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Mechanisms involved in SNP-induced relaxation and $[Ca^{2+}]_i$ reduction in piglet pulmonary and systemic arteries

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1 We have compared the mechanisms involved in sodium nitroprusside (SNP)-induced relaxation and $[Ca^{2+}]_i$ reduction in isolated piglet pulmonary (PA) and mesenteric (MA) arteries.

2 SNP (10^{-8} M -3×10^{-5} M) evoked a concentration-dependent relaxation of PA and MA ($pD_2=6.66\pm0.06$ and 6.74 ± 0.14, respectively) stimulated by noradrenaline, which was markedly reduced by the guanylate cyclase inhibitor ODQ. In fura 2-incubated PA and MA, SNP produced a parallel reduction in contractile force and in $[Ca^{2+}]_i$, expressed as the ratio of emitted fluorescence at 340 and 380 nm (F340/F380).

3 The inhibition of the Na^+/K^+-ATPase after the incubation in a K^+-free medium or the exposure to ouabain (10^{-6} M) inhibited SNP-induced relaxation in MA but not in PA. SNP-induced relaxation was not attenuated by 80 mM KCl plus nifedipine (10^{-6} M) but was inhibited by thapsigargin (2×10^{-6} M; $pD_2=5.69\pm0.19$ and 5.89 ± 0.19 for PA and MA, respectively).

4 Pretreatment of PA with thapsigargin and MA with thapsigargin plus ouabain induced a stronger inhibition on the reduction in $[Ca^{2+}]_i$ than on the relaxation induced by SNP, indicating the existence of Ca^{2+}-independent mechanisms.

5 The activation of the Na^+/K^+-ATPase by the addition of KCl after the incubation in a K^+-free medium similarly reduced $[Ca^{2+}]_i$ in PA and MA, whereas it relaxed with much less efficacy PA than MA.

6 We conclude that SNP reduces $[Ca^{2+}]_i$ and causes relaxation through the activation of SERCA in PA and SERCA and Na^+/K^+-ATPase in MA. However, Ca^{2+}-independent mechanisms also contribute to SNP-induced effects.

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Abbreviations: F340/F380, ratio of emitted fluorescence at two wavelengths of excitation (340 and 380 nm); MA, mesenteric arteries; NA, noradrenaline; PA, pulmonary arteries; PPHN, persistent pulmonary hypertension of the newborn; SNP, sodium nitroprusside; Thap, thapsigargin; SERCA, Sarcoplasmic reticulum Ca^{2+}-ATPase

Introduction

Nitric oxide (NO) is physiologically released from the endothelium and interacts with the surrounding vascular smooth muscle cells to produce vasorelaxation, playing an essential role in the regulation of arterial tone (Barnes & Liu, 1995; Moncada *et al*., 1991). Moreover, NO is a key factor during the transition from the foetal to the adult pulmonary circulation. In fact, NO synthase inhibitors produce inadaptation to extrauterine life (Kinsella & Abman, 1995). Inhaled NO causes selective pulmonary vasodilation and is currently employed in the treatment of persistent pulmonary hypertension of the newborn (PPHN) (Kinsella & Abman, 1995; Neonatal Inhaled Nitric Oxide Group, 1997; Roberts *et al*., 1997), but because of its short half-life, long-term use of gaseous NO demands continuous inhalation which may lead to potential toxicity of NO and its metabolites (Pearl, 1993). Recent evidence suggest that inhalation of NO-donor drugs such as sodium nitroprusside (SNP) could offer an alternative to NO (Palhares *et al*., 1998; Schütte *et al*., 1997). SNP effectively relaxes isolated neonatal pulmonary arteries (PA) (Pérez-Vizcaíno *et al*., 1996; 1998a), but its intravenous administration causes both pulmonary and systemic vasodilation with potentially dangerous systemic hypotension. In contrast, inhaled SNP causes sustained pulmonary vasodilation without altering systemic hemodynamics (Schütte *et al*., 1997). In addition, inhaled SNP protects from oleic acid-induced acute lung injury (Young *et al*., 2000) and reduces pulmonary hypertension induced by oxygen free-radical (Yu *et al*., 1999), lung-lavage (Yu & Saugstad, 1999) and infusion of a thromboxane analogue (Adrie *et al*., 1998; Schütte *et al*., 1997). Furthermore, inhalation of SNP increases O_2 arterial pressure levels in infants with PPHN (Palhares *et al*., 1998). These findings suggest that aerosolization of NO donors may offer a new therapeutic approach for the treatment of pulmonary hypertension.

NO donors induce vascular smooth muscle relaxation mainly through the activation of the soluble guanylate cyclase (sGC) and the subsequent increase in cyclic GMP (cGMP) levels (Barnes & Liu, 1995; Cogolludo *et al*., 1999; Lincoln *et al*., 1994), although cyclic GMP-independent mechanisms

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have also been reported (Bolotina *et al*., 1994; Plane *et al*., 1998). The relaxation induced by this cyclic nucleotide may involve a decrease in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) due to the activation of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA, Cohen *et al*., 1999; Khan *et al*., 1998), the plasma membrane Ca^{2+}-ATPase (Barnes & Liu, 1995), the Na^+/K^+-ATPase (Rapoport *et al*., 1985; Tamaoki *et al*., 1997) and different potassium channels (Archer *et al*., 1994; Murphy & Brayden, 1995; Robertson *et al*., 1993; Zhao *et al*., 1997) or to the closure of L-type Ca^{2+} channels (Ruiz-Velasco *et al*., 1998). In addition, Ca^{2+}-independent mechanisms involving a direct reduction of the sensitivity of the contractile apparatus to Ca^{2+} have also been reported to mediate cGMP-induced relaxation (Karaki *et al*., 1997).

