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Relaxation and modulation of cyclic AMP production in response to atrial natriuretic peptides in guinea pig tracheal smooth muscle

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Abstract

Relaxation and modulation of cyclic AMP production in response to atrial natriuretic peptides were investigated in epithelium-denuded guinea pig tracheal rings, treated with indomethacin (5 μM) and phosphoramidon (1 μM) and contracted with histamine (3 μM). Atrial natriuretic peptide (ANP) was a more potent relaxant than C-type natriuretic peptide whereas ANP-(4–23) was inactive suggesting the involvement of ANP_A receptors in the relaxant effect of ANP. ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*A*]quinoxalin-1-one, 10 μM), a selective inhibitor of soluble guanylyl cyclase, markedly inhibited the relaxant response to sodium nitroprusside. The relaxant response to ANP was not altered by ODQ demonstrating the involvement of particulate guanylyl cyclase. ANP-induced relaxations, as well as sodium nitroprusside-induced relaxations, were similarly potentiated by rolipram (4-(3-(cyclopentyloxy)-4-methoxyphenyl)pyrrolidin-2-one, 3 μM), a type IV phosphodiesterase inhibitor, and by zaprinast (2-(2-propyloxyphenyl)-8-azapurin-6-one, 10 μM), a type V phosphodiesterase inhibitor. ANP-mediated response was unaffected by glibenclamide (10 μM), a selective blocker of ATP-sensitive K^+ channels, and by apamin (1 μM), a selective blocker of small-conductance Ca^{2+}-activated K^+ channels. Iberiotoxin (100 nM) extensively prevented the relaxant effect of ANP suggesting the activation of large-conductance Ca^{2+}-activated K^+ channels. In addition, ANP (10 nM) and ANP-(4–23) (100 nM) significantly reduced forskolin (1 μM)-stimulated cAMP accumulation suggesting, for the first time, the presence of functional ANP_C receptors in guinea pig airway smooth muscle. However, relaxations to forskolin and to isoproterenol were not altered in the presence of ANP-(4–23) or ANP demonstrating that the inhibitory effect of ANP-(4–23) and ANP on adenylyl cyclase was not sufficient to alter the functional response induced by these two activators of adenylyl cyclase.

Keywords: Airway; Smooth muscle; Trachea, guinea pig; ANP (atrial natriuretic peptide); ANP receptor; Guanylyl cyclase, particulate; Adenylyl cyclase; K^+ channel; Phosphodiesterase inhibitor

1. Introduction

Atrial natriuretic peptide (ANP) or atrial natriuretic factor is one of a family of hormones involved in the regulation of a variety of physiological processes affecting mainly cardiovascular homeostasis. ANP is produced primarily in the heart but is also released in other tissues including the lungs (Perreault and Gutkowska, 1995). In airways, ANP has been localized in epithelium and ANP binding sites have been detected over both smooth muscle and epithelium (Fernandes et al., 1992; Perreault and Gutkowska, 1995). ANP caused relaxation of isolated airway smooth muscle preparations. However, marked species differences have been demonstrated with respect to the airway responsiveness to ANP, with a better responsiveness of isolated guinea pig trachea than human bronchus (Candenas et al., 1991; Fernandes et al., 1992). Several factors influence the responsiveness of guinea pig trachea to ANP. These include removal of airway epithelium and inhibition of neutral endopeptidase (endopeptidase 24-11) (Candenas et al., 1991; Fernandes et al., 1992). Neutral endopeptidase exists in the airway epithelium and submucosa and inactivates ANP explaining therefore the potentiating effects on ANP-induced relaxation of epithelium removal and enzyme inhibition.

In addition to ANP, two other members of the natriuretic peptide family are brain natriuretic peptide (BNP)

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and C-type natriuretic peptide (CNP) (Anand-Srivastava and Trachte, 1993). The natriuretic peptides act through activation of three types of natriuretic peptide receptors that have been identified by molecular cloning techniques: ANP_A (Chinkers et al., 1989; Lowe et al., 1989), ANP_B (Schultz et al., 1989; Chang et al., 1989) and ANP_C (Fuller et al., 1988; Anand-Srivastava et al., 1987). ANP_A and ANP_B receptors are membrane (particulate) forms of guanylyl cyclases that mediate the production of cGMP in response to atrial natriuretic peptides. The potency order of these peptides for ANP_A receptors is ANP > BNP ≫ CNP and for ANP_B receptors is CNP ≫ ANP = BNP whereas ANP, BNP and CNP bind with similar affinity to ANP_C receptors. In contrast with ANP_A and ANP_B, ANP_C receptors do not contain intrinsic guanylyl cyclase activity and have originally been considered in terms of clearance of bound ligand by internalization and degradation (ANP clearance receptors). The clearance receptors represent a major portion of total ANP receptors in bovine lung (Perreault and Gutkowska, 1995). In addition, ANP_C receptors are coupled to adenylyl cyclase inhibition or to phosphoinositide hydrolysis stimulation and have been shown to mediate various physiological effects of ANP (Anand-Srivastava and Trachte, 1993). The inhibitory effect of ANP_C receptors on adenylyl cyclase has been demonstrated in different cell types by the use of ring-deleted analogs of ANP, like ANP-(4–23), which stimulate selectively ANP_C receptors without increasing cGMP through stimulation of ANP_A or ANP_B receptors (Anand-Srivastava et al., 1990; Palaparti et al., 2000).

