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Effect of endopeptidase-24.11 inhibitors and C-ANP receptor ligand on responses evoked in arterioles of rat cremaster muscle by atrial natriuretic peptide

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1 The present study examined the effect of exogenous atrial natriuretic peptide (ANP), alone or in presence of inhibitors of the two major mechanisms for clearing ANP, metabolism by neutral endopeptidase-24.11 (NEP) and internalization by C-ANP receptors, on arteriolar responses using intravital microscopy on the rat cremaster muscle after intravenous or topical administration of the peptide.

2 Topical application of ANP (3×10^{-10} to 3×10^{-8} M) produced a gradual increase in arteriolar diameter. NEP inhibitors, thiorphan (30 mg kg^{-1}, i.v.), kelatorphan (10 mg kg^{-1}, i.v.) and retrothiorphan (25 mg kg^{-1}, i.v.) alone, did not significantly affect vascular tone but caused significant potentiation of the arteriolar responses to topically applied ANP.

3 When given as an i.v. bolus, ANP dilates skeletal arterioles at a high dose (20 $\mu g\ kg^{-1}$). At a lower dose (10 $\mu g\ kg^{-1}$), ANP alone or with retrothiorphan or the C-ANP receptor ligand C-ANP (4-23) did not produce any arteriolar responses, while after the combined administration of the two inhibitors, an increase in arteriolar diameter was induced.

4 These results indicate that low doses of topically applied ANP dilate rat cremaster arterioles and that the vasodilator responses can be potentiated by NEP inhibition. When given as an i.v. bolus, a high dose of ANP can also dilate skeletal arterioles. However at a lower dose the rapid metabolism of the peptide prevents it from producing its action.

Keywords: Atrial natriuretic peptide; endopeptidase-24-11 inhibitors; thiorphan; kelatorphan; retrothiorphan; C-ANP(4-23); rat cremaster arterioles

Introduction

Atrial natriuretic peptide (ANP) is a vasodilator and diuretic peptide with a potential significant role in the control of fluid volume and electrolyte homeostasis (Needleman *et al.*, 1989; Brenner *et al.*, 1990).

Neutral endopeptidase (NEP; EC 3-4-24-11) has been identified as the major enzyme responsible for the metabolism of ANP (Kenny & Stephenson, 1988). NEP is a metallopeptidase which cleaves ANP at position Cys^{105}-Phe^{106} in the peptide ring to yield an inactive metabolite (Kenny & Stephenson, 1988; Olins *et al.*, 1989). The enzyme is mostly concentrated in the brush border of proximal tubular cells, and small concentrations are also found in several other structures such as renal glomeruli, brain, lungs, intestines, skeletal and cardic muscle, neutrophils and fibroblasts (Gee *et al.*, 1985; Matsas *et al.*, 1986; Waksman *et al.*, 1986; Ronco *et al.*, 1988). NEP has also been shown to be located in endothelial cells, at least in large vessels (Tamburini *et al.*, 1989; Llorens-Cortes *et al.*, 1992; Soleilhac *et al.*, 1992) and in vascular smooth muscle cells of rabbit renal cortex (Dussaule *et al.*, 1993).

Circulating levels of ANP are also thought to be regulated by receptors which are not linked to guanylate cyclase, the C-ANP receptors. C-ANP receptors are widely distributed at high densities in several tissues and cells including kidney cortex (Martin *et al.*, 1989), endothelial (Leitman & Murad, 1986; Leitman *et al.*, 1986) and smooth muscle cells (Scarborough *et al.*, 1988; Nussenzweig *et al.*, 1990). In these tissues and cells, C-ANP receptors seem to account for the majority of ANP binding sites (reviewed in Anand-Srivastava & Trachte, 1993). The C-ANP receptors are thought to have a clearance function and to act in concert with NEP (Chiu *et al.*, 1991; Okolicany *et al.*, 1992). At low plasma levels, ANP is probably internalized through an interaction with the C-ANP receptors and degraded in the lysosomes, whereas at higher ANP concentrations, NEP inactivation also intervenes. Moreover, when only one of these pathways is eliminated, the other compensates (Koepke *et al.*, 1989).

Injection of ANP into dogs and rats results in hypotension, increases in cardiac output and decreases in calculated total peripheral resistance (Criscione *et al.*, 1987; Gardiner *et al.*, 1988; Shen *et al.*, 1991; Lappe *et al.*, 1986). These effects appear to be due to the vasodilator actions of ANP on peripheral resistance elements (Seymour *et al.*, 1985; Criscione *et al.*, 1987; Shen *et al.*, 1991). On the other hand, infusion of ANP almost always results in hypotension, reduced cardiac output and an increase in total peripheral resistance (Lappe *et al.*, 1986; Criscione *et al.*, 1987; Zimmerman *et al.*, 1987; Shen *et al.*, 1991). The reasons for the different vascular responses to bolus injection and infusion of ANP have not been fully elucidated. However, in most of the studies, the infusion rates of ANP resulted in plasma ANP levels lower than those found after i.v. bolus administration (Windquist & Hintze, 1990). Intravital microscopy has shown that intravenous infusion of ANP does not increase the diameter of rat cremaster arterioles (Barbee *et al.*, 1992), but a direct effect of ANP on skeletal microcirculation has not been studied after i.v. bolus administration of ANP.