Redundant mechanisms of action for the NO/cyclic GMP-induced vasodilation appear to be essential to provide for fine circulatory regulation (Lincoln *et al*., 1994). Furthermore, it is likely that the importance of a given mechanism may markedly differ not only among different vascular beds but also depending on the age of the animal. However, despite the effects of NO and NO donors having been extensively studied in the treatment of PPHN, little is known about the mechanisms mediating their relaxation in neonatal PA. In the present study we have, therefore, analysed the mechanisms involved in SNP-induced relaxation and $[Ca^{2+}]_i$ reduction in PA from newborn piglets, a well characterized animal model for pulmonary hypertension (Haworth & Hislop, 1981; Pérez-Vizcaíno *et al*., 1997; Yu *et al*., 1999; Yu & Saugstad, 1999). These mechanisms were compared with those present in mesenteric arteries (MA).

Methods

Tissue preparation

Two-week-old male piglets (3–4 kg) were used in this study. Piglets were killed in the local abattoir and the lungs and mesenteric 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. The PA (third branch) and MA (both with an internal diameter of about 0.5–1.5 mm) were carefully dissected free of surrounding tissue and cut into rings of 2–3 mm length (Pérez-Vizcaíno *et al*., 1996; 1997; 1998a; 1999). 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 (10^{-6} M) to relax arteries precontracted with noradrenaline (NA, 10^{-6} M).

Contractile tension recording

Endothelium-denuded pulmonary and mesenteric rings were mounted between two hooks under 0.5 and 2 g of tension, respectively, in a 5 ml organ bath filled with Krebs solution at 37°C gassed with a 95% O_2–5% CO_2 mixture as previously described (Pérez-Vizcaíno *et al*., 1997; 1998a). The contractile tension was measured by an isometric force transducer (model PRE 206-4, Cibertec, Madrid, Spain) and recorded by a REGXPC computer program (Cibertec, Madrid, Spain). Arteries were stimulated with NA (10^{-5} M and 10^{-6} M for PA and MA, respectively, which produced approximately 85 and 75% of the maximal response to NA, respectively) and once a stable contraction was reached a concentration-response curve to SNP (10^{-8} M -3×10^{-5} M) was constructed by cumulative addition of the drug. The role of the activation of the sGC in SNP-induced effects was studied by treating some arteries with ODQ (10^{-6} M, an inhibitor of the sGC) for 15 min before the addition of SNP. To further examine the mechanisms involved in the relaxation induced by SNP, arteries were exposed to different treatments before the challenge with NA. The involvement of the activation of the Na^+/K^+-ATPase in the SNP-induced relaxation was tested by incubating some rings with ouabain (10^{-6} M) for 30 min or with a K^+-free solution (without KCl and replacing KH_2PO_4 by NaH_2PO_4) just before the addition of NA. The participation of the activation of K^+ channels and the closure of L-type Ca^{2+} channels in SNP-induced effects was examined by the following protocol. Arteries were firstly stimulated with 80 mM KCl (isotonically replacing NaCl) to inhibit K^+ channel current, afterwards KCl-induced contraction was totally relaxed with nifedipine (10^{-6} M), a L-type Ca^{2+} channel blocker and finally the arteries were stimulated with NA. The possible role of the activation of the SERCA in the relaxation induced by SNP was tested by incubating arteries with Thapsigargin (Thap 2×10^{-6} M), an inhibitor of the SERCA, for 45 min. Finally, some arteries were incubated in a K^+-free solution containing Thap.

Simultaneous $[Ca^{2+}]_i$ and tension recording

Rings were inverted and incubated for 4–6 h at room temperature in Krebs solution containing the fluorescent dye Fura-2 acetoximethylester (5×10^{-6} M) and Cremophor EL (0.05%). Rings were mounted in a bath that was part of a fluorimeter (CAF 110, Jasco, Tokyo, Japan) which allows to estimate changes in the fluorescence intensity of Fura-2 simultaneously with force development (Pérez-Vizcaíno *et al*., 1998b; 1999). The luminal face of the rings was alternatively illuminated (128 Hz) with two excitation wavelengths (340 and 380 nm) from a xenon lamp coupled with two monochromators. The emitted fluorescent light at the two excitation wavelengths (F340 and F380) was measured by a photomultiplier through a 500 nm filter and recorded together with the force data by using data acquisition hardware (Mac Lab, model 8e) and data recording software (Chart v3.2, AD Instruments Pty Ltd., Castle Hill, Australia). The ratio of emitted fluorescence (F340/F380) obtained at the two excitation wavelengths was used as an indicator of $[Ca^{2+}]_i$. After equilibration for 30–45 min, vessels were initially stimulated with 80 mM KCl for 10–15 min which induced a sustained increase in $[Ca^{2+}]_i$ and force. After equilibration, exposure to 80 mM KCl and wash out in normal Krebs solution, the rings were stimulated with NA until a steady-state response in $[Ca^{2+}]_i$ and force was obtained (usually within 15 min) and then, a concentration-response curve to SNP was constructed by cumulative addition of the drug. Before the challenge with NA some arteries were treated with Thap (2×10^{-6} M) or Thap plus ouabain (10^{-6} M) for 15 min (to avoid an excessive Fura-2 loss the incubation time was shorter than in the conventional organ bath experiments). The effects of Thap, Thap plus ouabain and NA were expressed as a percentage, considering

the values at rest in normal Krebs solution (4.75 mM KCl) and after 80 mM KCl-induced stimulation to be 0 and 100%, respectively. SNP-induced effects on both $[Ca^{2+}]_i$ and force were expressed as a percentage of the reduction of responses elicited by NA plus treatments.

The effects of the Na^+/K^+-ATPase-activation on contractile force and $[Ca^{2+}]_i$ were assayed as previously reported (Pérez-Vizcaíno *et al*., 1999). Briefly, arteries were washed twice in a nominally K^+-free solution for 30 min and then stimulated with NA. Thereafter, KCl (0.2–8.8 mM) was added in a cumulative fashion. The effects of KCl on contractile force and $[Ca^{2+}]_i$ were expressed as a percentage of the reduction of the responses induced by NA in rings incubated in a K^+-free solution.