In an attempt to characterize the functional ANP receptors in guinea pig isolated tracheal smooth muscle, we have studied the effects of ANP, CNP and ANP-(4–23) in epithelium-denuded preparations treated with phosphoramidon. Firstly, we have compared the relaxant effects of ANP and CNP. In addition, since few studies have been devoted to the mechanisms of ANP-induced relaxation in airway smooth muscle preparations (Watanabe et al., 1990; Thirstrup et al., 1997), we have evaluated the effects of a selective soluble guanylyl cyclase inhibitor, of different K^+ channel blockers and of phosphodiesterase IV and V inhibitors on the relaxant response to ANP and to sodium nitroprusside used as a reference guanylyl cyclase activator. Secondly, we have evaluated the inhibitory effect of ANP-(4–23) and ANP on cAMP production and the functional consequences on the relaxation to forskolin and isoprenaline, two activators of adenylyl cyclase.

2. Materials and methods

2.1. Tracheal preparation for organ bath studies

Male guinea pigs weighing from 250 to 350 g were killed by intraperitoneal injection of pentobarbital (6 %). The trachea was rapidly excised and placed in oxygenated (95% O_2, 5% CO_2) Krebs solution (composition in mM: NaCl 118, KCl 5.4, $CaCl_2$, 2.5, $MgSO_4$ 0.6, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.7) at room temperature. The trachea was trimmed free of adherent fat and connective tissue and cut into four rings containing three adjacent cartilage plates. Each ring was gently rubbed using a cotton wool-coated probe to remove the epithelium as previously described (Devillier et al., 1988). Each ring was transferred to a 5-ml organ bath containing Krebs solution (37 °C, pH 7.4) gassed with 5% CO_2 in oxygen. Each ring was suspended under 1.5 g tension and equilibrated for 60 min during which the Krebs solution was changed every 15 min. In these conditions, the contractile responses to agonists were reproducible over several hours. Isometric force was measured with strain gauges (UF-1 Pioden, UK) and amplifiers (EMKA, France) and displayed on two-channel recorders (Linseis, Polylabo France). In all experiments, tracheal rings were first contracted maximally with acetylcholine (3 mM), then fully relaxed with aminophylline (3 mM) and allowed to equilibrate for 60 min during which the Krebs solution was changed every 15 min. Following this second equilibration period, the basal tone was in the range of 0.9 to 1.3 g. The rings were then pre-treated for 30 min with indomethacin (5 μM). The relaxant effect of natriuretic peptides and sodium nitroprusside were then assessed in preparations contracted with histamine (3 μM). The responses to natriuretic peptides were determined after a 15-min incubation (prior to exposure to histamine) of the preparations with phosphoramidon (1 μM), a neutral endopeptidase (NEP 24.11) inhibitor. Each concentration–response curve to natriuretic peptides or sodium nitroprusside was obtained by the cumulative addition of the compounds at intervals of 5–15 min to reach a stable level of relaxation before the next addition was made. After the maximal effect of each drug was obtained, aminophylline 3 mM was added to determine the maximum relaxation achievable. Only one concentration–response curve to natriuretic peptides or sodium nitroprusside was recorded for each tracheal ring.

The relaxant responses to ANP and to sodium nitroprusside were evaluated both in the absence and presence of a selective inhibitor of soluble guanylyl cyclase, 1*H*-[1,2,4]oxadiazolo[4,3-*A*]quinoxalin-1-one (ODQ; 10 μM), of selective K^+ channel blockers (iberiotoxin (100 nM), apamin (1 μM) or glibenclamide (10 μM)) or of selective phosphodiesterase inhibitors (rolipram (3 μM), zaprinast (10 μM)). Tracheal preparations were pretreated with these agents or the corresponding vehicles for 20 min prior to and during exposure to histamine and the construction of relaxant concentration–effect curves. In each set of experiments, the control rings were obtained from the same trachea as the treated rings and run in parallel.

The effects of ANP or ANP-(4–23) on the relaxant responses to forskolin or to isoproterenol were also investigated on preparations contracted with histamine. The two peptides were added to the histamine-precontracted prepa-

rations 15 min before the cumulative addition of forskolin or isoproterenol. Control preparations in the absence of peptides were run in parallel.