ANP produces dilatation by acting directly on the vascular smooth muscle and when given intravenously, it may bind to receptors on the endothelium before it reaches the muscle. In these conditions, metabolism by NEP and internalization by C-ANP receptors may moderate or even prevent the vascular effect of low doses of ANP. On the other hand, degradation of

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ANP must be reduced after topical application on the adventitial surface of the vessel. Several studies have examined the vasodilator effect of topically applied ANP and the results are conflicting. Sarelius & Huxley (1990) have shown that ANP can produce vasodilatation of terminal arterioles in the hamster cheek pouch. However, other investigators have found in the rat that terminal arterioles do not respond (Proctor & Bealer, 1987) or only minimally to topically applied ANP (Faber *et al.*, 1988).

Accordingly, in this study, we examined the effect of ANP on the diameter of skeletal vessels. The study was performed in the rat cremaster muscle by intravital microscopy. ANP was given at high doses as an i.v. bolus or at lower doses by topical application. We also assessed, in the same conditions, the influence of NEP inhibitors on the arteriolar response to ANP.

The NEP inhibitors used were thiorphan, retrothiorphan and kelatorphan. Thiorphan is a potent NEP inhibitor ($K_i = 4$ nM) but it is not completely specific because it also inhibits angiotensin-converting enzyme (ACE; $K_i = 140$ nM) (Roques *et al.*, 1980). In contrast, its retro-inverso-isomer, retrothiorphan is almost as potent as thiorphan for NEP ($K_i = 6$ nM) with a very low affinity for ACE ($IC_{50} > 10$ mM) (Roques *et al.*, 1983). Finally kelatorphan is a potent inhibitor of both endopeptidase 24-11 ($K_i = 1.8$ nM) and aminopeptidase ($K_i = 380$ nM), and is unable to interact with ACE ($IC_{50} > 10$ mM) (Bouboutou *et al.*, 1984). To test the role of C-ANP receptors in the failure of systemically injected ANP to dilate arterioles after NEP inhibition, we also investigated the effect of C-ANP(4-23), a truncated ANP analogue capable of selectively inhibiting the binding of ANP to the C-ANP receptor, alone or combined with a NEP inhibitor, on the response of rat cremaster arterioles to ANP.

Methods

Surgical procedure

Studies were performed on male Sprague-Dawley rats (120–180 g). Animals were anaesthetized with a mixture of urethane (425 mg kg^{-1}) and chloralose (100 mg kg^{-1}) administered i.p. Anaesthesia, thereafter was maintained by giving a supplement of the mixture (10–20% of the initial dose) when necessary. Animals breathed room air spontaneously via a tracheal cannula. Arterial blood pressure was monitored with a Gould P50 transducer through a cannula in the left common carotid artery. The right jugular vein was cannulated to allow i.v. injection. Body temperature was maintained at 36–37°C by a heating pad placed under the rat.

For observation of the skeletal muscle microvasculature, the left cremaster muscle was prepared using a modification of the method of Baez (1973) as described by Hill *et al.* (1990). Briefly, a midline incision was made in the scrotal skin to expose the testis with surrounding cremaster muscle. Connective tissue overlying the muscle was gently dissected away before opening the cremaster sac with a micro cautery. The ligament joining the cremaster muscle to the testis was disrupted, followed by ligature of the spermatic cord and removal of the testis.

The muscle was then secured in place over an optical port in a 85 ml tissue bath. A modified Krebs bicarbonated solution (composition (in mM) NaCl 113, $NaHCO_3$ 25.5, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ $7H_2O$ 1.2, KH_2PO_4 1.2 and dextrose 12, dissolved in distilled water) was continuously superfused over the cremaster muscle at 10 ml min^{-1}. A gas mixture of N_2 and CO_2 (95%–5%) was bubbled through both the tissue bath and the Krebs stock reservoir to provide mixing and to maintain tissue bath solution PO_2 (30–40 mmHg), PCO_2 (40–50 mmHg) and pH (7.35–7.45), measured with a Radiometer analyser (ABL 30).

The preparation was placed on the stage of a trinocular microscope (Jenalumar) equipped with a ×10 water immersion objective. Images of the circulation were recorded on a videotape using a closed circuit television system consisting of a Sony AXC120P color video camera, a Sony VO5630 Umatic videocassette recorder and a Sony trinitron color video monitor. A time reference accurate to 0.01 s was recorded on each video field. Vessel lumen diameters were measured from videotape recordings with a caliper on the video monitor or with a picture analysing system (Cyclope on IBM PC computer) at an overall magnification of ×1300.

The preparation was allowed to equilibrate for one hour after the completion of the surgery. During this time, it was examined and judged to be acceptable if, (i) mean arterial blood pressure and heart rate were stable, (ii) mean arterial blood pressure was >80 mmHg, (iii) no leucocyte adhesion or bleeding occured, (iv) vasodilator responses to 10^{-4} M adenosine and vasoconstrictor responses to 10^{-6} M noradrenaline were present. If any of these criteria were not met, the experiment was not carried out.

Protocols

Studies with topically applied ANP Two protocols were used. In the first one, the vessels were allowed to return to control diameter after each affusion of ANP in order to determine the time of peak activity. Two series of experiments were carried out in these conditions: concentration-response curves for ANP (10^{-9} M to 10^{-7} M in log concentration increments; $n = 5$) and studies with thiorphan and kelatorphan. NEP inhibitors were given by bolus injection (thiorphan, 30 mg kg^{-1} and kelatorphan, 10 mg kg^{-1} according to Ollins *et al.*, 1989). Vessels were exposed to ANP (10^{-9} M) before and 5 min after NEP inhibitor administration ($n = 5$ for each inhibitor).