Drugs

The following drugs were used in this study: (−)-noradrenaline bitartrate, ouabain, sodium nitroprusside, thapsigargin (Sigma Chemical Co., Alcobendas, Madrid, Spain), nifedipine (Bayer, Leverkusen, Germany), ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, Tocris Cookson Ltd, Bristol, U.K.) and Fura-2 acetoximethylester (1 mM solution in dimethyl sulphoxide, Calbiochem, La Jolla, CA, U.S.A.). Drugs were dissolved initially in distilled deionized water (except for thapsigargin and ODQ which were dissolved in dimethyl sulphoxide and nifedipine which was dissolved in ethanol) to prepare a 10^{-2} or 10^{-3} M stock solution and further dilutions were made in Krebs solution. The concentrations were expressed as final molar concentration in the tissue chamber.

Statistical analysis

Results are expressed as means ± s.e.mean, with n equal to the number of animals. 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 (pD_2). Statistically significant differences between groups were analysed using ANOVA followed by Newman-Keuls (*post hoc* test). $P<0.05$ was considered statistically significant.

Results

Mechanisms involved in SNP-induced relaxation

Stimulation of endothelium-denuded PA and MA with NA (10^{-5} and 10^{-6} M, respectively) induced a sustained contractile response of 821 ± 62 mg ($n=14$) and 1828 ± 360 mg ($n=9$), respectively. SNP (10^{-8} M – 3×10^{-5} M) fully relaxed both vessels in a concentration-dependent manner (Figure 1 and Table 1). The treatment with the sGC inhibitor ODQ (10^{-6} M) after NA exposure did not significantly modify the tone in PA (4 ± 2% of NA-induced response, $n=6$; $P>0.05$) but further contracted the MA (30 ± 7% of NA-induced response, $n=5$; $P<0.05$). In addition, ODQ markedly inhibited the relaxation induced by SNP in both vessels to a similar extent (Figure 1), indicating that the main mechanism of SNP-induced relaxation involves the activation of the sGC and the subsequent increase in cyclic GMP levels.

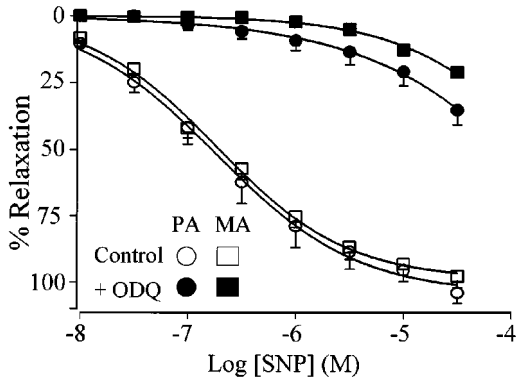

Figure 1 Vasodilation induced by sodium nitroprusside (SNP, 10^{-8} M – 3×10^{-5} M) in endothelium denuded pulmonary (PA) and mesenteric arteries (MA) stimulated by NA (10^{-5} and 10^{-6} M, respectively). The treatment with ODQ (10^{-6} M) markedly inhibited SNP-induced relaxation in PA and MA. Results are expressed as the mean ± s.e.mean ($n=5$–14).

In order to study the transduction pathways by which cyclic GMP produces relaxation several experimental approaches were carried out. The role of the Na^+/K^+-ATPase in SNP-induced relaxation was assessed by pretreating some arteries with ouabain (10^{-6} M) for 30 min or by the incubation in a K^+-free medium just before the challenge to NA. Ouabain induced a contractile response of 322 ± 101 mg ($n=6$) and 352 ± 145 mg ($n=5$) in PA and MA, respectively, even when ouabain plus NA or K^+-free solution plus NA produced a contractile response not significantly different than NA alone in PA (819 ± 167 and 977 ± 111 mg, respectively) or MA (2116 ± 331 and 1143 ± 265 mg, respectively). Furthermore, both treatments inhibited the relaxation induced by SNP in MA (Figure 2B and Table 1), whereas ouabain did not modify and the incubation in a K^+-free medium slightly potentiated SNP-induced relaxation in PA (Figure 2A and Table 1).

In another set of experiments the involvement of K^+ and L-type Ca^{2+} channels on SNP-induced relaxation was studied. The exposure to 80 mM KCl induced a contractile response of 899 ± 82 mg ($n=5$) and 1238 ± 184 mg ($n=5$) in PA and MA, respectively. Afterwards, arteries were treated with the L-type Ca^{2+} channel blocker nifedipine (10^{-6} M) that fully relaxed KCl-induced contraction. Under these conditions, NA induced a contraction that was similar in PA (1071 ± 71 mg, $P>0.05$) but lower in MA (587 ± 62 mg, $P<0.05$) than in untreated arteries. Pretreatment with 80 mM KCl plus nifedipine slightly potentiated the relaxation induced by SNP in PA (Figure 2A and Table 1) and significantly increased the maximal relaxant response in MA (Figure 2B and Table 1). These results suggest that the relaxation induced by SNP does not seem to be mediated through the activation of K^+ channels or the blockade of L-type Ca^{2+} channels.

