990). In VSM cells, increases in the cyclic AMP level can be obtained by stimulation of various receptors including β-adrenoceptors (Kukovetz *et al.*, 1981), A_2 purinoceptors (Fredholm & Sollevi, 1986) or prostacyclin receptors (Oliva & Nicosia, 1987). Endothelium-derived nitric oxide (NO) and natriuretic peptides, via activation of soluble (Gerzer *et al.*, 1981; Lüscher, 1991) and particulate guanylate cyclase (Garbers, 1989; Anand-Srivastava & Trachte, 1993), respectively, increase intracellular cyclic GMP in VSM cells.

Degradation of cyclic nucleotides by hydrolytic cleavage of the 3′-ribose-phosphate bond is catalysed by phosphodiesterases (EC 3.1.4.17.) (Beavo, 1990). Phosphodiesterases (PDE) have been classified into at least seven different isozyme families (Manganiello *et al.*, 1995) depending on the nucleotide preferentially hydrolysed and the regulatory properties of the enzyme. PDE 1 (Ca^{2+}-calmodulin-dependent PDE), PDE 2 (cyclic GMP-stimulated PDE) and PDE 3 (cyclic GMP-inhibited PDE) hydrolyse both cyclic AMP and cyclic GMP. PDE 4 (rolipram-sensitive PDE) and PDE 7 (rolipram-insensitive PDE) are cyclic AMP-specific whereas cyclic GMP is the substrate for PDE 5 and PDE 6. The fact that some PDE isozymes are regulated in both a positive or negative manner by cyclic nucleotides, phosphorylation and Ca^{2+}, highlights the pivotal role played by PDEs in the cross-talk between different second messenger systems, and in particular, between cyclic AMP and cyclic GMP. It has been demonstrated that nitrovasodilators can act synergistically with prostacyclin to inhibit platelet aggregation (Maurice & Haslam, 1990). This synergistic effect may result from the ability of cyclic GMP to enhance the accumulation of cyclic AMP in platelets, as the result of the inhibition of cyclic AMP breakdown by cyclic GMP-mediated inhibition of PDE 3. This mechanism has also been suggested to play a role in VSM (Maurice *et al.*, 1991). In rat VSM cells, four types of PDEs are present, namely PDE 1, PDE 3, PDE 4 and PDE 5 (Komas *et al.*, 1991). Cyclic GMP catabolism in VSM cells is mainly controlled by PDE 5 (Coste

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& Grondin, 1995). Therefore, selective inhibition of this enzyme will increase intracellular cyclic GMP. We recently described a potent and selective PDE 5 inhibitor, 1,3dimethyl-6-(2-propoxy-5-methanesulphonylamidophenyl) pyrazolo [3,4-d] pyrimidin-4-(5H)-one (DMPPO), which lowers blood pressure in different rat models and potentiates the relaxant effects of cyclic GMP-elevating agents in rat aortic rings (Delpy & le Monnier de Gouville, 1996).

Using rat cultured aortic smooth muscle cells (RASMC) and aortic rings, we have investigated the possibility of a synergistic action between cyclic AMP and cyclic GMP. The present study was designed in particular to determine whether cyclic GMP accumulation in RASMC could affect the cyclic AMP level and, if so, to investigate the underlying mechanism and the functional impact in rat isolated aortic rings.

A preliminary account of this work was presented to the B.P.S. meeting in Leicester (April, 1996).

Methods

Preparation of rat aortic rings

Male Sprague-Dawley rats (350–450 g) were killed by cervical dislocation. The thoracic aorta was quickly removed and placed in a physiological salt solution (PSS) containing (mM): NaCl 117, KCl 5, $CaCl_2$ 1.5, NaH_2PO_4 1.1, $NaHCO_3$ 25, $MgSO_4$ 1.2 and glucose 11.5. Aorta was cleaned of fat and connective tissue and cut into 3 mm rings. Rings were mounted in 20 ml organ baths with stainless steel hooks and bathed with PSS maintained at 37°C and gassed with 95% O_2-5% CO_2. All experiments were carried out under an initial tension of 2 g. Tension development was measured isometrically by a Grass transducer (FT 03) connected to a Graphtec linearcorder (WR3310). Data aquisition and analysis were done by the JAD vl.2 programme (Notocord systems, France).

Rings were allowed to equilibrate for a period of 1.5 h during which they were repeatedly washed with PSS. In some experiments endothelium was removed by gently rubbing the luminal surface of the vessel. Endothelium removal was confirmed by the lack of relaxation to 2 μM acetylcholine in rings precontracted with 1 μM phenylephrine. In contrast, acetylcholine (2 μM) relaxed, by more than 50%, phenylephrine-precontracted rings with a functional endothelium. Relaxation was calculated as a percentage of the maximal contraction induced by phenylephrine (1 μM). Phenylephrine and acetylcholine were dissolved in distilled water and were freshly prepared before each experiment.

