British Journal of Pharmacology (1997) 121, 1323–1333

Involvement of protein kinase C in reduced relaxant responses to the NO/cyclic GMP pathway in piglet pulmonary arteries contracted by the thromboxane A_2-mimetic U46619

[1]Francisco Pérez-Vizcaíno, *Eduardo Villamor, ‡Juan Duarte & Juan Tamargo

Department of Pharmacology, Institute of Pharmacology and Toxicology, School of Medicine, Universidad Complutense, 28040 Madrid; *Division of Neonatology, Department of Pediatrics, Hospital Universitario San Carlos, 28040 Madrid and ‡Department of Pharmacology, School of Pharmacy, University of Granada, 18071 Granada, Spain

1 Impairment of nitric oxide (NO)/cyclic GMP production and/or increased activities of thromboxane A_2 (TXA_2) and endothelin-1 (ET-1) have been associated with pulmonary hypertension. We have analysed the interactions of noradrenaline (NA), the TXA_2-mimetic U46619 and ET-1 with the relaxation induced via cyclic GMP in isolated piglet intrapulmonary arteries.

2 The contractions induced by NA were augmented by endothelium removal or by methylene blue and pre-contracted rings were fully relaxed by acetylcholine, sodium nitroprusside (SNP), atrial natriuretic peptide and 8-bromo-cyclic GMP. In contrast, U46619- and ET-1 induced contractions were endothelium-independent and only partially relaxed by the latter vasodilators. Whereas the reduced responses to SNP in arteries contracted by U46619 were independent of the U46619-induced tone, a higher concentration of ET-1 (tone higher than that induced by NA) was required to reduce the vasodilator responses to SNP. NA, U46619 and ET-1 had no effect on the SNP-induced increases in cyclic GMP.

3 The reduced relaxant responses to SNP in arteries pre-contracted by U46619 were specific for piglet pulmonary arteries since they were not observed in piglet mesenteric or coronary arteries or in rat pulmonary arteries. Furthermore, there were no differences in the relaxant response to the adenylate cyclase activator forskolin in piglet pulmonary arteries pre-contracted by either NA, U46619 or ET-1.

4 SNP-induced relaxation was inhibited by thapsigargin (but not by inhibition of the membrane Na^+/K^+ ATPase nor K^+ channels) indicating a role for Ca^{2+} sequestration by the Ca^{2+} ATPase in the effects of SNP.

5 The phorbol ester 12-myristate, 13-acetate inhibited the relaxant response to SNP. The inhibitory effect of U46619 on SNP-induced relaxation was abolished by the protein kinase C inhibitor (PKC) staurosporine suggesting that PKC may be a part of the signal transduction mechanism.

6 In summary, piglet pulmonary arteries when activated by a TXA_2-mimetic show abnormally reduced relaxant responses to the NO/cyclicGMP pathway. This effect appears to be mediated by activation of PKC.

Keywords: Thromboxane A_2; endothelin-1; nitric oxide; cyclic GMP; pulmonary artery

Introduction

The nitric oxide (guanosine 3′:5′-cyclic monophosphate) (NO/cyclic GMP) pathway plays a key role in the maintenance of vasodilator tone in the vascular bed (Moncada *et al*., 1991; Warner *et al*., 1994). Under physiological conditions, the endothelium is the main source of NO for vascular smooth muscle relaxation whereas in several pathological states the induction of the inducible NO synthase (iNOS) in smooth muscle cells and macrophages may account for a large production of NO (Moncada *et al*., 1991). NO acting as an autocrine or paracrine mediator, activates the soluble guanylate cyclase and increases intracellular levels of cyclic GMP in vascular smooth muscle cells (Warner *et al*., 1994; Barnes & Liu, 1995). Alterations of this pathway, at the level of NO and cyclic GMP synthesis or action in vascular smooth muscle have been associated with a number of vascular diseases. In the pulmonary system, NO is crucial for maintaining low vascular resistances and arterial pressure (Cremona *et al*., 1991; Barnes & Liu, 1995). Reduced NO or cyclic GMP activities have been related to the maintenance of high pulmonary pressure during foetal life (Abman *et al*., 1990), experimental persistent pulmonary hypertension of the newborn (McQueston *et al*., 1995), hypoxia-induced pulmonary vasoconstriction as well as primary and secondary pulmonary hypertension (Dinh-Xuan *et al*., 1991; Giaid & Saleh, 1995). In addition, in both adults and neonates, inhalation of NO has recently been introduced as a life-saving therapeutic approach with beneficial results in many subjects (Abman & Kinsella, 1995; Roberts *et al*., 1997; Neonatal Inhaled Nitric Oxide Study Group, 1997).

On the other hand, increased activity of the pulmonary vasoconstrictors thromboxane A_2 (TXA_2) and endothelin-1 (ET-1) has also been implicated in several forms of pulmonary hypertension. TXA_2 has been shown to be responsible for the early phase of sepsis-induced pulmonary hypertension (Weitzberg *et al*., 1995). It has also been implicated in other experimental models of pulmonary hypertension induced by heparin/protamine (Montalescot *et al*., 1990), leukotriene D_4 (Noonan & Malik, 1986), microembolism (García-Szabo *et al*., 1988) and ischaemia-reperfusion (Zamora *et al*., 1993). Furthermore, elevated levels of thromboxane B_2, the metabolite of TXA_2, have been found in neonatal pulmonary hypertension (Dobyns *et al*., 1994). Augmented levels of ET-1 have also been shown to be associated with several forms of experimental and clinical pulmonary hypertension, including that induced by sepsis, hypoxia and monocrotaline (Weitzberg *et al*., 1996), persistent pulmonary hypertension of the newborn (Rosenberg *et al*., 1993), primary pulmonary hypertension (Giaid *et al*., 1993) and

[1]Author for correspondence at: Department of Pharmacology, Institute of Pharmacology and Toxicology, School of Medicine, Universidad Complutense, 28040 Madrid, Spain.

pulmonary hypertension associated with congenital heart disease (Yoshibayashi *et al*., 1991) or congestive heart failure (Cody *et al*., 1992).

In physiological situations, pulmonary vascular tone results from the balance of vasoconstrictors and vasodilators. Whether the imbalance which occurs in pulmonary hypertension is due to alteration of a single factor or multiple vasoactive agents remains unclear. Therefore, the aim of the present investigation was to study the interactions of the vasoconstrictors noradrenaline (NA), the TXA_2 mimetic, U46619, and ET-1 with the NO/cyclic GMP pathway as well as the mechanisms involved in NO/cyclic GMP-induced relaxation in piglet isolated intrapulmonary arteries. The specificity of these interactions was analysed by comparing these results with those mediated through: (a) the cyclic AMP pathway in piglet pulmonary arteries, and (b) the NO/cyclic GMP pathway in piglet mesenteric and coronary arteries and in rat pulmonary arteries.

Methods

Tissue preparation

Two week old male piglets (10–17 days, 3–5 kg) were used in this study. Some experiments were also carried out on adult Wistar rats (250–300 g) and on 2–3 month old piglets (15–25 kg). Piglets were killed in the local abattoir by exsanguination and the lungs, hearts and mesenteric vascular beds were rapidly immersed in cold (4°C) Krebs solution (composition in mM: NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.0, KH_2PO_4 1.2 and glucose 11) and transported to the laboratory. Rats were killed in the laboratory by a sharp blow on the head followed by exsanguination. The intrapulmonary arteries (third branch), mesenteric and left descending coronary arteries from piglets (all with an internal diameter 1–2 mm) and the right and left branches of the main rat pulmonary artery were carefully dissected free of surrounding tissue and cut into rings of 2–3 mm length (Pérez-Vizcaíno *et al*., 1996; Villamor *et al*., 1996a,b). Except where stated otherwise, the endothelium was removed by gently rubbing the intimal surface of the rings with a metal rod. The endothelium removal procedure was verified by the inability of acetylcholine (ACh,10^{-6} M) to relax arteries precontracted with 10^{-6} M NA. Two L-shaped stainless-steel wires were inserted into the arterial lumen and the rings were introduced into Allhin organ chambers filled with Krebs solution (gassed with 95% O_2 and 5% CO_2 at 37°C). One wire was attached to the chamber and the other to an isometric force-displacement transducer coupled to a signal amplifier (Model PRE 206–4, Cibertec, Madrid) and connected to a Hewlett Packard computer via an A/D interface. Contractile tension was recorded by a REGXPC computer program (Cibertec, Madrid). The preparations were stretched to a resting tension of 0.5 g (pulmonary rings), 1 g (coronary rings) or 2 g (mesenteric rings) and allowed to equilibrate for 60–90 min. During this period tissues were re-stretched and washed every 30 min with warm Krebs solution.

Experimental protocols

After equilibration, rings were contracted with either NA, U46619 or ET-1. When the contractile response to each agonist reached a stable tension, cumulative concentration-response curves to methylene blue, ACh, sodium nitroprusside (SNP), atrial natriuretic peptide (ANP), 8-bromo-guanosine-3′-5′-cyclic monophosphate (8-Br-cyclic GMP), dipyridamole or forskolin, were carried out by cumulative addition of drugs after a steady-state response was reached after each increment. In some experiments the relaxant effect of SNP was tested in arteries precontracted with 3×10^{-7} M phorbol 12-myristate, 13-acetate (PMA). The relaxant effect of SNP was also analysed in arteries treated with thapsigargin (2×10^{-6} M), staurosporine (10^{-8} M and 10^{-7} M), PMA (3×10^{-8} M), meclofenamate (10^{-5} M) or K^+-free solution (without KCl and KH_2PO_4 replaced with NaH_2PO_4) for 45 min before a contraction was induced with NA, U46619 or ET-1. Some arteries were contracted with 80 mM KCl (replacing NaCl isotonically), then relaxed with 10^{-6} M nifedipine and, thereafter, NA or U46619 were added before the concentration-response curve to SNP was carried out. In other experiments, after a contraction had been induced with NA or U46619, arteries were treated with 10^{-6} M 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 3×10^{-7} M dipyridamole or 10^{-7} M charybdotoxin for 20–30 min before concentration-response curves were constructed to SNP.