Finally, we studied the role of the SERCA on SNP-induced relaxation. In these experiments, incubation for 45 min with the SERCA inhibitor Thap induced a contractile response with a time delay of about 20 min which averaged 379 ± 109 mg ($n=11$) and 57 ± 29 mg, ($n=7$) in PA and MA, respectively. The contraction induced by

Table 1 Relaxation induced by SNP (10^{-8} M -3×10^{-5} M) in control or treated pulmonary (PA) and mesenteric arteries (MA) stimulated by NA

		PA			*MA*	
Pretreatment	n	PD_2	E_{max} (%)	n	PD_2	E_{max} (%)
Control	14	6.66±0.06	106±2	9	6.74±0.14	108±3
$0K^+$	8	6.90±0.06*	110±6	9	5.89±0.27*	88±7
Ouabain	6	6.67±0.06	102±4	5	5.82±0.13*	112±4
80 mM KCl+Nif	5	7.06±0.05*	97±3	5	6.74±0.10	127±7*
Thap	11	5.69±0.19**	71±7**	7	5.89±0.19*	101±10
$0K^+$+Thap				6	5.27±0.19**	82±10*

Values are means±s.e.mean; n=number of animals. Arteries were pretreated with a K^+-free solution ($0K^+$), ouabain (10^{-6} M), 80 mM KCl plus nifedipine (Nif, 10^{-6} M), thapsigargin (Thap, 2×10^{-6} M) or K^+-free solution plus thapsigargin ($0K^+$+Thap). Results are expressed as a percentage of the precontracted tone just before the challenge with SNP. * and **$P<0.05$ and 0.01 vs control arteries, respectively.

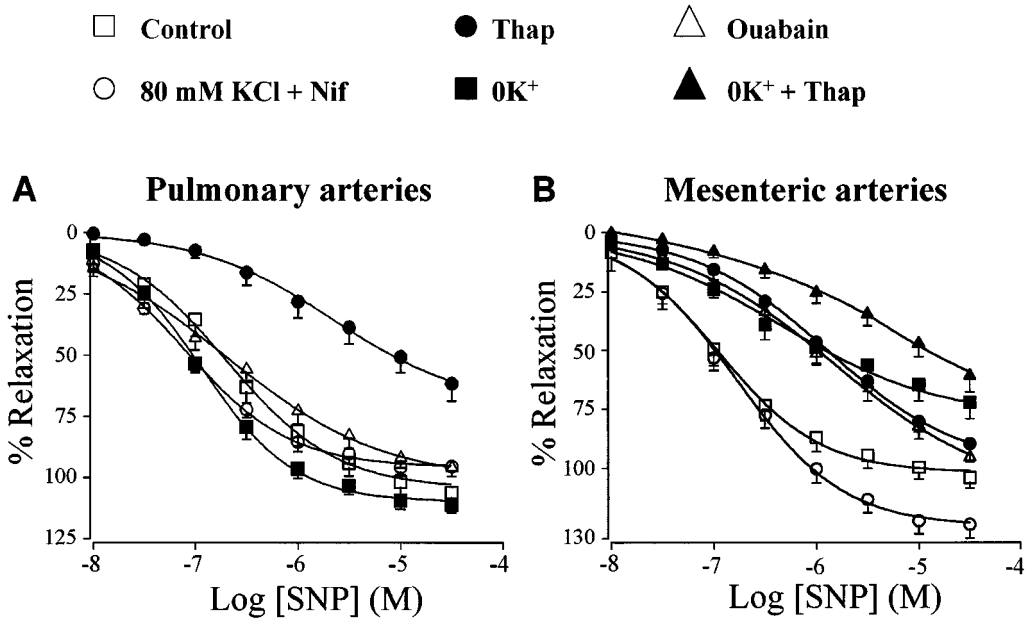

Figure 2 Vasodilation induced by SNP (10^{-8} M -3×10^{-5} M) in endothelium denuded pulmonary (A) and mesenteric (B) arteries treated with 80 mM KCl plus nifedipine (Nif, 10^{-6} M), thapsigargin (Thap, 2×10^{-6} M), K^+-free solution ($0K^+$), ouabain (10^{-6} M) or K^+-free solution plus thapsigargin ($0K^+$+Thap) before the stimulation with NA (10^{-5} and 10^{-6} M, respectively). Results are expressed as mean±s.e.mean (n=5–14).

Thap plus NA was not significantly different than that elicited by NA alone (969±92 and 1498±227 mg for PA and MA, respectively), but Thap markedly shifted to the right the concentration-response curve to SNP in PA and MA and significantly reduced ($P<0.01$) the maximal relaxant response in PA (Figure 2 and Table 1). Since in MA the relaxation induced by SNP was attenuated by SERCA and Na^+/K^+-ATPase inhibitors, we evaluated the effects of the combination of Thap plus K^+-free solution. Under these conditions a further inhibition of the relaxation induced by SNP was observed (Figure 2B and Table 1). These results suggested that the Na^+/K^+-ATPase is involved in SNP-induced relaxation in MA but not in PA, whereas the activation of SERCA seems to play an important role in the vasodilation induced by this NO donor in both PA and MA. Since these signalling mechanisms induce a reduction of $[Ca^{2+}]_i$, in the next group of experiments we analysed the effects of SNP on $[Ca^{2+}]_i$ changes by using the fluorescent dye Fura 2.

Mechanisms involved in SNP-induced $[Ca^{2+}]_i$ reduction

Figure 3 shows that in Fura 2-loaded arteries the stimulation with 80 mM KCl induced a sustained increase in the F340/F380 signal of 10.6±1.0 and 9.5±0.9% above resting values and a contractile response of 465±51 mg (n=14) and 483±57 mg (n=10) in PA and MA, respectively. After washing the preparations with normal Krebs the F340/F380 signal and force returned to basal levels. Under these conditions, NA induced a fast increase in F340/F380 and in contractile force which were followed by a slow decay to a lower steady state level in both vessels (Figure 3). The steady-state increase in F340/F380 and force induced by NA averaged 79±7 and 125±11% of KCl-induced response, respectively (n=9) in PA and 73±5 and 214±39% of KCl-induced response, respectively (n=5) in MA. Therefore, at the concentrations tested the relative force/F340/F380 ratio (per cent increase in force/per cent increase in F340/F380 of the response to 80 mM KCl) was 1.74±0.22 and 2.96±0.43