Relaxation of phenylephrine-precontracted rat aortic rings

Contraction of aortic rings was elicited by addition of 1 μM phenylephrine. After the contraction reached a plateau, isoprenaline was added cumulatively in the organ bath (10 nM to 10 μM). Relaxation caused by each addition of isoprenaline was allowed to achieve its full effect before addition of the subsequent dose. This experiment was performed under different conditions: with or without a functional endothelium and in the presence of various pharmacological agents. The following agents were incubated for 30 min before contraction to phenylephrine was initiated: 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM), methylene blue (10 μM), two soluble guanylate cyclase inhibitors, N^{ω}-nitro-L-arginine methyl ester (L-NAME, 300 μM), a NO synthase inhibitor, DMPPO (30 nM), cilostamide (100 nM) and rolipram (1 μM). Atrial natriuretic factor (ANF) and sodium nitroprusside (SNP), activators of particulate and soluble guanylate cyclase, respectively, were incubated at concentrations of 1 nM, 3 nM or 10 nM, 5 min before contraction to phenylephrine was initiated. In some experiments, isoprenaline was replaced by forskolin (0.01 μM to 1 μM), an adenylate cyclase activator.

Cilostamide, rolipram, DMPPO and forskolin were dissolved in dimethylsulphoxide (DMSO). The final concentration of DMSO in the organ bath never exceeded 0.1% (v/v). Isoprenaline, methylene blue, L-NAME, ANF and SNP were dissolved in distilled water and were freshly prepared before each experiment.

Cyclic nucleotide measurements in aortic rings

Rat aortic rings without endothelium were equilibrated in PSS as described above except that they were not under tension. Since an effective removal of endothelium could not be functionally tested, L-NAME (300 μM) was added to all organ baths. Rings were incubated for 30 min with the PDE inhibitors: cilostamide (10 μM), rolipram (10 μM) and DMPPO (0.1 μM). SNP (30 nM) and isoprenaline (10 μM), were added for the last 5 and 1 min, respectively. Rings were rapidly collected and frozen in liquid nitrogen to prevent degradation of cyclic nucleotides. Each ring was homogenized in cold trichloroacetic acid (6%) and the homogenate centrifuged (2000 g for 15 min at 4°C). Protein content in the pellet was determined by the BCA protein assay reagent (Pierce). The supernatant was washed 4 times with 5 volumes of water saturated diethyl ether. The upper ether layer was discarded after each wash. The aqueous phase was then evaporated to dryness with a Speed-vac system and the dried extract dissolved in a suitable volume of assay buffer prior to analysis. Cyclic nucleotides content was determined by scintillation proximity assay (Amersham).

Cell cultures

Rat aortic smooth muscle cells (RASMC) were prepared according to Chamley *et al.* (1977). Briefly, rats were killed and the thoracic aorta was quickly removed and placed in cold (4°C) phosphate buffered saline (PBS). Aorta was cleaned of fat and connective tissue and then incubated (30 min, 37°C) in the following mixture: collagenase type II (1 mg ml^{-1}), elastase (0.5 mg ml^{-1}) and trypsin inhibitor (0.5 mg ml^{-1}). Adventitia was removed and discarded. The remaining structure was placed in a second enzymatic bath (120 min, 37°C) of the same composition. After this second digestion, the cell suspension was washed twice with culture medium and centrifuged (500 g, 5 min). Then, the cell suspension was plated in a plastic flask previously coated with collagen. When cells became confluent, they were trypsinized and subcultured.

Cells were cultured in Dulbecco's modified Eagle medium (GIBCO) containing 10% foetal calf serum, 1% glutamine and 1% penicillin-streptomycin at 37°C in a 95% air – 5% CO_2 humidified atmosphere. They were used between the 5th and 15th passage.

Treatment of rat cultured aortic smooth muscle cells with pharmacological agents

Cells were seeded in 24-well culture dishes at a density of 2 to 5×10^4 cells per well. Experiments were performed after 3 to 5 days in culture when cells had reached confluence. Culture medium was aspirated and replaced by 0.5 ml of PBS containing the pharmacological agents. Treatments were performed in quadruplicate at 37°C. The type 3, 4 and 5 PDE inhibitors cilostamide (10 μM), rolipram (10 μM) and DMPPO (10 μM) (Table 1), respectively, were incubated with cells for 30 min before addition of adenylate or guanylate cyclase activators. Cyclic GMP and cyclic AMP were respectively increased in RASMC by stimulation of particulate guanylate cyclase with ANF (50 nM for 10 min) or β-adrenoceptors with isoprenaline (5 nM for 5 min). At the end of the incubation period, the medium was removed and intracellular cyclic nucleotides were extracted by two ethanolic (65%) washes at 4°C for 5 min. Ethanolic extracts were pooled, evaporated to dryness by a Speed-Vac system. The dried extract was dissolved in a suitable amount of assay buffer and cyclic nucleotide levels were measured by scintillation proximity assay (Amersham).

Cilostamide, rolipram and DMPPO were dissolved in dimethylsulphoxide (DMSO). Final concentration of DMSO in culture dishes never exceeded 0.1% (v/v).

Statistical analysis

Data are presented as means ± s.e.mean. Comparisons were performed by Student's two-tailed t test for unpaired data. Statistical significance was assumed when $P<0.05$.

Materials

−Phenylephrine, acetylcholine bromide, isoprenaline, sodium nitroprusside (SNP), rat atrial natriuretic peptide (ANF), forskolin, methylene blue and L-NAME were obtained from Sigma Chemical Co (St. Quentin Fallavier, France). ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) was obtained from Biomol (TEBU, France). DMPPO (1,3dimethyl-6-(2-propoxy-5-methanesulphonylamidophenyl) pyrazolo [3,4-d] pyrimidin-4-(5H)-one) was synthesized according to Dumaître & Dodic (1995). Cilostamide and rolipram were obtained from Glaxowellcome.