Cyclic GMP assay

Pulmonary rings were mounted in the organ chambers and tension was recorded as described above. After equilibration, they were exposed to vehicle, 10^{-5} M NA, 10^{-6} M U46619 or 3×10^{-9} M ET-1 for 40 min and finally, were either untreated or treated with 10^{-5} M SNP for 3 min. At the end of the 3 min, rings were rapidly removed from the organ chamber and quickly frozen on dry ice and stored at −80°C. The rings were then homogenized in 600 μl of 10% trichloroacetic acid, centrifuged at 10,000 *g* for 10 min at 4°C and the supernatant extracted 4 times in 3 volumes of water-saturated diethylether. The cyclic GMP concentrations were determined by radioimmunoassay by use of an acetylated Amersham [^{125}I]-cyclic GMP assay kit (Amersham International, Buckinghamshire, UK). The cyclic GMP was expressed as pmol g^{-1} wet tissue.

Drugs

The following drugs were used: (−)-noradrenaline bitartrate, acetylcholine chloride, sodium nitroprusside (SNP), human atrial natriuretic peptide (ANP), 8-bromo-cyclic GMP, 5-hydroxytryptamine (5-HT) creatine phosphate complex, adenosine 5′-trisphosphate magnesium salt (ATP), [Arg^8]-vasopressin, U46619 (9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$ methyl acetate solution), thapsigargin, endothelin-1 (ET-1), methylene blue, methoxamine hydrochloride, forskolin, staurosporine, phorbol 12-myristate, 13-acetate and dipyridamole (Sigma Chemical Co., London), charybdotoxin (RBI, Natick, MA), meclofenamate (Warner Lambert Co., U.S.A.), nifedipine (Bayer, Leverkussen, Germany) and ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (Tocris Cookson Ltd, Bristol, U.K.). All drugs were dissolved initially in distilled deionized water (except for dipyridamole, staurosporine, thapsigargin, PMA and forskolin which were dissolved in dimethyl sulphoxide and nifedipine in ethanol) to prepare a 10^{-2} M, 10^{-3} M or 10^{-4} M stock solution and further dilutions were made in PSS. The concentrations expressed are final molar concentrations in the tissue chamber.

Statistical analysis

Results are expressed as means ± s.e.mean of measurements in *n* arteries. Individual cumulative concentration-response curves were fitted to a logistic equation. The drug concentration exhibiting 50% of the maximal effect (E_{max}) was calculated from the fitted concentration-response curves for each ring and expressed as negative log molar concentration (pD_2). Statistically significant differences between groups were calculated by ANOVA followed by Newman Keuls test. $P<0.05$ was considered statistically significant.

Results

Contractile effects of NA, U46619, ET-1, vasopressin, 5-HT and ATP in piglet pulmonary arteries

In previous experiments in piglet endothelium-denuded pulmonary arteries, 10^{-5} M NA induced a maximally effective

contractile response (99±4% of the E_{max} to NA), 3×10^{-8} M, 10^{-7} M and 10^{-6} M U46619 induced a response of 56±9%, 80±9% and 96±4%, respectively, of the E_{max} to U46619 and 10^{-9} M and 3×10^{-9} M ET-1 induced a response of 65±5% and 86±4%, respectively, of the E_{max} to ET-1. The magnitude of the steady-state contractile responses induced by 10^{-4} M methoxamine, 3×10^{-8} M or 10^{-7} M U46619 or 10^{-9} M ET-1 were not significantly different from those induced by 10^{-5} M NA (Table 1). In endothelium-denuded rings NA (10^{-5} M), methoxamine (10^{-4} M), U46619 (10^{-6} M) and ET-1 (3×10^{-9} M) induced contractile responses with a clearly different time-course (Figure 1a). NA and methoxamine induced a rapid increase in tension which reached a peak in about 1–2 min and thereafter, slowly decreased to reach a lower steady-state tension at about 10 min. This response probably reflects an initial inositol 1,4,5-triphosphate(IP_3)-induced Ca^{2+} release followed by a secondary component due to Ca^{2+} entry. In contrast, U46619 and ET-1 induced a progressive monophasic contractile response which required about 20 and 40 min, respectively, to reach a plateau. Addition of NA 10^{-5} M on top of a steady-state contraction induced by 10^{-6} M U46619 (maximally effective concentration) was still able to increase tone (from 1192±190 mg to 1595±174 mg, $n=7$, $P<0.01$). 5-HT (up to 10^{-5} M), vasopressin (up to 10^{-6} M) and ATP (up to 10^{-3} M) produced no measurable contractile effects in resting pulmonary arteries ($n=4-6$).

In endothelium-intact arteries the steady-state contractile response to NA was significantly smaller than in endothelium-denuded arteries, whereas endothelium removal did not significantly affect the responses to U46619 and ET-1 (Figure 1b).

In endothelium-denuded arteries addition of the guanylate cyclase inhibitor methylene blue (10^{-6} M and 10^{-5} M) on top of a steady-state contraction induced by 10^{-5} M NA or 10^{-6} M U46619 produced a concentration-dependent contraction (Figure 1c). This contractile response was more marked in NA- than in U46619-precontracted vessels, so that 10^{-5} M methylene blue abolished the differences in the amplitude of the contractile responses between NA and U46619.

Vasorelaxant responses of ACh, SNP, ANP and 8-Br-cyclic GMP on NA-, U46619-, ET-1-and methoxamine-induced contractions in piglet pulmonary arteries

As shown in Figure 2a, in endothelium-intact arteries ACh induced a concentration-dependent relaxant response. However, ACh induced complete relaxation in 10^{-5} M NA-precontracted rings, while it relaxed only about 50% of the contractions induced by 10^{-6} M U46619. Similarly, the relaxant responses induced by SNP, ANP and 8-Br-cyclic GMP were significantly less marked in endothelium-denuded arteries precontracted with U46619 than with NA (Figure 2b, c and d).

To evaluate the role of the precontractile tone on SNP-induced relaxation, the effects of SNP (10^{-8} M – 3×10^{-5} M) were also compared in pulmonary arteries precontracted by NA (10^{-5} M), U46619 (3×10^{-8} M, 10^{-7} M and 10^{-6} M), ET-1 (10^{-9} M and 3×10^{-9} M) and the combination of 10^{-6} M U46619 plus 10^{-5} M NA. Parameters for the concentration-response curves are shown in Table 1. In rings precontracted by equieffective concentrations of U46619 and NA (10^{-7} M and 10^{-5} M, respectively), SNP produced complete relaxation of NA-induced contractions, while it only relaxed the contractions induced by U46619 by 66%. A similar result was observed in pulmonary arteries precontracted to lower tension levels by 3×10^{-8} M U46619. The relaxant effects of 8-Br-cyclic GMP were much more marked in arteries precontracted with 10^{-5} M NA than with 10^{-7} M U46619 (not shown). In rings contracted by the combination of 10^{-6} M U46619 plus 10^{-5} M NA, the relaxant response to SNP was not different from that obtained in arteries contracted by U46619 alone. In arteries contracted by 10^{-9} M ET-1 (equieffective to 10^{-5} M NA) the vasorelaxant response to SNP was similar to that observed in arteries contracted by 10^{-5} M NA, whereas with contractions induced by higher concentrations of ET-1 (3×10^{-9} M), SNP only induced a partial relaxation. Meclofenamate (10^{-5} M) did not modify the contractile response to 3×10^{-9} M ET-1 (1275±159 mg ($n=4$)) or the relaxant response to SNP ($pD_2=5.88\pm0.18$ and $E_{max}=68\pm7\%$, $P>0.05$ vs control values obtained with 3×10^{-9} M ET-1 in Table 1).

Pulmonary arteries contracted by the selective α_1-adrenoceptor agonist methoxamine (10^{-4} M) showed a similar relaxant response to SNP as when activated by the mixed α_1 and α_2 adrenoceptor agonist NA (Table 1).

Vasorelaxant responses of forskolin on NA-, U46619- and ET-1-induced contractions in piglet pulmonary arteries

Table 2 shows that the adenylate cyclase activator forskolin (10^{-9} M – 10^{-6} M) produced a similar full relaxation in pulmonary arteries contracted by either 10^{-5} M NA, 10^{-6} M U46619 or 3×10^{-9} M ET-1. Thus, in contrast to the results obtained with the vasodilators acting through the cyclic GMP pathway, the relaxant response to forskolin was independent of the agonist employed to induce tone.

Effects of SNP on NA-, U46619- and ET-1-induced contractions in piglet coronary and mesenteric arteries

In contrast to pulmonary arteries, SNP (10^{-8} M – 3×10^{-5} M) produced a complete relaxation in mesenteric and coronary arteries which was independent of the agonist employed to raise tone (10^{-6} M NA, 10^{-6} M U46619 or 3×10^{-9} M ET-1) and similar in both arteries (Table 3). As previously shown (Ohgushi *et al*., 1993), NA produced minimal contractile effects in pig coronary arteries and, therefore, the relaxant effects of SNP could not be evaluated.

Table 1 Relaxant effects of SNP in endothelium-denuded pulmonary arteries precontracted by NA, U46619, the combination of U46619 and NA, ET-1 and methoxamine

	n	*Tension* (mg)	pD_2	E_{max}(%)
NA (10^{-5} M)	13	654±41	6.62±0.08	104±2
U46619 (3×10^{-8} M)	6	561±43	5.65±0.07**	73±4**
U46619 (10^{-7} M)	12	720±40	6.20±0.10**	66±7**
U46619 (10^{-6} M)	14	1220±59**	5.92±0.10**	66±4**
U46619 (10^{-6} M)+NA (10^{-5} M)	7	1595±174**	5.92±0.11**	75±5**
ET-1 (10^{-9} M)	6	744±92	6.52±0.08	100±2
ET-1 (3×10^{-9} M)	11	1015±77**	5.68±0.17**	75±6**
Methoxamine (10^{-4} M)	4	575±64	6.49±0.17	107±2

Tension is the pre-contraction value induced by the vasoconstrictor and pD_2 and E_{max} values refer to the effects of SNP. Results are means±s.e.means of n number of experiments. *$P<0.05$, **$P<0.01$, respectively, vs 10^{-5} M NA. Data with 10^{-5} M NA and 10^{-6} M U46619 were calculated from Figure 2b.