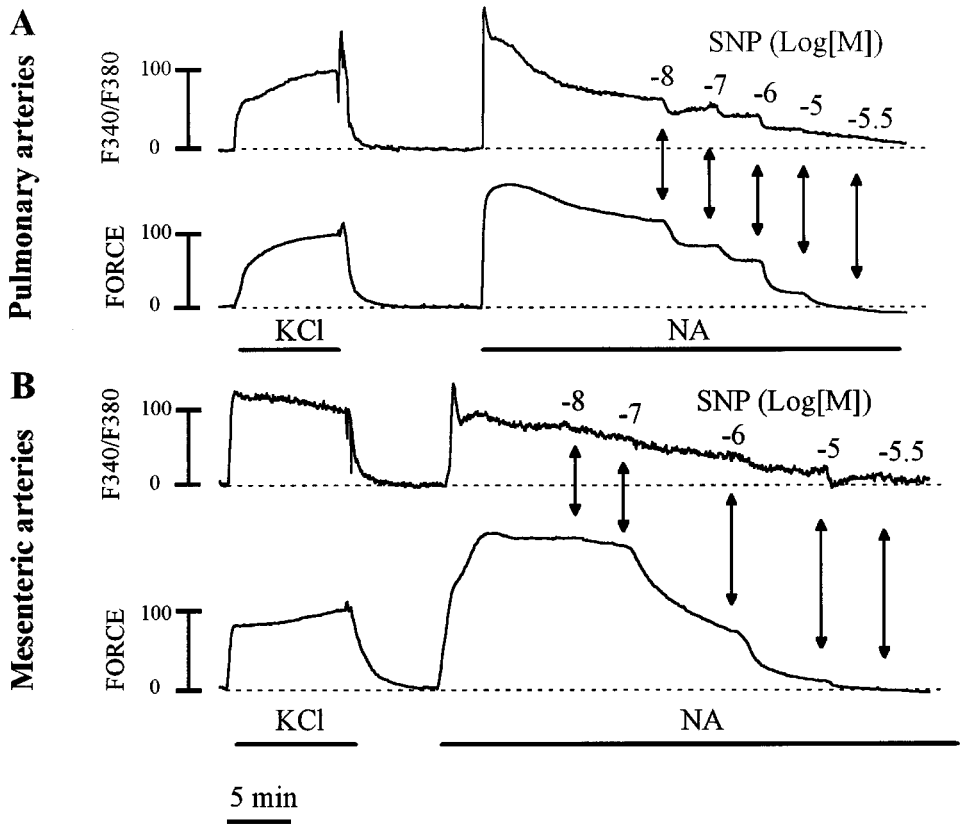

Figure 3 Original recordings of the simultaneous changes produced in F340/F380 (upper trace) and contractile force (lower trace) in pulmonary (A) and mesenteric (B) arteries. Arteries were firstly stimulated with 80 mM KCl and then washed with normal Krebs solution until both signals reached a basal level. Afterwards, rings were stimulated with NA and a concentration-response curve to SNP (10^{-8} M -3×10^{-5} M) was constructed. The vertical calibration bars represent the baseline (0%) and the response to 80 mM KCl (100%), respectively.

in PA and MA, respectively, indicating that NA induced a greater contractile response for the given increase in F340/F380 than that induced by 80 mM KCl in both vessels. After NA-induced responses reached steady-state cumulative addition of SNP produced a concentration-dependent and parallel reduction in F340/F380 and in contractile force (Figure 3), so that the curves showed similar parameters in PA ($pD_2=6.7\pm0.1$ and 6.6 ± 0.1, respectively, Figure 4A) and MA ($pD_2=6.9\pm0.2$ and 7.0 ± 0.2, respectively, Figure 4B). These results suggested that the mechanisms of SNP-induced relaxation basically imply a decrease in $[Ca^{2+}]_i$ in both vessels.

As indicated above, the activation of the SERCA seems to mediate SNP-induced relaxation in PA and MA, whereas the activation of the Na^+/K^+-ATPase appears to play a role only in MA. In order to assess if these were the main transduction mechanisms mediating SNP-induced $[Ca^{2+}]_i$ reduction, in another group of experiments we studied the effects of Thap in PA and of Thap plus ouabain in MA when simultaneously recording the changes in F340/F380 and force. After incubating PA with Thap for 15 min a slowly developing increase in F340/F380 was observed ($60\pm6\%$ of the response to KCl, $n=5$). A similar increase in F340/F380 was achieved in MA following exposure to Thap plus ouabain ($50\pm5\%$ of the response to KCl, $n=5$). However, these changes in $[Ca^{2+}]_i$ were not accompanied by changes in the contractile force, even when more prolonged incubation with Thap induced a contractile response (not shown) as occurred in conventional bath experiments. Under these conditions, NA increased F340/F380 to a final level of 130 ± 24 and $187\pm36\%$ of the response induced by 80 mM KCl, in PA and MA, respectively ($P>0.05$ vs NA in the absence of treatments) and this effect was accompanied by a contractile response which averaged 102 ± 15 and $285\pm34\%$ of the response induced by KCl, in PA and MA, respectively ($P>0.05$ vs NA in the absence of treatments). The time courses of the increases in F340/F380 and in contractile force induced by NA were not much different in the presence or absence of Thap or Thap plus ouabain (not shown). However, as shown in Figure 5A, Thap significantly inhibited the decrease in F340/F380 and in contractile force induced by SNP in PA ($E_{max}=10\pm6\%$, $P<0.01$ and $81\pm2\%$, $P<0.05$, respectively) as compared to untreated arteries. Similarly, Thap plus ouabain (Figure 5B) attenuated SNP-induced reduction of F340/F380 and contractile force in MA ($E_{max}=49\pm6\%$, $P<0.01$ and $72\pm8\%$, $P<0.05$, respectively, vs arteries in the absence of treatment). Furthermore, both pretreatments produced a stronger inhibition on the decrease of F340/F380 than on the relaxation induced by SNP and thus, a dissociation between F340/F380 decrease and relaxation was observed (Figure 5). This dissociation was much more marked in PA treated with Thap than in MA treated with Thap plus ouabain.