Results

Effect of isoprenaline on rat aortic rings

In phenylephrine-precontracted aortic rings with a functional endothelium, isoprenaline induced a concentration-dependent relaxation which attained a maximum of 59 ± 5% at 10 μM (Figure 1); a 15 ± 5% relaxation was observed at the end of the experiment in time-matched control preparations (not shown). In rings without endothelium, isoprenaline produced a weaker effect: 30 μM isoprenaline only relaxed by 19 ± 2%. Control endothelium-denuded preparations did not show any spontaneous relaxation. Phenylephrine-induced contraction was similar in preparations with or without endothelium (2.4 ± 0.2 g and 2.4 ± 0.1 g, respectively).

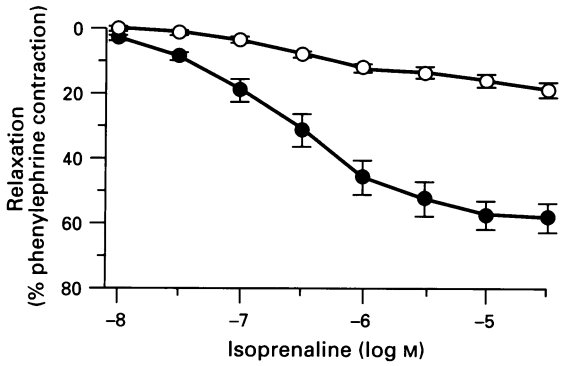

Figure 1 Concentration-response curve to isoprenaline in phenylephrine (1 μM) precontracted rat aortic rings in the presence (●, $n=8$) or in the absence of a functional endothelium (○, $n=10$). Values are mean and vertical lines show s.e.mean.

Mechanism of the potentiation of isoprenaline relaxation by endothelium

This set of experiments was designed to determine what in the endothelium was responsible for increased relaxation to isoprenaline.

In phenylephrine precontracted rings with endothelium, relaxation to isoprenaline was unchanged by indomethacin (10 μM), a cyclo-oxygenase inhibitor (data not shown). However, in rings with endothelium pretreated with an inhibitor of NO synthesis (L-NAME, 300 μM) or with an inhibitor of soluble guanylate cyclase (methylene blue, 10 μM) or with ODQ (10 μM), a more selective guanylate cyclase inhibitor, relaxation to isoprenaline was similar to that obtained in the absence of endothelium (Figure 2). Initial contractions to phenylephrine were not significantly different in control rings (2.6 ± 0.2 g), methylene blue (2.5 ± 0.2 g) and ODQ (3.2 ± 0.3 g) pretreated preparations, but slightly ($P<0.05$) increased after pretreatment with L-NAME (3.5 ± 0.6 g).

Effect of forskolin on rat aortic rings

In this experiment, we verified if the potentiation of relaxation to isoprenaline was related to β-adrenoceptor stimulation or stimulation of adenylate cyclase.

Forskolin induced a concentration-dependent relaxation of rings with endothelium ($pD_2 = -\log_{10} EC_{50} = 7.13 \pm 0.07$) (Figure 3). The dose-response curve to forskolin was shifted to the right in the absence of a functional endothelium ($pD_2 = 6.30 \pm 0.05$). A similar shift to the right of the dose-response curve to forskolin was observed when rings with endothelium were treated with methylene blue (10 μM) ($pD_2 = 6.34 \pm 0.02$). Initial contractions to phenylephrine were (g): 1.8 ± 0.05, 2.5 ± 0.2 and 2.3 ± 0.06 in rings with endothelium, without endothelium and with endothelium pretreated with methylene blue, respectively.

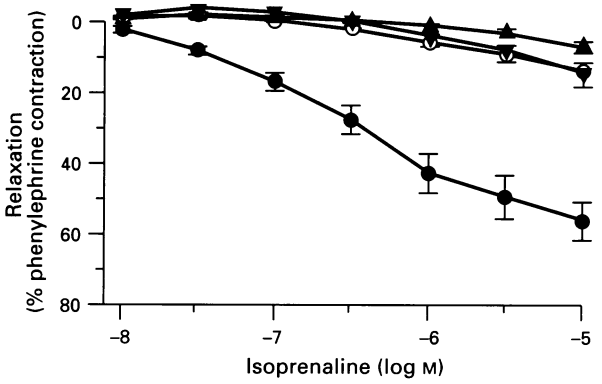

Figure 2 Concentration-response curve to isoprenaline in phenylephrine (1 μM) precontracted rat aortic rings with endothelium, without inhibitor (●, $n=6$), in the presence of 300 μM L-NAME (○, $n=6$), in the presence of 10 μM methylene blue (▲, $n=7$) and in the presence of 10 μM ODQ (▼, $n=5$). Values are mean and vertical lines show s.e.mean.