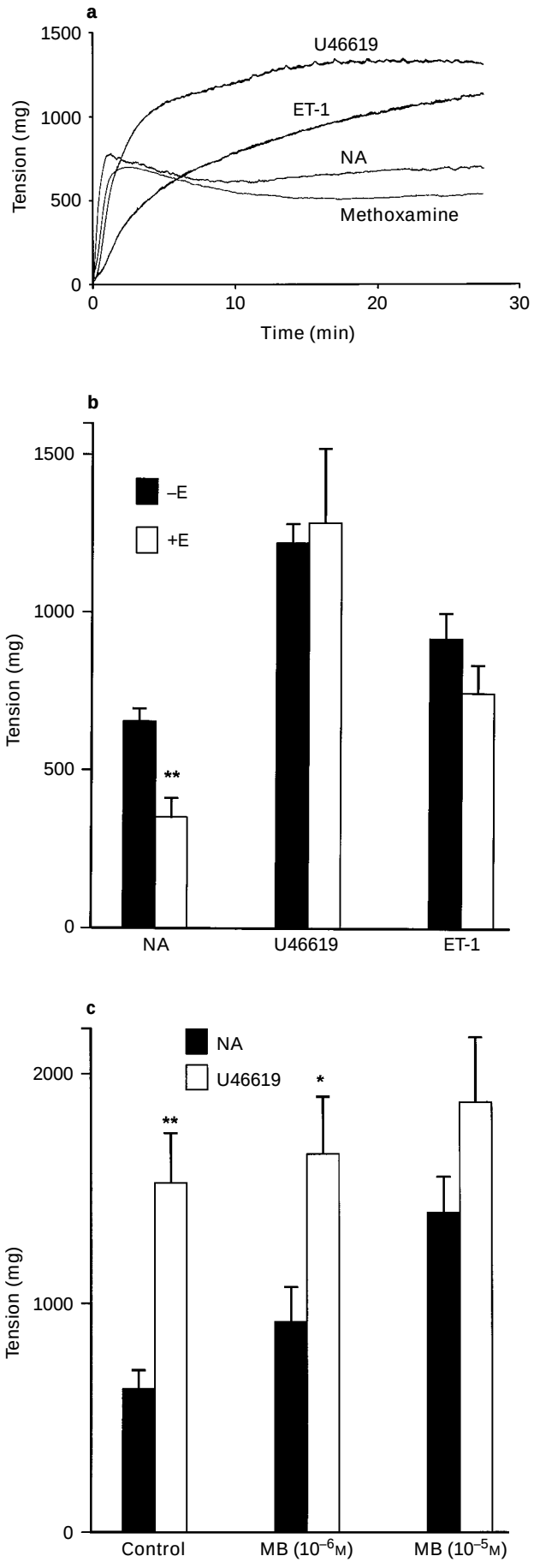

Figure 1 Time-course and role of endothelium and cyclic GMP in the contractile responses induced by NA (10^{-5} M), U46619 (10^{-6} M), ET-1 (3×10^{-9} M) and methoxamine (10^{-4} M) in piglet pulmonary arteries. (a) Time-course of the contractions induced by the four vasoconstrictors in endothelium-denuded arteries. Each trace represents the averaged recordings from 5–6 arteries. (b) Endothelial-dependence of the sustained contractions induced by the NA, U46619 and ET-1. Solid columns indicate endothelium-denuded arteries (−E) and open columns endothelium-intact arteries (+E). Each column represents the mean ± s.e.mean of 6–17 experiments. $^{**}P<0.01$ endothelium-denuded vs endothelium-intact arteries. (c) Effects of methylene blue (MB, 10^{-6} M and 10^{-5} M) on the contractions induced by NA and U46619 in endothelium-denuded arteries. Each column represents the mean ± s.e.mean of 7–8 arteries. $^{*}P<0.05$ and $^{**}P<0.01$ NA vs U46619.

Effects of SNP on NA- and U46619-induced contractions in adult rat and 2–3 months old piglet pulmonary arteries

Endothelium-denuded rat pulmonary arteries showed no differences in their maximal responses to NA and U46619 (351 ± 82 mg and 275 ± 29 mg, respectively, $P>0.05$, $n=5$). SNP (10^{-10} M – 10^{-6} M) was equally effective at relaxing the contractions induced by maximally effective concentrations of NA (10^{-7} M) and U46619 (3×10^{-6} M) (Table 4).

The relaxant effects of SNP were similar in pulmonary arteries from 2–3 month old (Table 4) or 2 week old piglets (Table 1), i.e. again U46619-induced contractions were less sensitive to SNP than those induced by NA.

Effects of NA, U46619 and ET-1 on SNP-induced increase in cyclic GMP in pulmonary arteries

Figure 3 shows the effects of SNP, alone or in combination with NA, U46619 and ET-1 on cyclic GMP levels in endothelium-denuded pulmonary arteries. SNP (10^{-5} M) increased the cyclic GMP content in resting arteries by about three fold after 3 min. The increase in cyclic GMP levels induced by SNP was unchanged in arteries pre-contracted with NA (10^{-5} M), U46619 (10^{-6} M) or ET-1 (3×10^{-9} M).

Effects of dipyridamole, ODQ, KCl, charybdotoxin, K^+-free solution and thapsigargin on the vasorelaxant responses to SNP in piglet pulmonary arteries

Addition of the cyclic GMP-dependent phosphodiesterase inhibitor dipyridamole (3×10^{-7} M) relaxed NA- and U46619-contracted arteries by 33 ± 4% and 9 ± 2%, respectively ($P<0.05$), and significantly shifted the concentration-response curves to SNP to the left (Figure 4a). This leftward shift was similar in arteries contracted by either NA or U46619 (6.3 and 6.6 fold, respectively). However, pretreatment with dipyridamole had no effect on the maximal vasodilator response to SNP. In NA-contracted arteries, addition of 10^{-6} M ODQ, a specific inhibitor of the soluble guanylate cyclase, raised tone by 102 ± 47% over previous tone and inhibited the relaxant response to SNP ($pD_2 = 5.96 \pm 0.22$, $E_{max} = 81 \pm 7\%$, $n=6$; $P<0.05$ as compared to ODQ-untreated arteries).

In resting arteries, 80 mM KCl (replacing NaCl isotonically) induced a sustained contraction averaging 876 ± 162 mg ($n=11$) which was relaxed by 74 ± 3% with 10^{-6} M nifedipine. Thereafter, a contractile response was induced by 10^{-5} M NA (final tension 1131 ± 107 mg, $n=6$) or 10^{-6} M U46619 (final tension 1585 ± 350 mg, $n=5$). As can be observed in Figure 4b, under these conditions, SNP induced a relaxant response in both NA-and U46619-contracted vessels which was slightly but significantly more potent ($P<0.05$) than in arterial rings not treated with KCl plus nifedipine (2.8 and 3.0 fold leftward shift in NA and U46619 contracted vessels, respectively). In NA-contracted arteries, addition of the Ca^{2+}-activated K^+ channel blocking agent charybdotoxin (10^{-7} M) induced a weak contractile response (16 ± 3% over previous tone) but did not modify the relaxant response to SNP ($pD_2 = 6.69 \pm 0.11$, $E_{max} = 103 \pm 3$, $n=4$, $P>0.05$ as compared to charybdotoxin-untreated arteries).