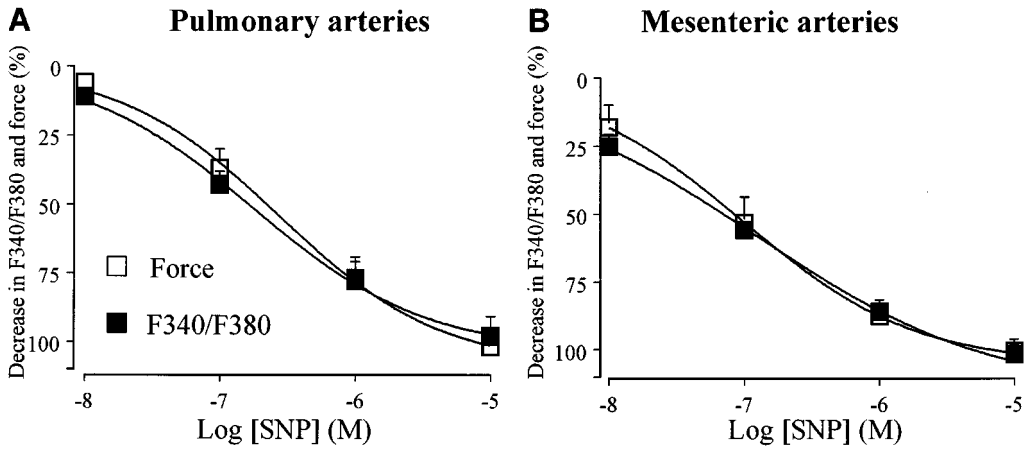

Figure 4 Effects of SNP (10^{-8} M -3×10^{-5} M) on $[Ca^{2+}]_i$ (F340/F380) and contractile force in pulmonary (A) and mesenteric (B) arteries. Results are expressed as the mean $\pm$ s.e.mean ($n=5-9$).

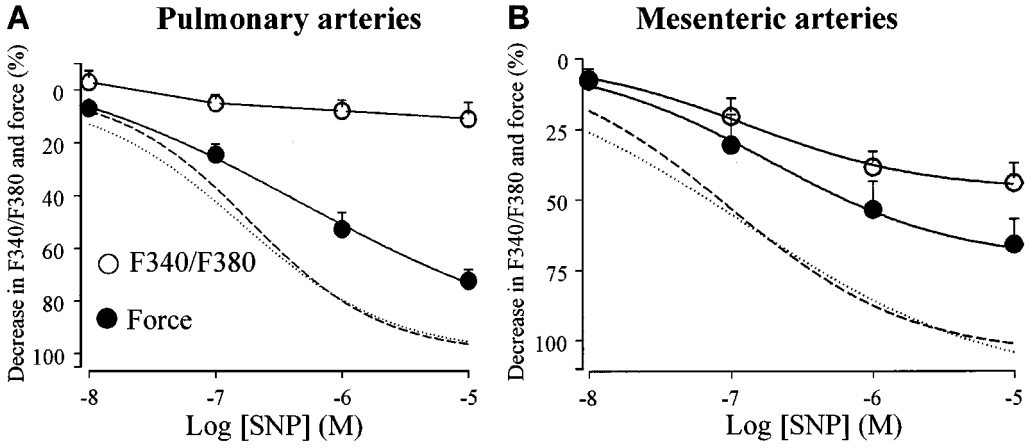

Figure 5 Effects of SNP (10^{-8} M -3×10^{-5} M) on $[Ca^{2+}]_i$ (F340/F380) and contractile force in pulmonary (A) and mesenteric (B) arteries pretreated with thapsigargin (2×10^{-6} M) and thapsigargin (2×10^{-6} M) plus ouabain (10^{-6} M), respectively. Control curves taken from Figure 4 are represented in dotted lines for comparison. Results are expressed as the mean $\pm$ s.e.mean ($n=5$).

Relaxation and $[Ca^{2+}]_i$ reduction induced by Na^+/K^+-ATPase activation

In Fura 2-loaded PA the exposure to K^+-free solution for 30 min produced a small increase in F340/F380 ratio and force (17 ± 6 and 36.6 ± 13% of the 80 mM KCl-induced response, respectively), whereas no changes were observed in MA. NA induced a further increase in F340/F380 ratio (45 ± 4 and 47 ± 13% of KCl-induced response for PA and MA, respectively) and force (126.7 ± 21 and 203 ± 41% of KCl-induced response, for PA and MA, respectively, $n=5$). Under these conditions, cumulative addition of KCl (0.2–8.8 mM) produced a progressive reduction of F340/F380 ratio and force in both PA and MA (Figure 6). The pD_2 and E_{max} of the KCl-induced effects are shown in Table 2. As it can be observed, KCl reduced the increase F340/F380 ratio induced by NA with similar pD_2 and E_{max} values in PA and MA, whereas it relaxed PA with much less efficacy than MA ($P<0.05$). KCl-induced responses were totally abolished in PA and MA pretreated with ouabain (10^{-6} M) but were unaffected by $BaCl_2$ (10^{-5} M, not shown).

Table 2 Effects of KCl (0.2–8.8 mM) on $[Ca^{2+}]_i$ (F340/F380) and contractile force in pulmonary and mesenteric rings incubated in a K^+-free solution and stimulated by NA

		F340/F380		*Force*	
Vessel	n	pD_2	E_{max} (%)	pD_2	E_{max} (%)
Pulmonary rings	5	3.06 ± 0.06	218 ± 53	2.87 ± 0.09	47 ± 11*
Mesenteric rings	5	2.88 ± 0.11	270 ± 39	3.17 ± 0.04	96 ± 2

Values are means $\pm$ s.e.mean; n = number of animals. Results are expressed as a percentage of the reduction of the responses elicited by NA in K^+-free solution. *$P<0.05$ vs mesenteric rings.