Table 1 K_i values (μM) of cilostamide, rolipram and DMPPO on the different phosphodiesterases (PDEs) present in vascular smooth muscle

	PDE 1	*PDE 3*	*PDE 4*	*PDE 5*	*Reference*
Cilostamide	114	0.042	80	30	Lugnier & Komas (1993)
Rolipram	>200	>200	0.76	>200	Lugnier & Komas (1993)
DMPPO	1	10	22	0.003	Coste & Grondin (1995)

Effect of guanylate cyclase activators on the response to isoprenaline in aortic rings without endothelium

This set of experiments was done in the absence of endothelium, i.e. in conditons where isoprenaline poorly relaxed phenylephrine precontracted rings. Before contraction with phenylephrine, guanylate cyclases (particulate or soluble) were pharmacologically stimulated by ANF or SNP in order to increase cyclic GMP levels and see whether the magnitude of isoprenaline-induced relaxation was correlated to the level of guanylate cyclase stimulation.

In the experiment described in Figure 4a, 10 μM isoprenaline induced 17±4% relaxation. In rat aortic rings pretreated with 1, 3 and 10 nM ANF and then contracted with phenylephrine, isoprenaline (10 μM) produced 31±7%, 69±7% and 94±1% relaxation, respectively. Initial contractions to phenylephrine were respectively (g): 3.4±0.2, 3.3±0.3, 3.1±0.2 and 3.0±0.4 in the above described groups. To ascertain whether cyclic GMP was involved in the effect of ANF, rings were pretreated with DMPPO (30 nM), a PDE 5 inhibitor in order to increase the level of cyclic GMP obtained after ANF stimulation (Figure 4b). In phenylephrine-contracted aortic rings, pretreatment with either 30 nM DMPPO or 1 nM ANF did not affect significantly isoprenaline-dependent relaxation (respectively 13±2% and 21±5% relaxation at 10 μM isoprenaline). However, when ANF and DMPPO were given in combination (when cyclic GMP is slightly increased and its breakdown inhibited), the relaxant response to isoprenaline was greatly increased and 10 μM isoprenaline induced 65±7% relaxation. Initial contractions to phenylephrine were (g): 3.5±0.4, 4.0±0.3 and 3.0±0.2 in ANF-treated group, DMPPO-treated group and ANF plus DMPPO-treated group, respectively.

Similarly, as shown in Figure 5a, pretreatment with the NO donor SNP concentration-dependently potentiated isoprenaline-induced relaxation in endothelium-denuded aortic rings. In the absence of SNP, 10 μM isoprenaline induced 17±4% relaxation. In rings pretreated with 1, 3 and 10 nM SNP, isoprenaline (10 μM) evoked 24±6%, 45±9% and 83±6% relaxation, respectively. Initial contractions to phenylephrine were (g): 3.4±0.2, 3.3±0.2, 3.3±0.1 and 2.6±0.2 for the groups described above, respectively. As shown in Figure 5b, DMPPO and SNP acted synergistically to potentiate isoprenaline relaxation. In rings treated with 30 nM DMPPO or 1 nM SNP, isoprenaline (10 μM) induced respectively 11±6% and 16±5% relaxation, whereas when DMPPO and SNP were added in combination the relaxation attained 53±7%. Initial contractions to phenylephrine were (g): 3.2±0.2, 3.1±0.2 and 2.9±0.1 in SNP-treated group, DMPPO-treated group and SNP plus DMPPO-treated group, respectively.

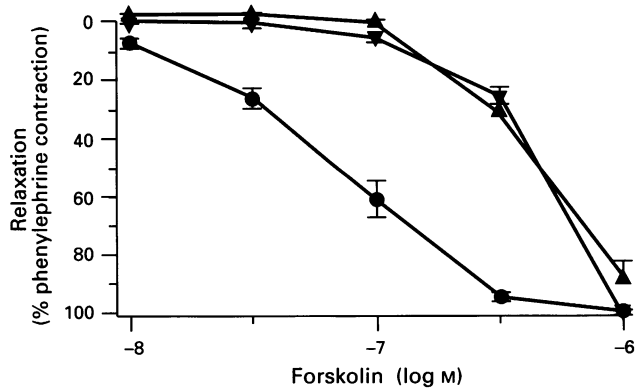

Figure 3 Concentration-response curve to forskolin in phenylephrine (1 μM) precontracted rat aortic rings in the presence (●, $n=6$) or in the absence of a functional endothelium (▼, $n=7$) and in the presence of endothelium in rings pretreated with 10 μM methylene blue (▲, $n=4$). Values are mean and vertical lines show s.e.mean.