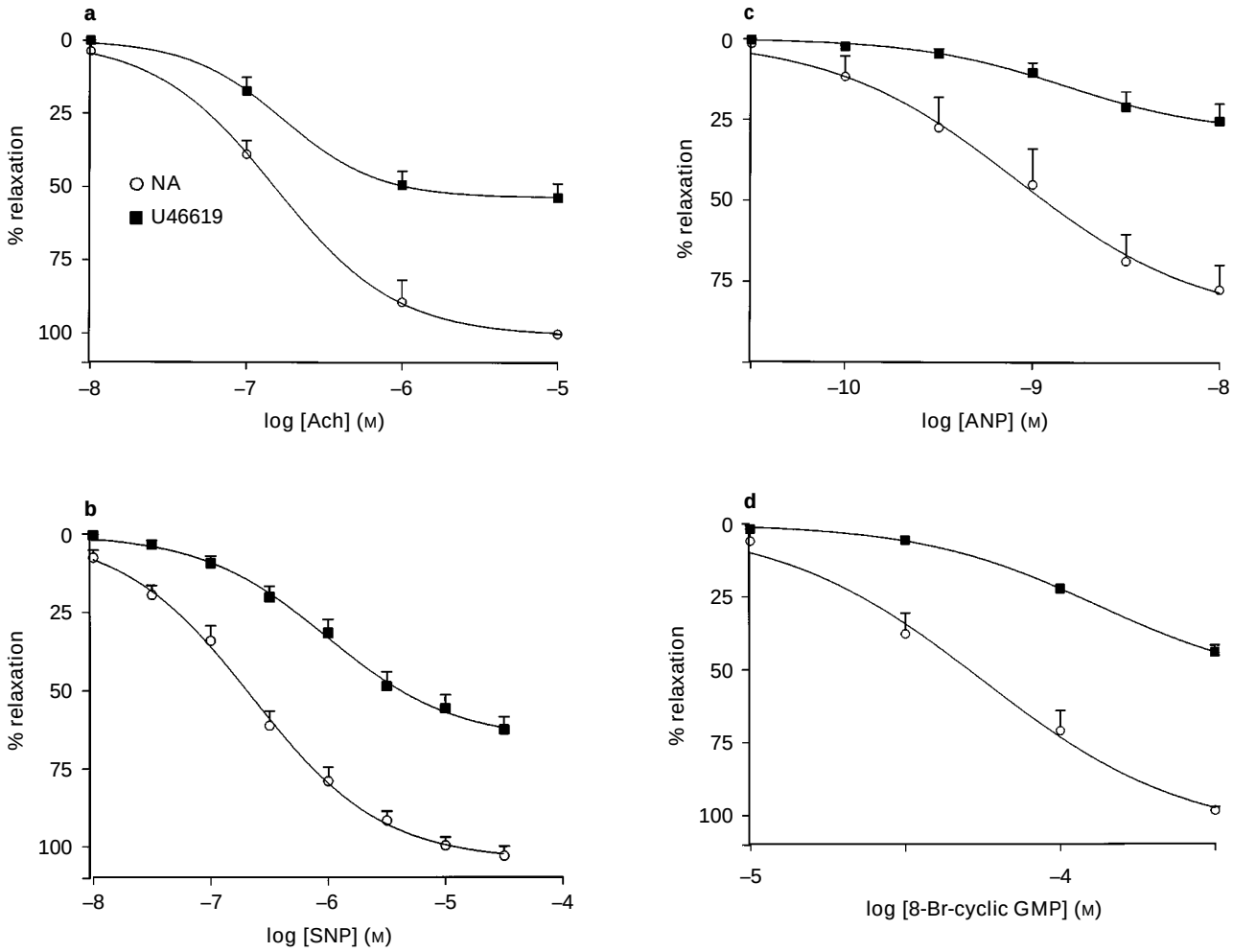

Figure 2 Effects of stimulation of the cyclic GMP pathway. Concentration-dependent relaxant effects of (a) ACh, (b) SNP, (c) ANP and (d) 8-Br-cyclic GMP in piglet pulmonary arteries precontracted with NA and U46619. The responses to ACh were studied in endothelium-intact arteries and to the other agonists in endothelium-denuded arteries. Each point represents the mean of 6–14 arteries; vertical lines show s.e.mean.

The sarcoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin (2×10^{-6} M) induced a slowly developing contractile response in resting arteries (322 ± 74 mg, $n=25$). In the presence of thapsigargin, the contractile responses induced by 10^{-5} M NA and 10^{-6} M U46619 averaged 964 ± 102 mg ($n=12$) and 1060 ± 153 mg ($n=13$), respectively. Figure 4c shows that under these conditions, the vasorelaxant response to SNP was strongly inhibited, thus E_{max} was reduced to $60\pm9\%$ and $28\pm4\%$, respectively ($P<0.01$).

Exposure of pulmonary arteries to a K^+-free solution induced a contractile response (112 ± 46 mg, $n=5$) but did not modify the contractile response to 10^{-6} M NA (779 ± 77 mg) or the relaxant effect of SNP ($pD_2=6.80\pm0.04$ and $E_{max}=105\pm4\%$).

Role of protein kinase C in the impaired response to SNP in piglet pulmonary arteries precontracted by U46619

The protein kinase C (PKC) activator PMA (3×10^{-8} M and 3×10^{-7} M) produced a contractile response which reached steady-state within 60–90 min (77 ± 17 mg, $n=5$ and 498 ± 61 mg, $n=10$, respectively). Figure 5a shows that in arteries contracted with 3×10^{-8}M PMA plus 10^{-5} M NA (final tension $=618\pm103$ mg), the relaxant response to SNP was significantly inhibited ($E_{max}=72\pm7\%$, $n=5$) as compared to NA alone ($P>0.05$), whereas in arteries precontracted with 3×10^{-7} M PMA alone, SNP induced only a small vasorelaxant effect ($E_{max}=25\pm4\%$).

Table 2 Relaxant effect of forskolin (10^{-9} M–10^{-6} M) on the contractions induced by NA, U46619 and ET-1 in piglet endothelium-denuded pulmonary arteries

	n	pD_2	E_{max} (%)
NA (10^{-5} M)	6	7.18 ± 0.10	99 ± 7
U46619 (10^{-6} M)	6	7.15 ± 0.04	96 ± 3
ET-1 (3×10^{-9} M)	7	6.97 ± 0.14	100 ± 4

Results are means$\pm$s.e.means of n number of experiments. No significant differences were found between the three groups.

The effects of pretreatment for 45 min with the PKC inhibitor staurosporine (10^{-8} M or 10^{-7} M) on the responses to SNP in arteries precontracted by 10^{-5} M NA or 10^{-6} M U46619 are shown in Figure 5b and c, respectively. This pretreatment had no effect on the contractile responses to NA (574 ± 98 mg, $n=7$, and 674 ± 84 mg, $n=7$, for 10^{-8} M and 10^{-7} M staurosporine, respectively, $P>0.05$ vs control in Table 1). Furthermore, staurosporine did not modify the relaxant responses to SNP in arteries precontracted with NA. In contrast, 10^{-8} M and 10^{-7} M staurosporine decreased the contractile response to U46619 (895 ± 121 mg, $n=8$, and 852 ± 176 mg, $n=6$ for 10^{-8} M and 10^{-7} M staurosporine, respectively, $P<0.01$ vs controls in Table 1) and augmented, in a concentration-dependent manner, the vasorelaxant response to

Table 3 Relaxant effect of SNP (10^{-8} M – 3×10^{-5} M) on the contractions induced by NA, U46619 and ET-1 in endothelium-denuded mesenteric and coronary arteries

	Mesenteric arteries				*Coronary arteries*			
	n	*Tension* (mg)	pD_2	E_{max} (%)	n	*Tension* (mg)	pD_2	E_{max} (%)
NA (10^{-6} M)	5	1480±394	6.55±0.24	107±3	5	–	–	–
U46619 (10^{-6} M)	7	1640±351	6.74±0.13	92±4	7	1190±145	6.57±0.11	96±2
ET-1 (3×10^{-9} M)	9	1957±230	6.42±0.10	99±3	6	1216±190	6.52±0.17	108±6

Tension is the pre-contraction value induced by the vasoconstrictor and pD_2 and E_{max} values refer to the effects of SNP. Results are means±s.e.means of *n* number of experiments. No significant differences were found between the three groups. NA produced very weak contractile effects in coronary arteries and, therefore, the effects of SNP were not studied.

Table 4 Relaxant effects of SNP on endothelium-denuded pulmonary arteries from adult rats or 2–3 month old piglets contracted by maximally effective concentrations of NA and U46619

		n	*Tension* (mg)	pD_2	E_{max} (%)
Adult rat					
	NA (10^{-7} M)	5	351±82	7.9±0.1	101±4
	U46619 (3×10^{-6} M)	5	275±29	7.8±0.1	100±4
2–3 month piglets					
	NA (10^{-5} M)	5	1081±129	6.5±0.3	107±4
	U46619 (10^{-6} M)	7	2034±169**	6.1±0.1*	64±6**

Tension is the pre-contraction value induced by the vasoconstrictor and pD_2 and E_{max} values refer to the effects of SNP. Results are mean±s.e.means of *n* number of experiments. *$P<0.05$, **$P<0.01$, respectively, vs 10^{-5} M NA.

SNP, increasing ($P<0.01$ vs controls in Table 1) both the pD_2 (6.26±0.15 and 6.83±0.19, respectively) and the E_{max} values (95±2% and 101±4%, respectively). Thus, in the presence of 10^{-7} M staurosporine the concentration-response curve to SNP was similar in arteries precontracted with NA and U46619. However, staurosporine (10^{-8} M) did not enhance the vasodilator effect of SNP when it was previously inhibited by 2×10^{-6} M thapsigargin ($pD_2=5.68\pm0.18$ and 5.49±0.10 and $E_{max}=62\pm9\%$ and 59±16% in the absence $n=13$, from Figure 4c, and in the presence of staurosporine, $n=5$, respectively).

Discussion

In the present study we have demonstrated that piglet pulmonary arteries when activated by the thromboxane A_2-mimetic U46619 show abnormally low relaxant responses to either NO or cyclic GMP. This effect was specific to piglet pulmonary arteries, since it was not present in rat pulmonary arteries or in piglet mesenteric or coronary arteries. The activation of protein kinase C (PKC) by the phorbol ester PMA inhibited the relaxant responses to SNP whereas the inhibition of PKC by staurosporine potentiated the relaxant response to SNP in U46619 pre-contracted arteries. The relaxant effect of SNP in piglet pulmonary arteries was inhibited by the sarcoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin but not by K^+-free solution, high KCl plus nifedipine or charybdotoxin.

Role of basal cyclic GMP

Resting pulmonary arteries have detectable basal levels of cyclic GMP which are reduced, but not abolished, when the endothelium is removed or by NO synthase inhibitors, indicating that endothelial release of NO partially accounts for the basal cyclic GMP concentration (Ignarro *et al.*, 1987). In accordance with previous studies (Levy *et al.*, 1995), in the piglet pulmonary artery the tonic contractile response to NA was augmented by endothelium removal. We found basal levels of cyclic GMP even in endothelium-denuded piglet pulmonary arteries, as detected by radioimmunoassay, and its inhibition by the guanylate cyclase inhibitor methylene blue augmented the contractile response induced by NA. Furthermore, dipyridamole induced a relaxant response in endothelium-denuded

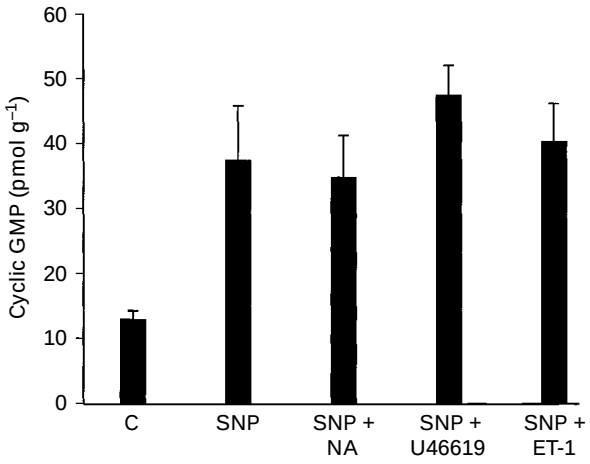

Figure 3 Effects of NA, U46619 and ET-1 on the formation of cyclic GMP stimulated by SNP. Results were obtained in endothelium-denuded arteries under control conditions (C) and in the presence of 10^{-5} M SNP alone or in combination with 10^{-5} M NA, 10^{-6} M U46619 or 3×10^{-9} M ET-1. Each column represents the mean±s.e.mean of 7–8 experiments.