In the second protocol, concentration-response curves were constructed by stepwise cumulative addition of the peptide to the cremaster bath (3×10^{-10} M to 3×10^{-8} M in log concentration increments). The tissue was exposed to each concentration for 30 s and vessel diameter was measured at the end of the period. Three experiments were performed with this protocol: (1) repetition of concentration-response curves for ANP to assess reproducibility of the response ($n = 5$). (2) Comparison of responses of large arterioles (54–62 μm; second order arterioles; $n = 5$) and small arterioles (16–24 μm; third order arterioles; $n = 5$) to ANP. (3) Effect of retrothiorphan: concentration-response curves for ANP were calculated before and 5 min after intravenous injection of retrothiorphan (25 mg kg^{-1} according to Pham *et al.*, 1992; $n = 5$).

At the end of each experiment, 3×10^{-5} M sodium nitroprusside or 10^{-4} M adenosine was added to the cremaster bath to produce maximal smooth muscle relaxation for determination of maximal diameter.

Drugs were topically applied in a 0.5 ml volume with a calibrated automatic pipette. During infusion of the drug, the flow of Krebs solution was not stopped so the exact concentration at the arteriole could not be calculated. As the error in concentration due to dilution did not exceed 6–8%, it was not taken into account. Therefore, results are expressed as the concentration determined for the bath volume of 85 ml.

Studies with intravenously injected ANP

For systemic administration, ANP was given as a bolus by rapid intrajugular injection (1 ml kg^{-1}). Vessel diameters were measured 60 and 30 s before (control measurements) and 60 s after ANP administration. At the end of each experiment, 3×10^{-5} M sodium nitroprusside or 10^{-4} M adenosine was added to the cremaster bath to assess the vasodilator responsiveness of the microvasculature.

ANP alone or in the presence of retrothiorphan Two groups of rats were used. In the first group, ANP (10 μg kg^{-1}; $n = 6$) was injected before and 5 min after retrothiorphan (25 mg kg^{-1}). In the second group, ANP (20 μg kg^{-1}; $n = 5$) was injected alone.

ANP in the presence of both retrothiorphan and C-ANP (4–23) Animals were randomly divided into two groups of 5 rats. C-ANP (4–23) (10 $\mu g\ kg^{-1}$) or vehicle (isotonic saline; 100 $\mu l\ 100\ g^{-1}$) was administered as a priming bolus followed by a sustaining infusion for 60 min (1 $\mu g\ kg^{-1}\ min^{-1}$ for C-ANP(4–23) and 50 $\mu l\ min^{-1}$ for isotonic saline). The dose of C-ANP(4–23) was selected from previous work on the *in vivo* efficacy of the peptide (Maack *et al.*, 1987). Retrothiorphan (25 mg kg^{-1}) or isotonic saline (100 $\mu l\ 100\ g^{-1}$) were given by an i.v. bolus injection 5 min before the end of C-ANP(4–23) infusion. All the animals received ANP (10 $\mu g\ kg^{-1}$) 15 min before the start of infusion and then 30 and 60 min after.

In all the experiments, only one vessel was studied in each animal. Diameter measurements were made on third order arterioles defined according to the classification of Hutchins *et al.* (1974) except in one experiment as previously indicated. Values obtained for diameter represent the average of 5 measurements taken at 5 locations on each vessel at the same time. The part of the vessel whose inside diameter was clearly visible on the video screen was traced onto transparent paper and the length of the vessel was divided into 4 approximately equal parts so that measurements were taken at the same locations during an experiment. Distance between 2 locations fluctuated from about 10 to 30 μm according to the length of the vessel selected.

In some experiments, drug responses were normalized by expressing data as a percentage of the maximal vasodilatation: responses = $[(Dx\text{-}Dc)/(Dmax\text{-}Dc)] \times 100$, where Dc is the control diameter, Dx is the diameter produced by concentration x of drug and Dmax is the diameter obtained during maximal vasodilatation produced by topical application of 10^{-4} M adenosine or 3×10^{-5} M sodium nitroprusside at the end of the experiment.

Data analysis

Data are presented as mean ± s.e.mean. Analysis of variance (ANOVA) for repeated measures followed by Dunett's *t* test was used to determine significant differences with respect to time within any treatment group. Responses of arterioles to ANP alone or in the presence of C-ANP(4–23) or C-ANP(4–23) + retrothiorphan were analysed by analysis of covariance (ANCOVA). Comparisons of blood pressure responses to ANP in the presence or absence of an NEP inhibitor were made by Wilcoxon's rank sum test applied to areas over curves (AOC) measured over the time period of observation following each injection. Statistical analysis of arteriolar responses to ANP alone (several doses given at different times) or in the presence or absence of an NEP inhibitor (several doses of ANP or one dose of ANP given at different times) were performed by a three way analysis of variance (treatments × doses × rats). Mean differences were assessed for statistical significance by using contrasts.

In other studies, data were analysed with paired or unpaired Student's *t* test as appropriate. Statistical significance was assumed when the *P* value was <0.05.