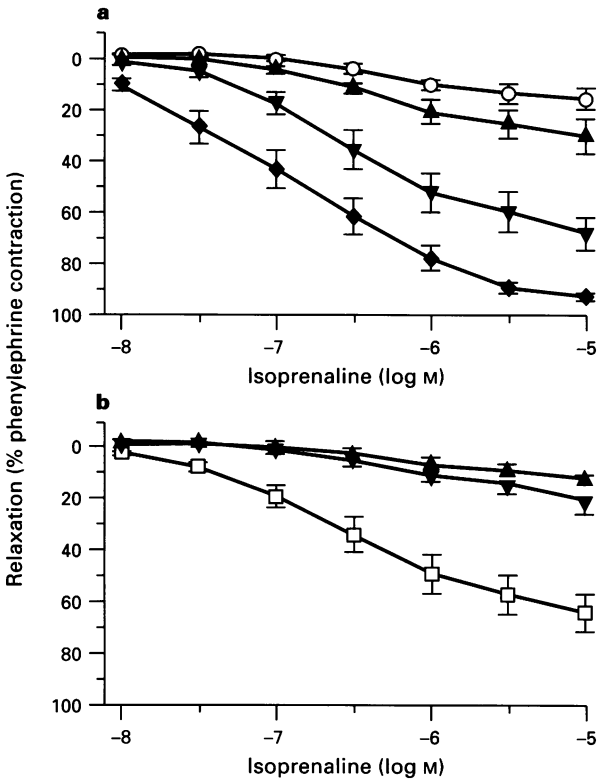

Figure 4 (a) Effect of pretreatment with atrial natriuretic factor (ANF) (▲, 1 nM, $n=8$; ▼, 3 nM, $n=8$; ◆, 10 nM, $n=4$) on isoprenaline-induced relaxation (○, control rings, $n=11$) in phenylephrine (1 μM) precontracted rat aortic rings without endothelium. (b) Effect of pretreatment with DMPPO 30 nM (▲, $n=5$), ANF 1 nM (▼, $n=5$) and a combination of both (□, $n=8$) on isoprenaline-induced relaxation in phenylephrine precontracted rat aortic rings without endothelium. Values are mean and vertical lines show s.e.mean.

Effects of PDE inhibitors on the relaxation mediated by isoprenaline in aortic rings without endothelium

In this experiment, we studied the effects of pretreatment with cilostamide or rolipram, PDE 3 and PDE 4 inhibitors, respectively. These isoforms of PDE are known to be involved in cyclic AMP degradation in VSM cells. Both inhibitors are used at concentrations that do not, on their own, relax phenylephrine precontracted rings.

The weak relaxant effect of isoprenaline in endothelium-denuded rat aortic rings was potentiated by cilostamide (100 nM) (Figure 6). Isoprenaline 10 μM which induced only 21±3% relaxation in the absence of the PDE inhibitor induced 79±6% relaxation in the presence of cilostamide. In contrast, rolipram (1 μM) had no effect on isoprenaline-induced relaxation (21±2%). Initial contractions to phenylephrine were (g): 3.2±0.1, 2.9±0.1 and 3.4±0.1 in control rings (without endothelium), cilostamide-treated rings and rolipram-treated rings, respectively.

Cyclic nucleotide measurements in rat aortic rings without endothelium

These experiments were designed to connect functional effects with intracellular levels of cyclic nucleotides.

In aortic rings without endothelium, the basal cyclic AMP level was 2.3 pmol mg^{-1} protein (Figure 7); basal cyclic GMP content was 0.12 pmol mg^{-1} protein. Therefore, in unstimulated conditions, the ratio between cyclic AMP and cyclic GMP was about 19. This value is close to the results usually found in the literature (Jang *et al.*, 1993). Neither cilostamide (10 μM) nor rolipram (10 μM) increased the basal cyclic AMP

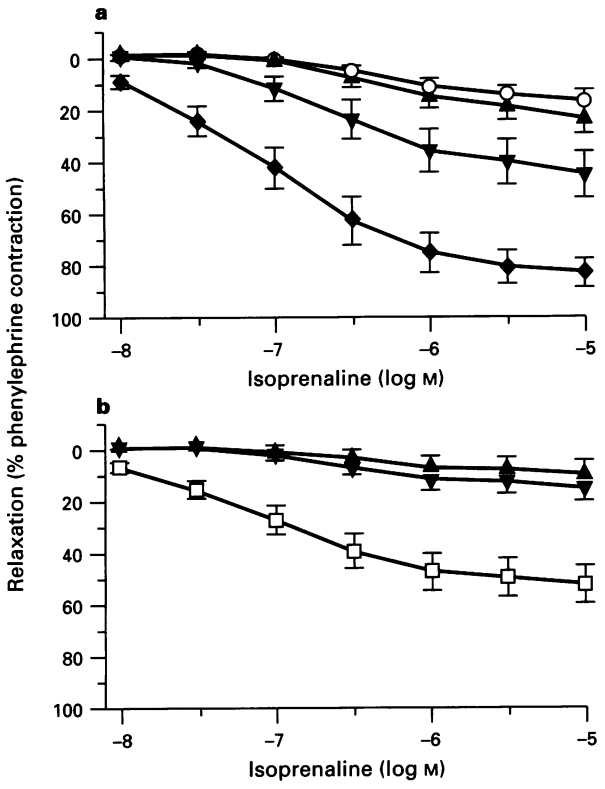

Figure 5 (a) Effect of pretreatment with sodium nitroprusside (SNP) (▲, 1 nM, $n=8$; ▼, 3 nM, $n=8$; ◆, 10 nM, $n=8$) on isoprenaline-induced relaxation (○, control rings, $n=11$) in phenylephrine (1 μM) precontracted rat aortic rings without endothelium. (b) Effect of pretreatment with DMPPO 30 nM (▲, $n=5$), SNP 1 nM (▼, $n=5$) and a combination of both (□, $n=8$) on isoprenaline induced relaxation in phenylephrine precontracted rat aortic rings without endothelium. Values are mean and vertical lines show s.e.mean.