pulmonary arteries. The vasodilator response to dipyridamole in the pulmonary circulation has been attributed to an increase in cyclic GMP by specifically inhibiting its degradation by cyclic GMP-dependent (type V) phosphodiesterase and not to its inhibitory effects of the uptake of adenosine (Ziegler *et al.*, 1995). However, this latter possibility cannot be fully excluded in our experiments. Thus, it can be concluded that basal formation of cyclic GMP modulates the contractile effects of NA and that the endothelium, by releasing NO, is partly responsible of cyclic GMP production. In contrast, the contractile responses to U46619 and ET-1 were unaffected by endothelium removal. The maximal U46619-induced tonic contractions were of higher magnitude than those induced by NA and this difference could be abolished by methylene blue, an inhibitor of soluble guanylate cyclase. From these results it seems that U46619 inhibits the synthesis or action of basal cyclic GMP in pulmonary smooth muscle cells. Alternatively, the differences

between NA and U46619 can be explained on the basis that NA might increase cyclic GMP and induce relaxation. This has been found following the activation of endothelial α_2-adrenoceptors in piglet pulmonary arteries by the non selective α-adrenoceptor agonist phenylephrine or the selective α_2-adrenoceptor agonist clonidine, but not by the selective α_1-adrenoceptor agonist methoxamine (Pepke-Zaba *et al*., 1993). However, this was not the case in our study, since the experiments were performed in endothelium-denuded arteries and even in these conditions methylene blue induced a contractile

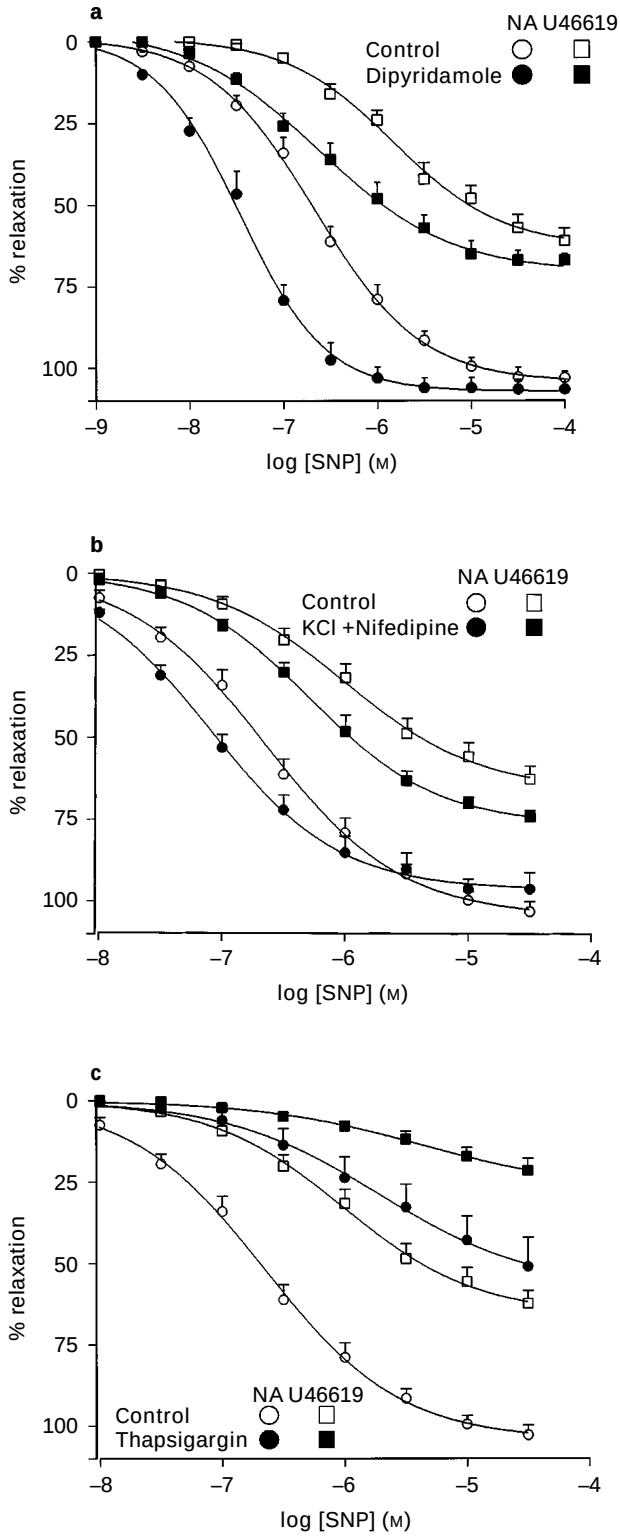

Figure 4 Effects of 3×10^{-7} M dipyridamole (a), KCl 80 mM plus 10^{-6} M nifedipine (b) and 2×10^{-6} M thapsigargin (c) on the responses to SNP in piglet endothelium-denuded pulmonary arteries contracted by 10^{-5} M NA and 10^{-6} M U46619. Each symbol represents the mean of 5–13 arteries; vertical lines show s.e.mean.

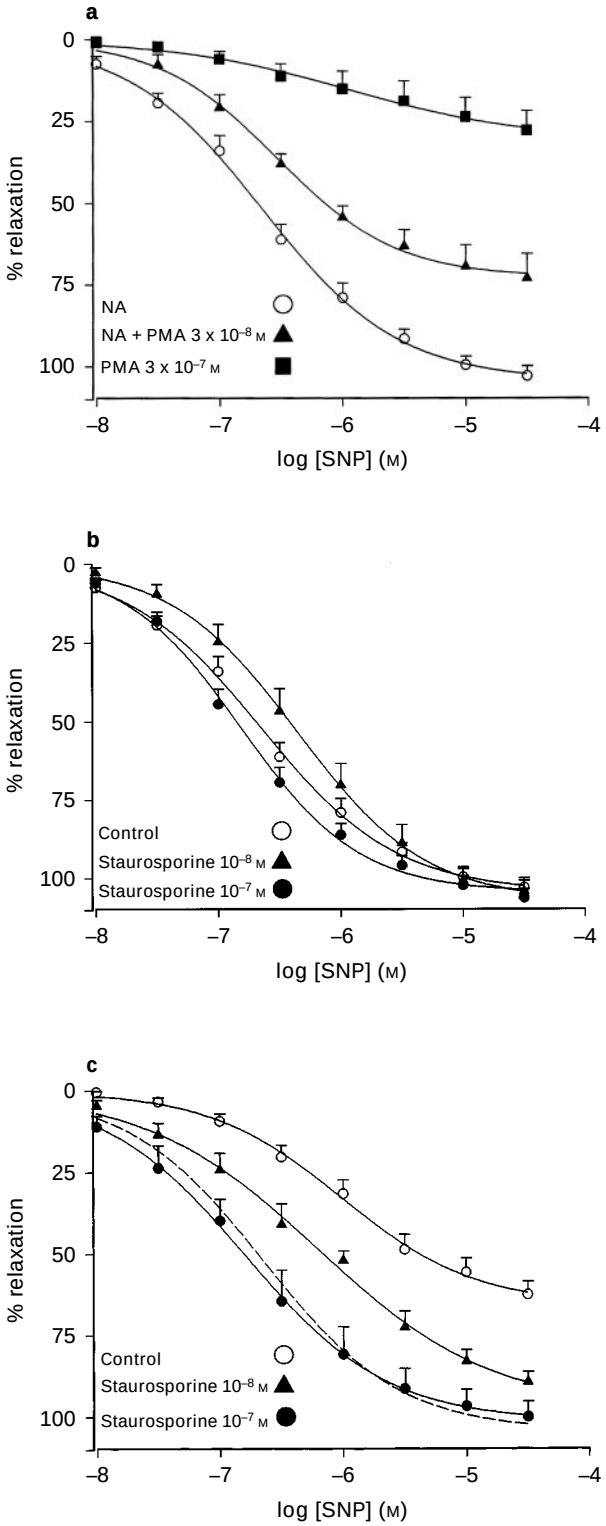

Figure 5 Role of protein kinase C pathway on U46619- and ET-1-induced inhibition of the relaxant effects of SNP in endothelium-denuded piglet pulmonary arteries. (a) The relaxant effects of SNP in arteries contracted by 10^{-5} M NA, 3×10^{-8} M PMA plus 10^{-5} M NA or 3×10^{-7} M PMA alone. (b) and (c) The effects of staurosporine 10^{-8} M and 10^{-7} M (controls are from Figure 2b) on the relaxant effects of SNP in arteries contracted by 10^{-5} M NA (b) and 10^{-6} M U46619 (c). In (c) the dashed line representing the fitted curve of the effects of SNP in NA-contracted arteries under control conditions (taken from (b)) is given for reference. Each symbol represents the mean of 6–10 arteries; vertical lines show s.e.mean.

response in arteries precontracted by NA. Furthermore, when NA was applied on top of a maximally effective U46619-induced contraction, it further contracted the artery.