Drugs and solutions

Sodium nitroprusside, adenosine and rat des-(Gln[18], Ser[19], Gly[20], Leu[21], Gly[22])-fragment 4–23 amide C-ANP (4–23) were purchased from Sigma chemical Co. (St Quentin-Fallavier, France). Thiorphan, kelatorphan, retrothiorphan and rANP (1–28) were synthesized in our laboratory (Laboratoire de Pharmacochimie Moléculaire, INSERM U266, CNRS D1500, Paris).

ANP was dissolved in distilled water and the solution was divided into aliquots and frozen (−30°C) until required. On the day of the experiment, ANF stock aliquots were thawed and diluted in Krebs solution. Stock solutions of sodium nitroprusside and adenosine were prepared with saline and diluted with Krebs solution. C-ANP(4–23), kelatorphan and retrothiorphan were dissolved in saline; thiorphan in sodium hydroxide (4×10^{-2} M) and diluted in saline.

Results

Effects of neutral endopeptidase inhibitors

Bolus injection of NEP inhibitors alone did not modify arteriolar diameters as shown in Table 1. However thiorphan (30 mg kg^{-1}) and retrothiorphan (25 mg kg^{-1}) caused a modest fall in mean arterial blood pressure which started 30 s after administration and returned to control levels 5 min later. In contrast kelatorphan (10 mg kg^{-1}) induced a significant rise in mean arterial blood pressure (Table 1).

Effects of topically applied ANP on arteriolar tone

ANP alone Topical application of ANP (10^{-9} to 10^{-7} M) produced a transient increase in vessel diameters which was maximum 25–30 s after peptide administration and then gradually returned to the baseline during the recovery period (Table 2). Maximal responses were reached for concentrations of ANP $> 10^{-8}$ M.

Table 1 Effects of thiorphan, retrothiorphan and kelatorphan on arteriolar tone and mean arterial blood pressure (MABP)

Treatments	n	*Time after inhibitor* (s)	*Diameter* (μm)	*MABP* (mmHg)
Control	8		35.5 ± 1.7	113 ± 5.4
Thiorphan		30	36 ± 1.6	97 ± 5.4**
(30 mg kg^{-1}, i.v.)		60	35.5 ± 1.7	89 ± 5.3**
		300	36 ± 1.8	116 ± 7
Control	5		33 ± 1.8	93 ± 3.4
Kelatorphan		30	32.5 ± 1.2	88 ± 3.4
(10 mg kg^{-1}, i.v.)		60	33 ± 1.5	86 ± 8
		300	31 ± 2	137 ± 10**
Control	6		42 ± 4.6	111 ± 4.6
Retrothiorphan		60	43 ± 4	101 ± 3*
(25 mg kg^{-1}, i.v.)		300	43 ± 4.7	116 ± 1.5

Values are mean ± s.e.mean; *n* = number of rats. *$P<0.05$, **$P<0.01$ when compared to the appropriate control (ANOVA for repeated measures followed by Dunnett's *t* test).

Table 2 Effects of increasing doses of topically applied ANP on arteriolar diameters and mean arterial blood pressure (MABP)

		ANP									
		10^{-9} M			10^{-8} M			10^{-7} M			*Adenosine* 10^{-4} M
		Time after ANP			*Time after ANP*			*Time after ANP*			*30s after*
	Control	*30s*	*60s*	*Control*	*30s*	*60s*	*Control*	*30s*	*60s*	*Control*	*adenosine*
Diameter (μm)	35± 1.3	42± 1.6***	38± 2.3***	36± 1.4	45.5± 2***	40.5± 2.6***	36± 1.4	46.5± 1.7***	41± 3***	35.5± 2.3	54.5± 1.7***
MABP (mmHg)	110± 4.5		112± 4.6	110± 4.3		109± 7	115± 4		115± 5.7	107± 6.2	106± 5

Values are mean ± s.e.mean of 5 rats; ***$P<0.001$ when compared to their respective control (three way ANOVA using contrasts for statistical significance).

The effects of ANP were reproducible since no difference was found in three successive concentration-response curves performed in the same vessel at 20 min intervals (Figure 1).

Topically applied ANP never produced maximal vasodilatation equivalent to that produced by application of 10^{-4} M adenosine and its effect seemed to depend on the size of the vessel. Thus, in the largest arterioles studied (54–62 μm; $n=5$), ANP at the highest concentration tested (3×10^{-8} M) increased vessel diameters by 67±9% of the maximal vasodilatation achieved by 10^{-4} M adenosine. In the smallest arterioles (16–24 μm; $n=5$), the increase only amounted to 37±9% of maximal vasodilatation ($P<0.02$ between large and small arterioles).

ANP in presence of neutral endopeptidase inhibitors Arteriolar responses to topical application of ANP were potentiated 5 min after injection of thiorphan or kelatorphan (Figure 2). Maximum responses of vessels to 10^{-9} M ANP without and with NEP inhibitors represent respectively 33 and 45% of maximal dilatation produced by topical application of 10^{-4} M adenosine in animals treated with kelatorphan and 33 and 54% in animals treated with thiorphan. Moreover the duration of the response was increased since a significant vasodilatation was still observed 5 min after administration of the peptide in the presence of kelatorphan (Figure 2). The vasodilator responses of arterioles to ANP were also enhanced by previous administration of retrothiorphan, a more specific NEP inhibitor (Figure 3).