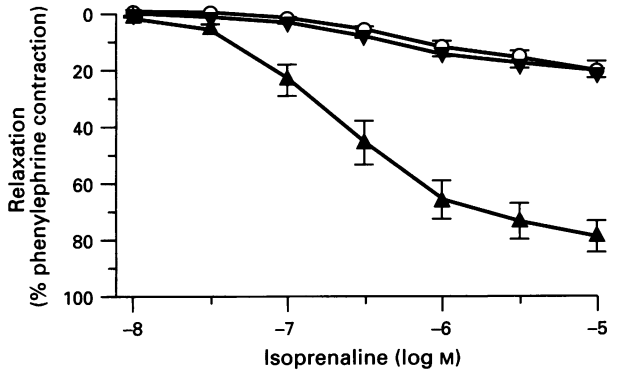

Figure 6 Concentration-response curve to isoprenaline in phenylephrine (1 μM) precontracted endothelium-denuded rat aortic rings, in the absence (○, $n=12$) and presence of 100 nM cilostamide (▲, $n=6$) or 1 μM rolipram (▼, $n=6$). Values are mean and vertical lines show s.e.mean.

level; however, when added together, they increased cyclic AMP content by 3.5 fold. A combination of SNP (30 nM) and DMPPO (100 nM) increased by 5.8 fold the cyclic GMP content without changing the cyclic AMP levels. When rolipram was added to the latter combination, cyclic AMP levels were increased by a further 1.7 fold whereas the addition of cilostamide to SNP- and DMPPO-treated rings did not further increase cyclic AMP.

Isoprenaline 10 μM increased cyclic AMP by 1.4 fold. This level was further enhanced 1.4 fold by cilostamide (10 μM) or rolipram (10 μM), and 2.7 fold by the combination of both

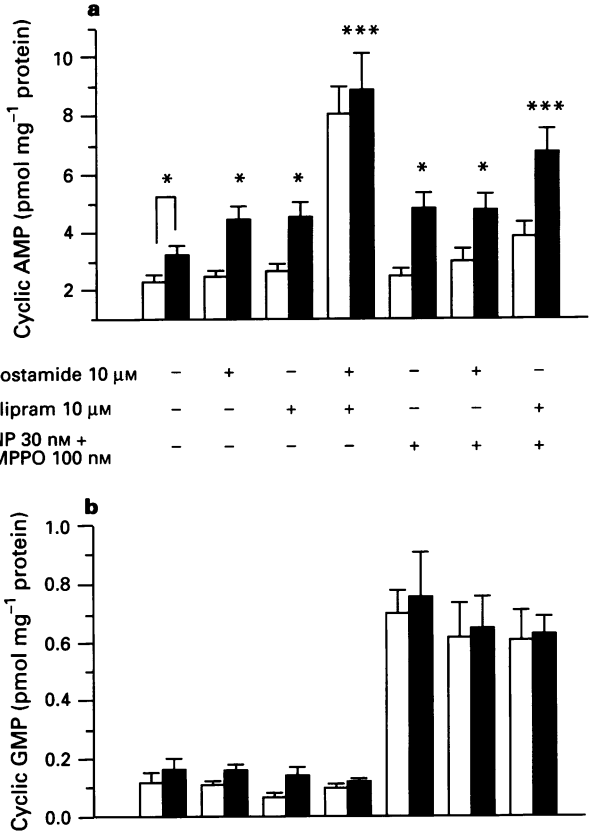

Figure 7 Effect of sodium nitroprusside (SNP) and phosphodiesterase (PDE) inhibitors on the cyclic nucleotide levels in endothelium-denuded rat aortic rings, either in the absence (open columns) or in the presence of isoprenaline (10 μM, solid columns). (a) Cyclic AMP and (b) cyclic GMP were measured by scintillation proximity assay. Values are mean ± s.e.mean for $n=17-29$ rings. $^{*}P<0.005$; $^{***}P<0.001$ in comparison with isoprenaline alone. DMPPO: 1,3dimethyl-6-(2-propoxy-5-methanesulphonylamidophenyl) pyrazolo [3,4-d] pyrimidin-4-(5H)-one.

PDE inhibitors. Similarly, the cyclic GMP-increasing agents, SNP plus DMPPO, which increased cyclic GMP levels by 5 fold, also produced a 1.5 fold augmentation of cyclic AMP accumulation. The potentiation of the isoprenaline-induced increase in cyclic AMP level obtained by incubation of rings with SNP plus DMPPO remained unchanged by further addition of cilostamide whereas it was increased 2.1 fold in the presence of rolipram. As with the results obtained in cultured cells, elevated levels of cyclic GMP, or a PDE 3 inhibitor, are capable of potentiating isoprenaline-stimulated cyclic AMP accumulation. However, the effects of cyclic GMP-increasing agents and of the PDE 3 inhibitor are not additive. This is not the case in rings treated with both a PDE 4 inhibitor and a PDE 3 inhibitor or cyclic GMP-elevating agents.

Cyclic nucleotide measurements in rat cultured aortic smooth muscle cells

In these experiments, we attempted to verify that the cross-talk between cyclic AMP and cyclic GMP is taking place in vascular smooth muscle cells and not in another cell type present in aortic tissues.