Effects of stimulation of cyclic GMP

Cyclic GMP can be synthesized by the soluble and the membrane-bound isoforms of guanylate cyclase which are mainly activated by NO and ANP, respectively (Warner *et al*., 1994). NO can be raised by stimulating its release from endothelial cells (e.g. with ACh) or by the administration of NO donors such as SNP. Most of the effects of cyclic GMP are mediated by stimulation of the cyclic GMP-dependent protein kinase (PKG) (Warner *et al*., 1994; Lincoln *et al*., 1994), which can also be activated by membrane permeable and more stable cyclic GMP analogues such as 8-Br-cyclic GMP. In the present study, various activators of the cyclic GMP pathway (i.e., ACh, SNP, ANP and 8-Br-cyclic GMP) fully relaxed NA-contracted arteries, while only a partial relaxation was observed on the contractions induced by U46619. Likewise, expression of inducible nitric oxide synthase (iNOS) by endotoxin in these arteries depressed the contractile responses to NA but did not affect the responses to U46619 (Pérez-Vizcaíno *et al*., 1996). Dipyridamole, an inhibitor of type V phosphodiesterase, produced a similar leftward shift of the concentration-response curve to SNP in NA- and U46619-contracted vessels, but was unable to enhance its maximal response, which suggests that U46619 was not increasing the degradation of cyclic GMP. Moreover, U46619 did not affect SNP-induced increase in cyclic GMP synthesis. Taken together, these results suggest that U46619 inhibits the NO/cyclic GMP pathway for smooth muscle relaxation beyond the level of cyclicGMP synthesis, probably by inhibiting the activity of PKG or the mechanisms by which PKG induces its relaxant effects. An alternative explanation for the reduced relaxant responses to NO/cyclicGMP is that U46619 might induce a contractile response through a different signal transduction pathway from NA. This pathway could be less sensitive to inhibition by cyclic GMP. Assuming this possibility, U46619 would not actively inhibit the NO/cyclicGMP pathway but the contractions induced by U46619 would just be less sensitive to it. Because U46619 induced greater maximal contractile responses than NA, the effects of SNP were also studied (Table 1) in arteries contracted with lower concentrations of U46619 which produced a contractile response similar to that induced by NA. Under these conditions, arteries pre-contracted by U46619 (3×10^{-8} M or 10^{-7} M) still showed a reduced relaxant response to SNP as compared to NA. Therefore, the inhibitory action of U46619 on SNP-induced relaxation is independent of previous tone. Since the relaxant response to SNP was similar in arteries treated with U46619 alone or in combination with NA, differences in the relaxant response to SNP between NA and U46619 must be due to an U46619-induced impairment of the SNP-induced relaxation, rather than to a NA-induced potentiation of SNP-induced relaxation. A possible interference of NA with α_2-adrenoceptors can be ruled out since similar results were obtained with methoxamine (selective α_1-adrenoceptor agonist). Pulmonary arteries precontracted with ET-1 (10^{-9} M) showed similar relaxations to SNP as NA pre-contracted arteries, whereas at 3×10^{-9} M ET-1, which induced a contraction higher than 10^{-5} M NA, the relaxant response to SNP was greatly reduced, therefore, this higher tone might be responsible for the inhibitory action. However, it should be noted that 10^{-5} M NA-induced contractions were maximal or near maximal and they could be fully inhibited by SNP, i.e. an activation of the NO/cyclicGMP pathway can abolish vascular tone in the presence of any amount of NA. In contrast, if ET-1 concentrations were raised over a certain level, the increased vascular tone could not be fully inhibited even with a maximal activation of the NO/cyclic GMP pathway.

Specificity of U46619-and ET-1-induced effects

In order to assess the specificity of the impaired response to SNP observed in 2 week old piglet pulmonary arteries, we also studied the effects of SNP in endothelium-denuded mesenteric and coronary arteries from the same animals, pulmonary arteries from older piglets (2–3 months old) and pulmonary arteries from adult rats. SNP fully relaxed mesenteric and coronary arteries precontracted by NA, U46619 or ET-1 as well as those induced by NA and U46619 in adult rat pulmonary arteries. However, in pulmonary arteries from 2–3 month old piglets the vasorelaxant response induced by SNP was similar to that observed in 2 week old piglets contracted by either NA or U46619 (i.e. again U46619-induced contractions were partially resistant to the relaxant effects of SNP).

The adenylate cyclase activator forskolin (Seamon & Daly, 1986), which increases cyclic AMP levels, produced complete vasorelaxation of the contractions induced by NA, U46619 and ET-1. The mechanism by which cyclic AMP causes smooth muscle relaxation is not fully understood. While activation of the specific cyclic AMP-dependent protein kinase has been shown to be involved, more recent data suggest that cyclic AMP could also stimulate PKG and relax smooth muscle (Lincoln *et al*., 1990). Due to the differences in the relaxant response between cyclic AMP- and cyclic GMP-dependent vasodilators, our data might suggest that cyclic AMP- and cyclic GMP act via different pathways in these arteries. However, this requires further investigation. All these results showed that the abnormal relaxant response to the NO/cyclic GMP pathway is a singular feature of piglet pulmonary arteries when activated by U46619.

Mechanism of action of the vasorelaxant effect of SNP in piglet pulmonary arteries

SNP causes vascular smooth muscle relaxation by releasing NO which, in turn, activates guanylate cyclase and increases intracellular cyclic GMP levels (Rapoport *et al*., 1985; Ignarro *et al*., 1986; Kowaluk *et al*., 1992). In the present study, SNP raised intracellular cyclic GMP by about three fold and its vasorelaxant effect was potentiated by dipyridamole and inhibited by ODQ, which indicates that SNP-induced relaxation in piglet pulmonary arteries is mediated by stimulation of cyclic GMP synthesis.

Cyclic GMP and PKG may control a large number of cellular activities to regulate vascular smooth muscle tone (Lincoln *et al*., 1994), including inhibition of the synthesis and/or action of IP_3 (Hirata *et al*., 1990), activation of the sarcoplasmic reticulum Ca^{2+}-ATPase (Rashatwar *et al*., 1987; Luo *et al*., 1993), activation of Ca^{2+}-dependent K^+ channels (Archer *et al*., 1994), inhibition of Ca^{2+} entry and possibly activation of the Na^+/K^+-ATPase (Rapoport *et al*., 1985). The role of each of these mechanisms may be different depending on the vascular bed (Ferrer *et al*., 1995). All these effects may lead to decreased cytosolic Ca^{2+} levels and/or Ca^{2+} sensitivity of the contractile apparatus of vascular smooth muscle leading to relaxation (Lincoln *et al*., 1994; Warner *et al*., 1994). Using pharmacological tools we have investigated the role of several of these mechanisms in the piglet pulmonary artery. The role of K^+ channel opening was studied in preparations exposed to high KCl concentrations, thus eliminating the chemical gradient for K^+ efflux so that the opening of K^+ channels would not result in net K^+ flow and hyperpolarization. Nifedipine was included in these experiments to avoid an excessive intracellular Ca^{2+} load induced by KCl depolarization. Under these conditions, the relaxant effect of SNP was not inhibited and instead a weak but significant increase was observed, suggesting that K^+ channel activation is unlikely to mediate the vasodilator effects of SNP. Moreover, the specific inhibitor of large conductance Ca^{2+}-activated K^+ channels charybdotoxin (10^{-7} M) had no effect on SNP-induced relaxation which is consistent with a lack of involvement of Ca^{2+}-activated K^+ channels in SNP-induced relaxation. Ca^{2+} sequestration by the

sarcoplasmic reticulum Ca^{2+}-ATPase leads to decreased cytosolic Ca^{2+} levels and smooth muscle relaxation. Thapsigargin, a specific inhibitor of this Ca^{2+}-ATPase (Thastrup *et al*., 1990), markedly suppressed SNP-induced relaxation in arteries contracted by either NA or U46619, which indicated that increased Ca^{2+} uptake by the sarcoplasmic reticulum is an important mechanism by which SNP decreases $[Ca^{2+}]_i$ and induces its relaxant effects in piglet pulmonary arteries. Activation of the membrane electrogenic Na^+/K^+ ATPase leads to vascular smooth muscle hyperpolarization and relaxation, whereas its inhibition produces the opposite effects (Rapoport *et al*., 1985). Incubation in a K^+-free solution, which inhibits the Na^+/K^+-ATPase, had no effect on SNP-induced relaxation, suggesting that an activation of this pump is not involved in its vasorelaxant effect in piglet pulmonary arteries. This is in accordance with previous results from our group (Villamor *et al*., 1996a), showing that the relaxant effects of ACh in these arteries were not modified by incubation with a Mg^{2+}-free solution which also inhibits the activity of the pump.

Possible mechanisms of reduced NO/cyclic GMP relaxation in U46619 pre-contracted arteries

We further investigated the mechanisms involved in the inhibition of the relaxant effects of SNP by U46619. Because both U46619 and ET-1 inhibit several K^+ channels (Miyoshi *et al*., 1992; Scornik & Toro, 1992) this effect might be mediating the inhibition of SNP-induced relaxation. However, as described above, K^+ channels do not appear to be involved in SNP-induced relaxation in pulmonary arteries. Furthermore, the inhibitory action of U46619 and ET-1 on K^+ channels has been shown in porcine coronary artery myocytes (Miyoshi *et al*., 1992; Scornik & Toro, 1992) which showed normal relaxation to SNP in our experiments.

G-protein linked membrane receptors for several vasoconstrictors including NA, thromboxane A_2 (TXA_2) and ET-1 (α_1-adrenoceptors, TP and ET_A receptors, respectively) are coupled with phospholipase C (Strader *et al*., 1995). Activated phospholipase C catalyzes hydrolysis of phosphoinositol 1,4,5-diphosphate (PIP_2) into IP_3, which releases Ca^{2+} from intracellular stores, and diacylglycerol, which activates PKC. In our experiments, activation of PKC by the phorbol ester PMA, alone or in combination with NA, inhibited the relaxant response to SNP, suggesting that one potential mechanism of inhibition of cyclic GMP-induced vasodilatation by U46619 might be related to the activation of PKC. This has also been shown in the rat aorta where SNP was more effective in relaxing methoxamine- than phorbol ester-induced contractions (Morrison & Pollock, 1990). Staurosporine, a potent inhibitor of protein kinase C (Hidaka & Kobayashi, 1992), did not modify the relaxant response to SNP in arteries precontracted by NA, but abolished the inhibition induced by U46619. Thus, these results suggest that U46619 may activate PKC and thus, inhibit the relaxant effects of cyclic GMP. Staurosporine also depressed the contractile response to U46619, indicating that inhibition of PKC might increase the sensitivity not only to stimulated cyclic GMP but also to basal cyclic GMP. ET-1 has been shown to stimulate the release of TXA_2 in guinea-pig lung (De Nucci *et al*., 1988), so that ET-1 might inhibit SNP-induced relaxation by releasing TXA_2. However, this was not the case in the present study, because the cyclo-oxygenase inhibitor meclofenamate did not change either the contractile effect of ET-1 or the relaxant effect of SNP.