Discussion

In this study we have studied the mechanisms involved in the SNP-induced relaxation and $[Ca^{2+}]_i$ reduction in isolated neonatal piglet PA and MA. Our results indicate that SNP-induced effects are mainly mediated by the activation of the sGC and the subsequent increase in cyclic GMP levels in both vessels. This cyclic nucleotide seems to produce vasorelaxation by two main mechanisms: (1) a decrease of

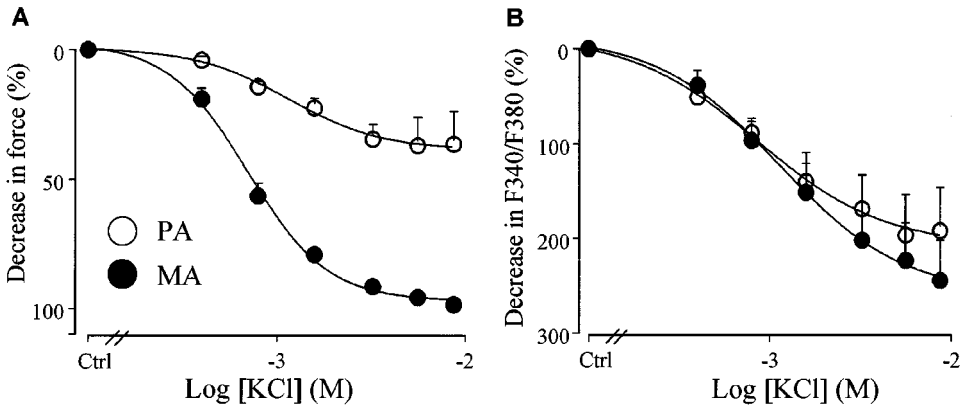

Figure 6 Effects of KCl (0.2–8.8 mM) on contractile force (A) and $[Ca^{2+}]_i$ (F340/F380) (B) in pulmonary (PA) and mesenteric (MA) arteries previously incubated in a K^+-free medium and stimulated with NA. Results are the mean ± s.e.mean ($n=5$).

the $[Ca^{2+}]_i$ mediated through the activation of the SERCA (in PA and MA) or the Na^+/K^+-ATPase (in MA); and (2) a reduction of the sensitivity of the contractile apparatus to Ca^{2+}. The involvement of the Na^+/K^+-ATPase on SNP-induced relaxation in MA, but not in PA, could be attributed to the fact that this mechanism is much less effective to relax PA than MA although reduces $[Ca^{2+}]_i$ with similar efficacy in both vessels.

In our experimental preparations the sGC inhibitor ODQ markedly inhibited the vasodilation induced by SNP. We have previously reported that in these arteries SNP increases cyclic GMP levels by about 3 fold and the cyclic GMP-dependent (type V) phosphodiesterase inhibitor dipyridamol potentiated the relaxation induced by this NO donor (Pérez-Vizcaíno *et al*., 1997). Altogether, these results strongly suggest that the relaxation induced by SNP is mediated mainly through the elevation of cyclic GMP levels. In Fura 2-loaded PA and MA, SNP fully reduced both $[Ca^{2+}]_i$ and force in parallel suggesting that the relaxation was secondary to a mechanism/s that produced a decrease in the $[Ca^{2+}]_i$. The most important mechanisms involved in the cyclic GMP-mediated $[Ca^{2+}]_i$ reduction included the activation of SERCA and Na^+/K^+-ATPase, the opening of K^+ channels, and the inhibition of Ca^{2+} influx through L-type channels (Barnes & Liu, 1995; Karaki *et al*., 1997; Plane *et al*., 1998).

An increase in Na^+/K^+-ATPase activity may induce vasorelaxation by increasing Na^+/Ca^{2+} exchange and reducing the Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Fleming, 1980). Treatment with ouabain or the incubation in a K^+-free medium inhibit the Na^+/K^+-ATPase activity (Pérez-Vizcaíno *et al*., 1999; Rapoport *et al*., 1985; Webb & Bohr, 1978). Under both conditions, the relaxation induced by SNP was markedly inhibited in MA but not in PA, which suggested that the activation of the Na^+/K^+-ATPase plays an important role in the vasodilation induced by SNP in MA but not in PA. In contrast to the present results, Tamaoki *et al*. (1997) have shown a link between cyclic GMP-mediated vasorelaxation and activation of Na^+/K^+-ATPase in canine PA smooth muscle cells. The reason for the discrepancy is unknown, but could be due to the difference in animal species.

The elevation in cyclic GMP levels may activate different types of K^+ channels including Ca^{2+}-activated channels (Archer *et al*., 1994; Bolotina *et al*., 1994; Robertson *et al*., 1993; Zhao *et al*., 1997), delayed rectifier channels (Zhao *et al*., 1997) and ATP-sensitive channels (Murphy & Brayden, 1995). In the present study, we evaluated the relaxation induced by SNP when the extracellular K^+ concentration ($[K^+]_o$) was augmented to 80 mM. Under these conditions, the membrane potential approaches the potassium equilibrium potential (E_K), so that the activation of potassium channels is relatively ineffective to induce vasodilation (Quayle *et al*., 1997). Afterwards, KCl-induced contractions were totally relaxed with nifedipine, a L-type Ca^{2+} channel blocker, before vessels were finally exposed to NA. NA-induced contraction was not modified in PA but it was moderately attenuated in MA, probably because the contraction induced by NA is more sensitive to nifedipine in MA than in PA (Pérez-Vizcaíno *et al*., 1996). The pretreatment with KCl plus nifedipine did not inhibit the relaxant response to SNP. In fact, this treatment slightly potentiated the relaxation induced by SNP in PA and augmented the maximal relaxant response in MA, suggesting that neither the opening of K^+ channels nor the inhibition of Ca^{2+} influx through L-type channels were involved in the vasorelaxant effect of SNP. However, we cannot rule out the possibility that SNP may inhibit L-type Ca^{2+} channels in MA but if so, this mechanism does not seem to play an important role. The greater relaxant response induced by SNP in MA could be attributed to the lower preexisting contractile tone as well as to the fact that the elevation of $[K^+]_o$ could increase the Na^+/K^+-ATPase activity (Vasilets & Schwarz, 1993), which, as discussed above, is one of the main mechanisms mediating the relaxation induced by SNP in MA.