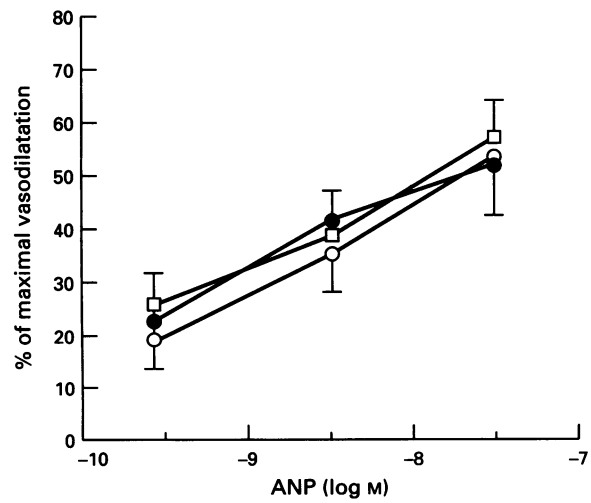

Figure 1 Concentration-response curves of rat cremaster arterioles for ANP. Curves were performed at 20 min intervals on the same arteriole. Mean control diameters were 35±2.6μm at t0 (●). 34.5±2.5μm at t20 (□) and 34.3±2.7μm at t40 (○) respectively. Values are mean±s.e.mean of 5 rats and are expressed as % of maximal vasodilatation produced by 10^{-4}M adenosine (mean maximal response: 50±6.2μm).

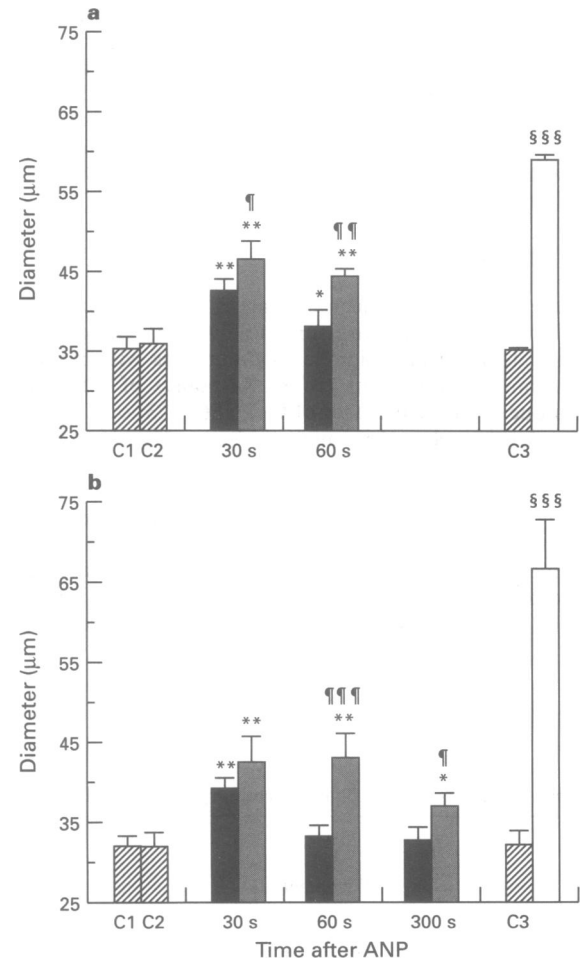

Figure 2 Vasodilator responses of rat cremaster arterioles to ANP (10^{-9}M) topically applied before (■) and 5 min after administration of a neutral endopeptidase (NEP) inhibitor (▨) in (a) thiorphan (30 mg kg^{-1}, i.v. $n=5$) and (b) kelatorphan (10 mg kg^{-1}, i.v. $n=5$). Controls (▨): C1, mean control diameter before inhibitor; C2, after inhibitor; C3 at the end of the experiment before adenosine (□, 10^{-4}M). Values are mean±s.e.mean of 5 rats. *$P<0.05$, **$P<0.01$ when compared to the respective control (ANOVA for repeated measures followed by Dunnett's t test). ¶$P<0.05$, ¶¶$P<0.01$ when compared to ANP before inhibitor for each time (three way ANOVA). §§§$P<0.001$ when compared to its own control (paired t test).

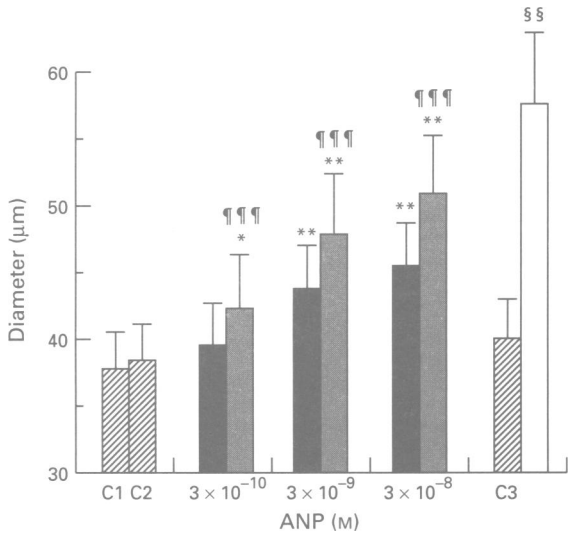

Figure 3 Effect of increasing concentrations of ANP topically applied before (■) and 5 min after retrothiorphan (25 mg kg^{-1}, i.v., ▨) on rat cremaster arterioles. Controls (▨): C1, mean control diameter before retrothiorphan; C2, after retrothiorphan; C3 at the end of the experiment before sodium nitroprusside (□, 3×10^{-5} M). Values are mean ± s.e.mean of 5 rats. $^{*}P<0.05$, $^{**}P<0.01$ when compared to their respective control (ANOVA for repeated measures followed by Dunnett's t test). $^{¶¶¶}P<0.001$ when compared to ANP before inhibitor for each dose (three way ANOVA). $^{§§}P<0.01$, when compared to its own control (paired t test).