Implications of TXA_2, ET-1 and impaired NO/cyclic GMP pathway in the pulmonary vascular bed

The finding that U46619 inhibits the NO/cyclic GMP pathway in the pulmonary vasculature may help to explain why: (a) The induction of iNOS by endotoxin or Group B *Streptococcus* and the subsequent large increase in NO concentrations did not modify the contractile responses to U46619 in piglet isolated pulmonary arteries despite reducing the contractions to NA (Villamor *et al*., 1996b; Pérez-Vizcaíno *et al*., 1996). (b) The administration of inhaled NO to piglets did not modify (10 p.p.m) (Weitzberg *et al*., 1993) or only slightly reduced (50 p.p.m) (Klemm *et al*., 1995) the TXA_2-mediated early phase but inhibited the TXA_2-independent late phase of endotoxin-induced pulmonary hypertension (Weitzberg *et al*., 1993; Klemm *et al*., 1995). (c) In piglets with endotoxin-induced pulmonary hypertension, the administration of NO synthase inhibitors did not further increase pulmonary vascular resistance (Klemm *et al*., 1995; Weitzberg *et al*., 1995). (d) Inhaled NO (50 p.p.m) had no effect on the pulmonary hypertension induced by continuous i.v. administration of U46619 in dogs (Welte *et al*., 1995). In contrast, inhaled NO (5–80 p.p.m) inhibited U46619-induced vasoconstriction in sheep (Frostell *et al*., 1991). In our study, species-dependent differences were also observed, since U46619 inhibited the vasodilator effect of the NO donor SNP in piglet but not in rat pulmonary arteries.

Furthermore, our results showed that ET-1 only impaired the NO/cyclic GMP induced relaxation when inducing a relatively high contraction. In addition, very high concentrations of ET-1 (2×10^{-8} M and 2×10^{-7} M) inhibited SNP-induced relaxation in human pulmonary arteries and veins but, in contrast to the present study, it decreased cyclic GMP synthesis (Pussard *et al*., 1995). To our knowledge it is not known if inhaled nitric oxide can reduce pulmonary hypertension induced by infusion of ET-1. However, the late phase of pulmonary hypertension induced by endotoxin in piglets, which has been related to increased production of ET-1 (Weitzberg *et al*., 1996), can be inhibited by inhaled NO (Weitzberg *et al*., 1993; Klemm *et al*., 1995). We have also recently demonstrated that iNOS induction by Group B *Streptococci* reduces the contractile responses to ET-1 (Villamor *et al*., 1996b).

Pulmonary hypertension is accompanied by an increase in pulmonary vascular resistances due to an imbalance between vasoactive mediators. Increased ET-1 and TXA_2 levels and decreased activity of NO/cyclic GMP are the most recognized mechanisms in the vasoconstrictor and vasodilator responses, respectively (see Introduction). It is becoming clear that no single mechanism can be responsible for the different clinical forms of pulmonary hypertension. Impairment of the NO/cyclic GMP pathway at the level of NO synthesis, activation of guanylate cyclase and/or the activity of cyclic GMP may depend on several factors, including the species, age and the factor causing the pulmonary insult. Our results provide evidence for a novel mechanism of NO/cyclic GMP impairment mediated by PKC through TXA_2 receptor activation at the level of cyclic GMP activity. Inhaled NO has been shown to be effective in vasodilating the ventilated lung areas and improving gas exchange in newborn and adult patients with pulmonary hypertension (Kinsella *et al*., 1993; Falke, 1993; Abman & Kinsella, 1995). However, about half of the patients do not respond to inhaled NO (Roberts *et al*., 1997; Neonatal Inhaled Nitric Oxide Study Group, 1997). A possible theoretical explanation for this lack of response is an alteration of the smooth muscle responsiveness to NO (Cremona *et al*., 1991; Abman & Kinsella, 1995). Because anatomical development of neonatal pig lung is similar to that of human lung and neonatal piglets have been widely used as an experimental model of pulmonary hypertension of the newborn (e.g. Gibson *et al*., 1987), it is tempting to speculate that an impairment of the NO/cyclic GMP pathway similar to that herein described may play a role in human nonresponders to inhaled NO. Understanding the precise mechanisms regulating the NO/cyclic GMP signalling under physiological and pathological conditions will certainly help in the therapeutic management of patients with pulmonary hypertension.

In conclusion, piglet pulmonary arteries pre-contracted by the TXA_2-mimetic U46619 showed reduced relaxant responses to the NO/cyclic GMP-pathway. This effect was not associated with changes in cyclic GMP content and was not observed in

piglet mesenteric or coronary arteries or in rat pulmonary arteries. SNP-induced relaxation in piglet pulmonary arteries was inhibited by the sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (but not by inhibition of either the membrane Na^+/K^+-ATPase or K^+ channels) indicating a role for Ca^{2+} uptake by the sarcoplasmic reticulum in these relaxant effects. The effects of U46619 on the relaxation of SNP could be abolished by inhibition of PKC, suggesting that PKC may be a part of signal transduction in the U46619-induced inhibitory effect.

The authors are grateful to C. Rivas for excellent technical assistance. This work was supported by a CICYT (SAF 96/0042) and FIS (95/0308 and 95/0318) Grants.

References

ABMAN, S.H., CHATFIELD, B., HALL, S.L. & MCMURTRY, I.F. (1990). Role of endothelium-derived releasing factor during the transition of the pulmonary circulation at birth. *Am. J. Physiol.*, **259,** H1921 – H1927.

ABMAN, S.H. & KINSELLA, J.P. (1995). Inhaled nitric oxide for persistent pulmonary hypertension of the newborn: The physiology matters! *Pediatrics*, **96,** 1153 – 1155.

ARCHER, S.L., HUANG, J.M.C., HAMPL, V., NELSON, D.P., SHULTZ, P.J. & WEIR, E.K. (1994). Nitric oxide and cyclic GMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cyclic GMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.*, **91,** 7583 – 7587.

BARNES, P.J. & LIU, S.F. (1995). Regulation of pulmonary vascular tone. *Pharmacol. Rev.*, **47,** 87 – 131.

CODY, R.J., HASS, G.J., BINKLEY, P.F., CAPERS, Q. & KELLEY, R. (1992). Plasma endothelin correlates with the extent of pulmonary hypertension in patients with congestive heart failure. *Circulation*, **85,** 504 – 509.

CREMONA, G., DINH XUAN, A.T. & HIGENBOTTAM, T.W. (1991). Endothelium-derived relaxing factor and the pulmonary circulation. *Lung*, **169,** 185 – 202.

DE NUCCI, G., THOMAS, R., D'ORLEANS-JUSTE, P., ANTUNES, E., WALDER, C., WARNER, T. & VANE, J.R. (1988). Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U.S.A.*, **85,** 9797 – 9800.

DINH-XUAN, A.T., HIGENBOTTAM, W., CLELLAND, T.W., PEPKE-ZABA, J., CREMONA, G., BUTT, A.Y., LARGE, S.R., WELLS, F.C. & WALLWORK, J. (1991). Impairment of endothelium-dependent pulmonary-artery relaxation in chronic obstructive lung disease. *N. Engl. J. Med.*, **324,** 1539 – 1547.

DOBYNS, E.L., WESCOTT, J.Y., KENNAUGH, J.M., ROS, M.N. & STENMARK, K.R. (1994). Eicosanoids decrease with successful extracorporeal membrane oxygenation therapy in neonatal pulmonary hypertension. *Am. J. Respir. Crit. Care Med.*, **149,** 873 – 880.

FALKE, K.J. (1993). Long term inhalation with evaluated low doses of nitric oxide for selective improvement of oxygenation in patients with adult respiratory distress syndrome. *Intensive Care Med.*, **19,** 443 – 449.

FERRER, M., ENCABO, A., CONDE, M.V., MARIN, J. & BALFAGON, G. (1995). Heterogeneity of endothelium-dependent mechanisms in different rabbit arteries. *J. Vasc. Res.*, **32,** 339 – 346.

FROSTELL, C., FRATACCI, M.D., WAIN, J.C., JONES, R. & ZAPOL, W.M. (1991). Inhaled nitric oxide. A selective pulmonary vasodilator reversing hypoxic pulmonary vasoconstriction. *Circulation*, **83,** 2038 – 2047.

GARCIA-SZABO, R.R., JOHNSON, A. & MALIK, A.B. (1988). Thromboxane increases pulmonary vascular resistance and transvascular fluid and protein exchange after pulmonary microembolism. *Prostaglandins*, **35,** 707 – 721.

GIAID, A. & SALEH, D. (1995). Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N. Engl. J. Med.*, **333,** 214 – 221.

GIAID, A., YANAGISAWA, M., LANGLEBEN, D., MICHEL, R.P., LEVY, R., SHENNIB, H., KIMURA, S., MASAKI, T., DUGUID, W.P. & STEWART, D.J. (1993). Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. *N. Engl. J. Med.*, **328,** 1732 – 1739.

GIBSON, R.L., TRUOG, W.E. & REDDING, G.J. (1987). Thromboxane-associated pulmonary hypertension during three types of Gram-positive bacteriemia in piglets. *Pediatr. Res.*, **23,** 553 – 556.

HIDAKA, H. & KOBAYASHI, R. (1992). Pharmacology of protein kinase inhibitors. *Annu. Rev. Pharmacol. Toxicol.*, **32,** 377 – 397.

HIRATA, M., KOHSE, K.P., CHANG, C.-H., IKEBE, T. & MURAD, R. (1990). Mechanism of cyclic GMP inhibition of inositol phosphate formation in rat aorta segments and cultured bovine aortic smooth muscle cells. *J. Biol. Chem.*, **265,** 1268 – 1273.

IGNARRO, L.J., BYRNS, R.E. & WOOD, K.S. (1987). Endothelium-dependent modulation of cyclic GMP levels and intrinsic smooth muscle tone in isolated bovine intrapulmonary artery and vein. *Circ. Res.*, **60,** 89 – 92.

IGNARRO, L.J., HARBISON, R.G., WOOD, K.S. & KADOWITZ, P.J. (1986). Dissimilarities between methylene blue and cyanide on relaxation and cyclic GMP formation in endothelium-intact intrapulmonary artery caused by nitrogen oxide-containing vasodilators and acetylcholine. *J. Pharmacol. Exp. Ther.*, **236,** 30 – 36.