Thap, a SERCA inhibitor, produced a contractile response with a time delay of about 15–20 min, although an increase in $[Ca^{2+}]_i$ was evident within a few minutes. This dissociation between $[Ca^{2+}]_i$ and contractile force following the inhibition of the SERCA has been attributed to a local increase of Ca^{2+} in a compartment unrelated to the contractile apparatus (Karaki *et al*., 1997). Thap markedly reduced the relaxation induced by SNP in PA and MA, indicating that the activation of the SERCA plays an important role in this effect. Our results in piglet PA are in good agreement with previous reports obtained in rabbit and mouse aorta (Cohen *et al*., 1999) and in dog coronary arteries (Khan *et al*., 1998). In MA, the treatment with K^+-free solution plus Thap induced a further inhibitory effect over that observed in the

presence of either treatment alone, suggesting that both SERCA and Na^+/K^+-ATPase independently participate in SNP-induced relaxation.

The results obtained in Fura 2-loaded arteries confirmed our findings in conventional organ baths. Thap markedly inhibited the relaxation and the reduction of the $[Ca^{2+}]_i$ induced by SNP, an observation that has been previously reported for NO and other NO donors (Cohen *et al*., 1999; Karaki *et al*., 1997). However, an important dissociation between both effects (i.e. decrease in $[Ca^{2+}]_i$ and contractile force) was observed in PA, so that although Thap almost abolished the reduction in $[Ca^{2+}]_i$, there was still an important relaxant effect after the addition of SNP. These results strongly suggest that some Ca^{2+}-independent mechanisms can mediate, at least partly, the relaxant effect of SNP in PA. In fact, it has been reported that SNP reduced the Ca^{2+} sensitivity of the contractile proteins (Karaki *et al*., 1997). SNP-induced desensitization of the contractile proteins to Ca^{2+} may involve the phosphorylation of different proteins such as myosin light chain kinase (inhibiting its activity) or telokin (which activates myosin light chain phosphatase); both events lead to a decrease of the phosphorylation of the myosin light chains (Woodrum *et al*., 1999). However, myosin light chain phosphorylation-independent mechanisms may also participate in cyclic GMP-induced relaxation (Woodrum *et al*., 1999). A dissociation between the effects of SNP on contractile force and $[Ca^{2+}]_i$ was also observed in MA pretreated with Thap plus ouabain, but it was less marked than in PA pretreated with Thap alone, suggesting that Ca^{2+}-independent mechanisms could play a less important role in MA. Moreover, in contrast to PA, where the activation of the SERCA seems to mediate most, if not all, SNP-induced $[Ca^{2+}]_i$ reduction, in MA the combination of ouabain plus Thap produced only a partial inhibition of SNP-induced $[Ca^{2+}]_i$ reduction. Thus, we cannot rule out that other mechanisms different from the activation of the SERCA and the Na^+/K^+-ATPase (i.e. plasma membrane Ca^{2+}-ATPase) may contribute to SNP-induced reduction of $[Ca^{2+}]_i$ in MA.

Our results suggested that the activation of the SERCA and the decrease of Ca^{2+}-sensitivity of the contractile proteins represent the two main mechanisms of SNP-induced relaxation in PA, whereas in MA, the activation of the Na^+/K^+-ATPase also plays a significant role. The lack of involvement of the Na^+/K^+-ATPase activation on SNP-induced relaxation in PA could imply that this pump is not activated by the NO/cyclic GMP pathway or if activated, it has a minor relaxant efficacy. To further analyse these possibilities, we compared the effects of the Na^+/K^+-ATPase activation on $[Ca^{2+}]_i$ and contractile force in PA and MA following the addition of KCl to the extracellular medium in PA and MA incubated in a K^+-free medium as previously reported (Pérez-Vizcaíno *et al*., 1999). The vasodilation induced by KCl under these conditions was totally abolished by ouabain indicating that this effect was due to the activation of the Na^+/K^+-ATPase. The activation of the pump reduced the $[Ca^{2+}]_i$ to a similar extent in PA and MA, even when the relaxation was much less marked in PA than in MA. These results suggest that the $[Ca^{2+}]_i$ reduction may play a less important role to relax PA than MA. In fact, it has been previously observed that vasodilatory drugs that basically act by decreasing $[Ca^{2+}]_i$, such as L-type Ca^{2+} channel blockers or K^+ channel openers, relax PA less effectively than systemic arteries (Kwan *et al*., 1999; Magnon *et al*., 1994; Pérez-Vizcaíno *et al*., 1998a). Thus, the reduced relaxant response induced by Na^+/K^+-ATPase activation in PA suggests that this pump plays a minor role in SNP-induced relaxation in these arteries, whereas it represents an important mechanism in MA.

In conclusion, SNP relaxes PA and MA through different mechanisms. The activation of SERCA participate in SNP-induced reduction of $[Ca^{2+}]_i$ and contractile force in both arteries, while the activation of Na^+/K^+-ATPase only plays a role in MA. In addition to the reduction in $[Ca^{2+}]_i$, Ca^{2+}-independent mechanisms also seem to contribute to SNP-induced effects. The lack involvement of the Na^+/K^+-ATPase activation in SNP-induced effects in PA could be attributed to the smaller efficacy of this mechanism to relax PA than MA.

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