Effects of intravenously injected ANP

ANP alone As expected, ANP bolus injection (10 μg and 20 μg kg^{-1}) lowered mean arterial blood pressure (Table 3). However, the magnitude of the maximal depressor response was not significantly different between the two doses of ANP (22 ± 2 mmHg versus 29.5 ± 4.9 mmHg for 10 μg kg^{-1} and 20 μg kg^{-1}, respectively).

An i.v. bolus injection of 10 μg kg^{-1} ANP did not affect the arteriolar diameter. However increasing the dose of ANP to 20 μg kg^{-1} caused a significant dilatation of the arterioles which reached 40% of maximal dilatation produced by topical application of 3×10^{-5} M sodium nitroprusside (Table 4).

ANP in the presence of retrothiorphan When ANP (10 μg kg^{-1}) was administered as an i.v. bolus 5 min after retrothiorphan, no changes were observed in arteriolar diameter. Yet sodium nitroprusside added to the cremaster bath at the end of the experiment caused a significant vasodilatation (Table 4). No significant change was observed in the hypotensive effect of 10 μg kg^{-1} ANP after retrothiorphan administration (Table 3) (AOC = 71 ± 6.5 mmHg min versus 64 ± 12 mmHg min before and after retrothiorphan respectively).

Table 4 Effects of intravenous ANP administration on arteriolar diameter with and without retrothiorphan (25 mg kg^{-1}, i.v.)

	n	*Vessel diameter* (μm) *Control*	*60 s after drug administration*
ANP (10 μg kg^{-1})	6		
Before retrothiorphan		26 ± 3.5	27 ± 3
After retrothiorphan		25 ± 3.5	26 ± 3
Nitroprusside (3×10^{-5} M)[a]		25 ± 3.5	39 ± 3**
ANP (20 μg kg^{-1})	5	36 ± 5	44 ± 4.5**
Nitroprusside (3×10^{-5} M)[a]		38 ± 5	58 ± 4.5**

Values are mean ± s.e.mean of n rats; [a]topically applied. $^{**}P<0.01$, when compared to the respective control (paired t test).

ANP in the presence of both retrothiorphan and C-ANP (4–23) Administration of C-ANP(4–23) alone or in combination with retrothiorphan had no significant effect on arteriolar diameter (Table 5). Moreover, arteriolar diameters were not affected by bolus injection of ANP (10 μg kg^{-1}) alone as previously observed or after a 30 min infusion of C-ANP(4–23) (Table 5). In contrast, significant vasodilatation was observed when 10 μg kg^{-1} ANP was injected after co-administration of C-ANP(4–23) and retrothiorphan (Table 5). In the control experiments, arteriolar responses to ANP were not modified when administered after a 30 or 60 min saline infusion (Table 5).

The modest fall in mean arterial blood pressure induced by 10 μg kg^{-1} ANP (Table 6) was not significantly modified by C-ANP(4–23) infusion, or by a combination of C-ANP(4–23) and retrothiorphan when compared to the saline-treated group (AOC = 321 ± 56 mmHg min versus 259 ± 62 mmHg min, respectively before treatment; 190 ± 29 mmHg min versus 133 ± 14 mmHg min 30 min after C-ANP(4–23) infusion or saline infusion; 180 ± 45 mmHg min versus 132 ± 38 mmHg min 60 min after (C-ANP(4-23) + retrothiorphan or saline infusion).

Discussion

The present study indicates that topically applied ANP can dilate small arterioles of the innervated rat cremaster. Moreover we have found that the vasodilator properties of ANP differ according to the size of the vessels. When compared to the complete smooth muscle relaxation induced by sodium nitroprusside, the maximal dilatation produced by ANP was greater in large vessels than in small arterioles, possibly because small arterioles exhibit a high degree of intrinsic tone which is not affected by ANP (Faber *et al.*, 1988; Faber, 1990). Our results are in agreement with those of Sarelius & Huxley (1990) who showed that local application of ANP into arterioles of the hamster cheek pouch of diameter 25–35 μm produced vasodilatation. However, they differ somewhat from

Table 3 Effects of intravenous ANP on mean arterial blood pressure with and without retrothiorphan (25 mg kg^{-1}, i.v.)

	n	*Mean arterial blood pressure* (mmHg) *Control*	*Time after ANP* (min) 1	2	5	10
ANP (10 μg)	6					
Before retrothiorphan		125 ± 7	116 ± 7.4**	110 ± 8**	105 ± 8**	
After retrothiorphan		112 ± 7	101 ± 6**	97 ± 7**	98 ± 5**	
ANP (20 μg)	5	126 ± 6	111 ± 6*	106 ± 6**	93 ± 7**	103 ± 10

Values are mean ± s.e.mean of n rats; $^{*}P<0.05$, $^{**}P<0.01$ when compared to their respective control (ANOVA for repeated measures followed by Dunnett's t test).