In cultured RASMC, the basal level of cyclic AMP was about 750 fmol/well, whereas the cyclic GMP level was < 50 fmol/well (Figure 8). Isoprenaline (5 nM for 5 min) increased cyclic AMP content by 4.7 fold. This level was increased a further 2.3 fold by the PDE 3 inhibitor cilostamide (10 μM), 2.9 fold by the PDE 4 inhibitor rolipram (10 μM) and 6.5 fold by the combination of both compounds suggesting that both PDE 3 and 4 are responsible for cyclic AMP hy-

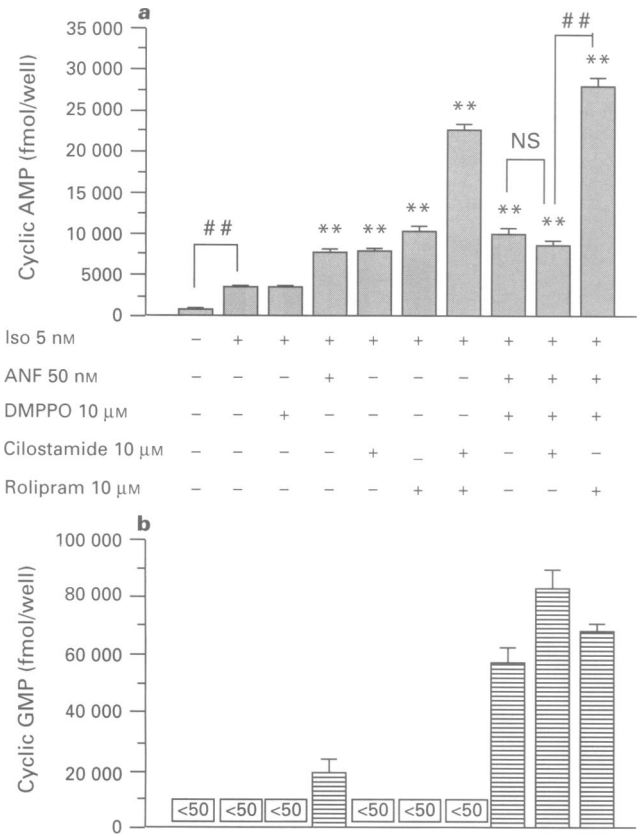

Figure 8 Cyclic nucleotide levels in cultured smooth muscle cells from the rat aorta after incubation with isoprenaline (Iso, 5 nM) in the presence of various pharmacological agents. Incubation with drugs was performed as described in the experimental section. $^{\#\#}P<0.001$; $^{**}P<0.001$ in comparison with isoprenaline alone. Values are mean ± s.e.mean. In (b), < 50 means that cyclic GMP levels were below the sensitivity of the assay (50 fmol/well). ANF: atrial natriuretic factor; DMPPO: 1,3dimethyl-6-(2-propoxy-5-methanesulphonylamidophenyl) pyrazolo [3,4-d] pyrimdin-4-(5H)-one.

drolysis. In contrast, the PDE 5 inhibitor DMPPO (10 μM) did not affect isoprenaline-stimulated cyclic AMP levels. ANF (50 nM for 10 min) enhanced by 2.2 fold the isoprenaline-stimulated cyclic AMP accumulation. At this concentration ANF elevated the cyclic GMP level to 2,000 fmol/well. The latter effect of ANF was potentiated by DMPPO (10 μM): cyclic GMP levels attained 6,000 fmol/well and the increase in cyclic AMP induced by isoprenaline (5 nM) was further enhanced by 2.9 fold. The potentiation of the isoprenaline-induced increase in cyclic AMP level obtained by coincubation with ANF and DMPPO was unchanged by further addition of cilostamide (2.5 fold) whereas it was increased by 7.8 fold in the presence of rolipram.

In basal conditions without isoprenaline stimulation, cilostamide and rolipram did not affect cyclic AMP and cyclic GMP levels (data not shown). Similarly, DMPPO and ANF, either alone or in combination, did not change basal cyclic AMP levels (Coste & Grondin, 1995).

Discussion

Type 2 and type 3 PDE are both cyclic GMP-regulated, in a positive and negative manner, respectively (Sonnenburg & Beavo, 1994). Since those enzymes hydrolyse both cyclic AMP and cyclic GMP (Manganiello *et al.*, 1995), it is suggested that cross-talk between cyclic nucleotides could occur depending on the level of cyclic GMP. We have investigated how cyclic GMP could regulate cyclic AMP content in VSM where both cyclic nucleotides are implicated in vascular relaxation (Murad, 1986; Murray, 1990).