KINSELLA, J.P., NEISH, S.R., IVY, D.D., SCHAFFER, E. & ABMAN, S.H. (1993). Clinical responses to prolonged treatment of persistent pulmonary hypertension of the newborn with low doses of inhaled nitric oxide. *J. Pediatr.*, **123,** 103 – 108.

KLEMM, P., THIEMMERMAN, C., WINKLMAIER, G., MARTORANA, P.A. & HENNING, R. (1995). Effects of nitric oxide synthase inhibition combined with nitric oxide inhalation in a porcine model of endotoxic shock. *Br. J. Pharmacol.*, **114,** 363 – 368.

KOWALUK, E.A., SETH, P. & FUNG, H.-L. (1992). Metabolic activation of sodium nitroprusside to nitric oxide in vascular smooth muscle. *J. Pharmacol. Exp. Ther.*, **262,** 916 – 922.

LEVY, M., TULLOH, R.M., KOMAI, H., STUART-SMITH, K. & HAWORTH, S.G. (1995). Maturation of the contractile response and its endothelial modulation in newborn porcine intrapulmonary arteries. *Pedriatr. Res.*, **38,** 25 – 29.

LINCOLN, T.M., CORNWELL, T.L. & TAYLOR, A.E. (1990). cyclic GMP-dependent protein kinase mediates the reduction of Ca^{2+} by cAMP in vascular smooth muscle cells. *Am. J. Physiol.*, **258,** C399 – C407.

LINCOLN, T.M., KOMALAVILAS, P. & CORNWELL, T.L. (1994). Pleiotropic regulation of vascular smooth muscle tone by cyclic GMP-dependent protein kinase, *Hypertension*, **23,** 1141 – 1147.

LUO, D.-L., NAKAZAWA, M., ISHIBASHI, T., KATO, K. & IMAI, S. (1993). Putative, selective inhibitors of sarcoplasmic reticulum Ca^{++}-pump ATPase inhibit relaxation by nitroglycerin and atrial natriuretic factor of the rabbit aorta contracted by phenylephrine. *J. Pharmacol. Exp. Ther.*, **265,** 1187 – 1192.

MCQUESTON, J.A., KINSELLA, J.P., IVY, D.D., MCMURTRY, I.F. & ABMAN, S.H. (1995). Chronic pulmonary hypertension in utero impairs endothelium-dependent vasodilation. *Am. J. Physiol.*, **268,** H288 – H294.

MIYOSHI, Y., NAKAYA, Y., WAKATSUKI, T., NAKAYA, S., FUJINO, K., SAITO, K. & INOUE, I. (1992). Endothelin blocks ATP-sensitive K^+ channels and depolarizes smooth muscle cells of porcine coronary artery. *Circ. Res.*, **70,** 612 – 616.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.*, **43,** 109 – 142.

MONTALESCOT, G., LOWENSTEIN, E., OGLETREE, M.L., GREENE, E.M., ROBINSON, D.R., HARTL, K. & ZAPOL, W.M. (1990). Thromboxane receptor blockade prevents pulmonary hypertension induced by heparin-protamine reactions in awake sheep. *Circulation*, **82,** 1765 – 1777.

MORRISON, K.J. & POLLOCK, D. (1990). Impairment of relaxations to acetylcholine and nitric oxide by a phorbol ester in rat isolated aorta. *Br. J. Pharmacol.*, **101**, 432 – 436.

NEONATAL INHALED NITRIC OXIDE STUDY GROUP. (1997). Inhaled nitric oxide in full-term and nearly full-term infants with hypoxic respiratory failure. *New Engl. J. Med.*, **336,** 597 – 604.

NOONAN, T.C. & MALIK, A.B. (1986). Pulmonary vascular response to leukotriene D_4 in unanesthetized sheep: Role of thromboxane. *J. Appl. Physiol.*, **60,** 765–769.

OHGUSHI, M., YASUE, H., KUGIYAMA, K., MUROHARA, T. & SAKAINO, N. (1993). Contraction and endothelium-dependent relaxation via alpha adrenoceptors are variable in various pig arteries. *Cardiovasc. Res.*, **27,** 779–784.

PEPKE-ZABA, J., HIGENBOTTAM, T.W., DINH-XUAN, A.T., RIDDEN, C. & KEALEY, T. (1993). Alpha-adrenoceptor stimulation of porcine pulmonary arteries. *Eur. J. Pharmacol.*, **235,** 169–175.

PEREZ-VIZCAINO, F., VILLAMOR, E., FERNANDEZ DEL POZO, B., MORO, M. & TAMARGO, J. (1996). Lack of endotoxin-induced hyporesponsiveness to U46619 in isolated neonatal porcine pulmonary but not mesenteric arteries. *J. Vasc. Res.*, **33,** 249–257.

PUSSARD, G., GASCARD, J.-P., GORENNE, I., LABAT, C., NOREL, X., DULMET, E. & BRINK, C. (1995). Endothelin-1 modulates cyclic GMP production and relaxation in human pulmonary vessels. *J. Pharmacol. Exp. Ther.*, **274,** 969–975.

RAPOPORT, R.M., SCHWARTZ, K. & MURAD, F. (1985). Effect of sodium-potassium pump inhibitors and membrane-depolarizing agents on sodium nitroprusside-induced relaxation and cyclic guanosine monophosphate accumulation in rat aorta. *Circ. Res.*, **57,** 164–170.

RASHATWAR, S.S., CORNWELL, T.L. & LINCOLN, T.M. (1987). Effects of 8-bromo-cyclic GMP on Ca^{2+} levels in vascular smooth muscle cells: Possible regulation of Ca^{2+}-ATPase by cyclic GMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.*, **84,** 5685–5689.

ROBERTS, J.D., FINEMAN, J.R., MORIN III, F.C., SHAUL, P.W., RIMAR, S., SCHREIBER, M.D., POLIN, R.A., ZWASS, M.S., ZAYEC, M.M., GROSS, I., HEYMANN, M.A. & ZAPOL, W.M. (1997). Inhaled nitric oxide and persistent pulmonary hypertension of the newborn. *New Engl. J. Med.*, **336,** 605–610.

ROSENBERG, A.A., KENNAUGH, J., KOPPENHAFER, S.L., LOOMIS, M. & ABMAN, S.H. (1993). Elevated immunoreactive endothelin-1 levels in infants with persistent pulmonary hypertension of the newborn. *J. Pediatr.*, **123,** 109–114.

SCORNIK, F.S. & TORO, L. (1992). U46619, a thromboxane A_2 agonist, inhibits K_{Ca} channel activity from pig coronary artery. *Am. J. Physiol.*, **262,** C708–C713.

SEAMON, K.B. & DALY, J.W. (1986). Forskolin: Its biological and chemical properties. *Adv. Cyclic Nucleotide Res.*, **20**, 1–150.

STRADER, C.D., FONG, T.M., GRAZIANO, M.P. & TOTA, M.R. (1995). The family of G-protein-coupled receptors. *FASEB J.*, **9,** 745–754.

THASTRUP, O., CULLEN, P.J., DROBAK, B.K., HANLEY, M.R. & DAWSON, A.P. (1990). Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+} ATPase. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 2466–2470.

VILLAMOR, E., PEREZ-VIZCAINO, F., RUIZ, T., TAMARGO, J. & MORO, M. (1996a). *In vitro* effects of magnesium sulfate in isolated intrapulmonary and mesenteric arteries of piglets. *Pediatr. Res.*, **39,** 1107–1112.

VILLAMOR, E., PEREZ-VIZCAINO, F., TAMARGO, J. & MORO, M. (1996b). Effects of *group B Streptococcus* on the responses to U46619, endothelin-1 and noradrenaline in isolated pulmonary and mesenteric arteries of piglets. *Pediatr. Res.*, **40,** 827–833.

WARNER, T.D., MITCHELL, J.A., SHENG, H. & MURAD, F. (1994). Effects of cyclic GMP on smooth muscle relaxation. *Adv. Pharmacol.*, **26,** 171–194.

WEITZBERG, E., HEMSEN, A., RUDEHILL, A., MODIN, A., WANECEK, M. & LUNDBERG, J.M. (1996). Bosentan-improved cardiopulmonary vascular performance and increased plasma levels of endothelin-1 in porcine endotoxin shock. *Br. J. Pharmacol.*, **118,** 617–626.

WEITZBERG, E., LUNDBERG, J.M. & RUDEHILL, A. (1995). Inhibitory effects of diclofenac on the endotoxin shock response in relation to endothelin turnover in the pig. *Acta Anaesthesiol. Scand.*, **39,** 50–59.

WEITZBERG, E., RUDEHILL, A. & LUNDBERG, J.M. (1993). Nitric oxide inhalation attenuates pulmonary hypertension and improves gas exchange in endotoxin shock. *Eur. J. Pharmacol.*, **233**, 85–94.

WELTE, M., ZWISSLER, B., HABLER, O., KLEEN, M. & MESSMER, K. (1995). Prostacyclin aerosol and inhaled nitric oxide fail to reverse pulmonary vasoconstriction induced by thromboxane analogue in dogs. *Acta Physiol. Scand.*, **154,** 395–405.

YOSHIBAYASHI, M., NISHIOKA, K., NAKAO, K., SITO, Y., MATSUMURA, M., UEDA, T., TEMA, S., SHIRAKAMI, G., IMURA, H. & MIKAWA, H. (1991). Plasma endothelin concentrations in patients with pulmonary hypertension associated with congenital heart disease. *Circulation*, **84,** 2280–2285.

ZAMORA, C.A., BARON, D.A. & HEFFNER, J.E. (1993). Thromboxane contributes to pulmonary hypertension in ischaemia-reperfusion lung injury. *J. Appl. Physiol.*, **74,** 224–229.

ZIEGLER, J.P., IVI, D.D., FOX, J.J., KINSELLA, J.P., CLARKE, W.R. & ABMAN, S.H. (1995). Dipirydamole, a cGMP phosphodiesterase inhibitor, causes pulmonary vasodilation in the ovine fetus. *Am. J. Physiol.*, **269,** H473–479.

(Received November 11, 1996
Revised March 18, 1997
Accepted April 17, 1997)