Table 5 Effect of C-ANP (4–23) alone or in combination with retrothiorphan on arteriolar responses to intravenous ANP (10 $\mu g\,kg^{-1}$)

	Diameter (μm)		
	Control	*ANP*	*Nitroprusside* 3×10^{-5} M [(d)]
Before treatment	26±4	26±4	
30 min after C-ANP (4–23) infusion[(a)]			
60 min after C-ANP (4–23) infusion and	26±4.3	28±3.9	
5 min after retrothiorphan[(b)]	28±4	34±3.5**	
15 min after the end of infusion	27±3.7		39.5±3.3¶¶
Before treatment	32±3.2	33±3.2	
30 min after saline infusion[(c)]	31±3.5	32±3.4	
60 min after saline infusion	32±3.5	32±3.5	
15 min after the end of infusion	31±3.6		45±2.6¶¶

Values are mean±s.e.mean of 5 rats. [(a)]Bolus injection of 10 $\mu g\,kg^{-1}$ C-ANP (4–23) followed by a constant infusion of 1 $\mu g\,kg^{-1}\,min^{-1}$. [(b)]Retrothiorphan 25 $mg\,kg^{-1}$ as a bolus injection. [(c)]Bolus injection of 1 $ml\,kg^{-1}$ followed by a constant infusion of 50 $\mu l\,kg^{-1}\,min^{-1}$. [(d)]Topically applied.
**$P<0.01$ when compared to ANP+C-ANP or ANP alone (ANCOVA). ¶¶$P<0.01$ when compared to its own control (paired t test).

Table 6 Effect of ANP (10 $mg\,kg^{-1}$, i.v.) before and after C-ANP (4–23) alone and in combination with retrothiorphan or saline infusion on mean arterial blood pressure

	Mean arterial blood pressure (mmHg)					
	Control	*Time after ANP administration* (min)				
		1	2	5	10	15
Before treatment	120±7	114±8	108±8	100±8*	92±8**	89±6**
30 min after C-ANP (4–23) infusion[(a)]	108±6	103±5.5	100±5*	97±6**	89±5.5**	90±5**
60 min after C-ANP (4–23) infusion and 5 min after retrothiorphan[(b)]	103±3	96±5.5	93±5**	91±5**	90±4**	90±3.5**
Before treatment	115±3	110±3	105±4*	100±5**	92±5**	93±4**
30 min after saline infusion[(c)]	104±6	112±6.5	110±7*	106±7**	100±7**	100±7**
60 min after saline infusion	103±6	99±7	96±8	94±8*	91±6.5**	98±4.6

Values are mean±s.e. mean of 5 rats. [(a)]Bolus injection of 10 $\mu g\,kg^{-1}$ C-ANP (4–23) followed by a constant infusion of 1 $\mu g\,kg^{-1}\,min^{-1}$. [(b)]25 $mg\,kg^{-1}$ retrothiorphan as bolus injection. [(c)]Bolus injection of 1 $ml\,kg^{-1}$ followed by a constant infusion of 50 $\mu l\,kg^{-1}\,min^{-1}$.
*$P<0.05$, **$P<0.01$ when compared to the respective control (ANOVA for repeated measures followed by Dunnett's t test).

those obtained in the rat by other investigators. Proctor & Bealer (1987) found that topically applied ANP (30 nM) failed to dilate third order rat spinotrapezius arterioles directly (mean diameter 25±2 μm). Similarly Faber *et al.* (1988) using the rat cremaster showed that while bath-added ANP (10^{-11} to 10^{-7}M) caused a concentration-dependent dilatation of large arterioles (mean diameter 127±16 μm), it did not produce any vasodilatation of terminal arterioles (mean diameter 17±1 μm) except when applied at a concentration $>10^{-7}$ M. Differences in tissues and experimental conditions may account for these apparent discrepancies. Proctor & Bealer (1987) performed their experiments on the rat spinotrapezius in the presence of cyclo-oxygenase inhibitors. On the other hand, in the study of Faber *et al.* (1988), the rat cremaster muscle was acutely denervated and vascular tone was induced by addition of exogenous noradrenaline. Moreover the bath solution contained propranolol (1 μM), desipramine (10 nM) and normetanephrine (10 μM). The denervated preparation certainly seemed to have different behavioural characteristics since the maximal effect of ANP was observed several minutes after application, whereas, under our conditions, responses reached a maximum within 30 s after application of the peptide, in accordance with the results of Sarelius & Huxley (1990) in the hamster cheek pouch.

In the present work, i.v. bolus injection of NEP inhibitors did not affect arteriole diameters at doses which have been demonstrated to be effective in preventing the degradation of ANP *in vitro* and in reducing the metabolism of the exogenously administered peptide *in vivo* (Ollins *et al.*, 1989; Trapani *et al.*, 1989).

In the presence of NEP inhibitors, we observed an increase in the dilator responses of arterioles to topically applied ANP. This suggests that NEP present on the smooth muscle cells (Dussaule *et al.*, 1993) and on the cells that surround the vascular wall, skeletal muscle cells, neutrophils and fibroblasts (Gee *et al.*, 1985; Ronco *et al.*, 1988) may contribute to the degradation of ANP. In agreement with this, Piedimonte and collaborators (1994) have shown in coronary circulation that NEP localized on cardiac myocytes modulates the activity of vasodilator peptides, mainly bradykinin, released by myocardial ischaemia. Further, in the hamster cheek pouch, pretreatment by phosphoramidon, a NEP inhibitor, was associated with significant potentiation of arteriolar dilatation induced by topical application of bradykinin (Gao *et al.*, 1994).