In rat aortic rings precontracted with phenylephrine, isoprenaline induced a relaxation of greater magnitude in the presence than in the absence of endothelium. This effect does not seem to be mediated by prostacyclin since it was unaffected by indomethacin. In preparations with endothelium pretreated with L-NAME, methylene blue or ODQ, the relaxation to isoprenaline was similar to that obtained in preparations without endothelium. These results imply an involvement of endothelium in the relaxation to isoprenaline via generation of NO and subsequent production of cyclic GMP in VSM cells. The PD_2 value of forskolin-dependent relaxation was also higher in preparations with endothelium compared with those without endothelium. Again, in preparations with endothelium, methylene blue displaced to the right the amplitude of forskolin-mediated relaxation to a level similar to that observed in preparations without endothelium. These results suggest that cyclic GMP potentiates cyclic AMP-dependent relaxation by a mechanism downstream of the β-adrenoceptor signalling. The cross-talk between both second messenger systems could be achieved by inhibition, by cyclic GMP, of the PDE hydrolysing cyclic AMP, PDE 3. Moreover, we showed that the PDE 3 inhibitor cilostamide potentiates the relaxation induced by isoprenaline. On the contrary, the PDE 4 inhibitor rolipram, which enhances isoprenaline-induced cyclic AMP accumulation, does not potentiate the relaxation evoked by isoprenaline. Such a lack of correlation between cyclic AMP content and functional effects has been demonstrated previously and can be explained by the possible existence of different intracellular pools of cyclic AMP, as suggested in cardiomyocytes (Hayes *et al.*, 1980; Buxton & Brunton, 1983; Hohl & Li, 1991).

The hypothesis of a cyclic GMP pathway to explain the greater relaxation induced by isoprenaline in intact rings compared with endothelium-denuded preparations is further stressed by the fact that ANF and SNP concentration-dependently potentiate the isoprenaline-induced relaxation in preparations without endothelium. Accordingly, addition of the cyclic GMP-specific PDE 5 inhibitor DMPPO to endothelium-denuded rings further enhanced the potentiating effect of ANF or SNP on the cyclic AMP-mediated relaxation. Cyclic nucleotide measurements further stressed these results. In rat isolated aortic rings without endothelium the β-adrenoceptor agonist isoprenaline increased cyclic AMP levels. This effect was potentiated by the PDE 3 inhibitor, cilostamide, the PDE 4 inhibitor, rolipram and by the cyclic GMP-elevating agent SNP in combination with the PDE 5 inhibitor, DMPPO. In addition, the level of cyclic AMP obtained in isoprenaline plus rolipram-treated cells was further potentiated by cilostamide or by cyclic GMP-elevating agents. In contrast, when given together, cilostamide and cyclic GMP-elevating agents did not produce an additional effect suggesting that their mechanism of action is similar. Since PDE 3 is negatively regulated by cyclic GMP, the enhancement of isoprenaline-dependent cyclic AMP accumulation by SNP plus DMPPO can be explained by cyclic GMP-induced inhibition of PDE 3. In cultured RASMC, cyclic nucleotide measurements carried out under similar experimental conditions gave qualitatively similar results. These experiments allow us to confirm that the cross-talk between cyclic AMP and cyclic GMP is a vascular smooth muscle phenomenon and does not originate from another cell type present in aortic rings such as remaining endothelial cells or fibroblasts.

Our results are in agreement with those of Maurice *et al.* (1991) and Jang *et al.* (1993) which have shown that nitrovasodilators or atriopeptin II could act synergistically with isoprenaline to inhibit contraction of rat aortic rings. Similarly, Komas *et al.* (1991) and Grace *et al.* (1988) suggested that cyclic GMP could enhance cyclic AMP-mediated relaxation of rat aortic rings. More recently, Satake *et al.* (1995) demonstrated a similar effect of nicorandil, a dual potassium opener and NO donor, which potentiates the relaxation to isoprenaline.

An alternative explanation for our results could be that an increase in endothelial cyclic AMP induced by isoprenaline could stimulate NO synthesis and release, as suggested by Gray & Marshall (1992). These authors recently hypothesized that isoprenaline acts through β-adrenoceptors on the endothelium to raise cyclic AMP and that this may activate NO release and thus evoke vascular relaxation. However, data in the literature are not consistent with this: neither endothelial β-adrenoceptor stimulation (Macdonald *et al.*, 1987) nor endothelial cyclic AMP (Kuhn *et al.*, 1991) regulate the release of endothelium-derived relaxing factor (EDRF). Graier *et al.* (1992) also found that forskolin, adenosine and isoprenaline amplified bradykinin- and ATP-induced biosynthesis and release of EDRF. However, basal release of EDRF was not affected by the cyclic AMP-increasing agents. Moreover, in our experiments with endothelium-denuded preparations, isopranaline still evoked a relaxation and increased cyclic AMP levels confirming the existence of functional β-receptors on VSM cells, as further shown by the results in cultured RASMC.

We conclude that isoprenaline-dependent vasorelaxation is potentiated by endothelial constitutive release of NO and subsequent increase in smooth muscle cyclic GMP. The elevated level of cyclic GMP in VSM cells is probably responsible for inhibition of PDE 3 and thereby an increase in cyclic AMP level.

We have recently shown (Delpy & le Monnier de Gouville, 1996) that PDE 5 inhibitors could represent a new class of therapeutic agents useful in the treatment of cardiovascular disorders such as essential hypertension. We have demonstrated in the present study that augmenting cyclic GMP levels by a PDE 5 inhibitor could also be an interesting strategy to potentiate the vasodilator effects of adenylate cyclase activators such as endogenous catecholamines and prostacyclin. This mechanism will be limited to tissues where PDE 5 and PDE 3 are colocalized such as in vascular beds. Consequently, a PDE 5 inhibitor will be devoid of direct inotropic effects since PDE 5 is not found in cardiomyocytes (Reeves *et al.*, 1987; Muller *et al.*, 1992).

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