As in the endothelium, more than 90% of the total population of ANP receptors consist of C-ANP receptors in cultured vascular smooth muscle and in fibroblasts (for review see Maack, 1992). Moreover, it has been shown that except in the presence of very high plasma levels of ANP, the C-ANP receptor mechanism may prevent any increase in ANP levels that could result from NEP inhibition (reviewed in Rushkoao, 1992). In our study, the potentiation of the vasodilator effect of topically applied ANP, suggests that the C-ANP receptor activity did not compensate for the increase in peptide concentrations resulting from NEP inhibition. It seemed unlikely that in our conditions, the increased concentration of ANP achieved by NEP inhibition was such that it would saturate the C-ANP receptor activity. The lowest concentration of ANP used in our study (0.9 ng ml^{-1}) was comparable with ANP

plasma levels obtained for rats with experimental heart failure (1.2 ± 0.2 ng ml^{-1}, Chien *et al.*, 1988). When ANP was infused or intravenously injected at a low dose, resulting in physiological or pathophysiological plasma levels of ANP, pretreatment by an NEP inhibitor such as thiorphan did not further increase the plasma levels of ANP (Trapani *et al.*, 1986; Koepke *et al.*, 1989; Smits *et al.*, 1990). A more likely explanation could be that arterioles express low levels of the C-receptor. Indeed Suga *et al.* (1992) have raised a problem concerning the expression of C-ANP receptors *in vitro* when compared with *ex vivo* conditions. Like other authors, they observed that first passaged, cultured aortic smooth muscle cells expressed about 96% C-ANP receptors with few A-ANP receptors and the B-ANP receptor constituting the remainder of the receptor population. On the other hand they showed that the rat isolated aortic medial layer contained mainly the A-ANP receptor and only about 5% of total binding was attributed to C-ANP receptors.

As expected, ANP induced a fall in blood pressure when given as an i.v. bolus at 10 and 20 $\mu g\ kg^{-1}$ but hypotension was associated with an increase in arteriole diameter only in the presence of the highest dose. Criscione *et al.* (1987) have shown that an i.v. bolus injection of ANP (7 $\mu g\ kg^{-1}$) in the anaesthetized rat causes a transient fall in blood pressure accompanied by a decrease in total calculated peripheral resistance. However, depending on the dose, ANP can display marked regional variability in relaxing the vascular bed (reviewed in Windquist & Hintze, 1990). Lappe *et al.* (1986) have shown in the conscious rat that at lower bolus doses ($< 10\ \mu g\ kg^{-1}$), ANP may cause dilatation only in the kidney while at higher doses (11 to 27.5 $\mu g\ kg^{-1}$), the regional selectivity is lost and mesenteric and to a lesser degree, hindquarter vasodilatation is observed.

In our study, prior treatment with retrothiorphan did not modify either the magnitude or the duration of the depressor effect of an i.v. bolus injection of 10 $\mu g\ kg^{-1}$ ANP. This result differs from that obtained by others in the conscious rat. Pretreatment with the NEP inhibitor SQ 28603 prolonged the duration of the depressor effect of ANP given as an i.v. bolus at a comparable dose (9 $\mu g\ kg^{-1}$) without affecting the peak response in the spontaneously hypertensive rat (Seymour *et al.*, 1992) or in the normotensive rat (Gardiner *et al.*, 1992). Further, in the presence of a very high dose of ANP (30 $\mu g\ kg^{-1}$, i.v. bolus) an increase in the duration and also in the magnitude of the hypotensive effect of ANP was observed in spontaneously hypertensive rats pretreated with the NEP inhibitor SCH 39370 (Sybertz *et al.*, 1989). Anaesthesia, the doses of ANP used and the use of different NEP inhibitors may be responsible for the failure of retrothiorphan to influence the hypotensive effect of ANP in the present study.

No change was observed in arteriolar diameter after a bolus injection of 10 $\mu g\ kg^{-1}$ ANP in the presence of an NEP inhibitor or the C-ANP receptor ligand C-ANP(4–23). However, the combined administration of retrothiorphan and C-ANP receptor ligand unveils the vasodilator properties of ANP. Thus is appears that the regional variability in relaxing vessels shown by ANP might be linked, at least in part, to the metabolism of the peptide. Similarly, it can be speculated that given a sudden and strong increase in plasma ANP levels, as may result after the bolus i.v. administration of a high dose of ANP alone, the two main metabolic pathways could be saturated and the peptide, therefore, reaches the smooth muscle cells in concentrations adequate to cause vasodilatation of skeletal arterioles, as found by Lappe *et al.* (1986) in the conscious spontaneously hypertensive rat and by us in the anaesthetized normotensive rat.

In summary, topically applied ANP dilates rat cremaster arterioles at low doses and this effect is potentiated by NEP inhibitors. When given as an i.v. bolus at high doses, ANP also dilates skeletal arterioles. At a lower dose, ANP did not affect arteriolar diameter and the two main metabolic pathways need to be inhibited before the vasodilator properties of the peptide become apparent.

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