2.2. Measurement of cyclic AMP

Measurement of cAMP was performed as previously described in vessel rings (Cracowski et al., 2000). Guinea pig tracheal rings were placed in glass tubes and incubated free-floating in oxygenated Krebs solution at 37 °C for a 60-min equilibration period during which the Krebs solution was changed every 15 min. All experiments were conducted in the presence of indomethacin (1 μM, 30 min), 3-isobutyl-1-methyl-xanthine (IBMX; 0.1 mM, 30 min), a non-selective phosphodiesterase inhibitor, and of phosphoramidon (1 μM, 15 min). Histamine (3 μM) was added to the preparations for 15 min. The tissues were then incubated for a further 15 min in the presence or absence of ANP (10 nM) or ANP-(4–23) (10 and 100 nM) to investigate the effects of these peptides on basal cAMP levels. The effects of ANP and ANP-(4–23) on stimulated cAMP levels were also studied by the addition of forskolin (1 μM) for 15 min after the incubation period with the peptides. The results were compared to the cAMP levels stimulated with forskolin in the absence of ANP or ANP-(4–23). At the end of the incubation period, the tracheal rings were rapidly removed, blotted dry and frozen by immersion in liquid nitrogen. Frozen tissues were finely chopped prior to homogenization and incubation in 6% trichloroacetic acid for 24 h at 4 °C in the presence of IBMX (0.1 mM). Precipitated protein was separated from the soluble extract by centrifugation at $10{,}000 \times g$ for 30 min at 4 °C. Trichloracetic acid was removed from the supernatants with four successive water ethyl ether extractions (5 ml). After the last ether extraction, the tubes were placed in a temperature-controlled bath at 70 °C (5 min) to eliminate all traces of ether. The concentrations of cAMP were determined after acetylation, using a commercially available enzyme-immunoassay kit (Cayman, Ann Arbor, USA). Precipitates were used for protein determination by the method of Bradford using bovine serum albumin as standard. cAMP levels were expressed as pmol cAMP per mg protein.

2.3. Data analysis and statistics

All data are expressed as means ± standard error of the mean (S.E.M.). Relaxant responses were expressed as a percentage of the maximal relaxation produced by aminophylline 3 mM. E_{max} represents the maximal effect obtained with the maximal concentrations of drugs. The pD_2 value represents the negative logarithm of the concentration of agent which induces a relaxation equal to 50% of its own maximal effect (EC_{50}). EC_{50} was determined from each relaxation by a logistic curve fitting equation. The probability of differences between the mean results was determined using either paired or unpaired Student's t test or two-way analysis of variance as appropriate and was considered significant if $P < 0.05$.

2.4. Drugs

The following compounds were used: acetylcholine, indomethacin, 3-isobutyl-1-methyl-xanthine, sodium nitroprusside, histamine dihydrochloride, glibenclamide, phosphoramidon (*N*-(α-rhamnopyranosyloxyhydroxyphosphinyl)-Leu-Trp) and isoproterenol were from Sigma (Saint Quentin Fallavier, France); ANP (Thr-Ala-Pro-Arg-atrial natriuretic peptide (1–28)) was from Bachem (Voisins le Bretonneux, France) and from Euromedex (Strasbourg, France); CNP (C-type natriuretic peptide) was from Peninsula (Belmont, USA) and ANP-(4–23) (des-(Gln^{18}, Ser^{19}, Gln^{20}, Leu^{21}, Gly^{22})-fragment (4–23)-NH_2) was from Euromedex; iberiotoxin and apamin were from RBI (Saint Quentin Fallavier, France); ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*A*]quinoxalin-1-one), rolipram (4-(3-(cyclopentyloxy)-4-methoxyphenyl)pyrrolidin-2-one) and zaprinast (2-(2-propyloxyphenyl)-8-azapurin-6-one) were from Tocris (Illkirch, France); aminophylline was obtained from Pharmacie Centrale des Hôpitaux (Paris, France). The water-saturated ethyl ether and trichloracetic acid were obtained from Prolabo (Fontenay sous Bois, France).

Indomethacin, rolipram and glibenclamide were prepared in ethanol. ODQ was dissolved in dimethylsulfoxide (DMSO). Stock solutions of the drugs were held frozen (−20 °C) in aliquots and were freshly diluted in distilled water to the appropriate concentrations expressed as final molar concentrations.

3. Results

3.1. Relaxant effects of ANP, CNP and ANP-(4–23)

ANP and CNP caused concentration-dependent relaxations of histamine-contracted guinea pig tracheal preparations. Significantly greater relaxant responses were obtained with ANP ($E_{max} = 68 \pm 5\%$, $pD_2 = 8.2 \pm 0.2$, $n = 7$, $P < 0.05$) than with CNP ($E_{max} = 31 \pm 7\%$, $n = 6$) as assessed by analysis of variance (Fig. 1). Accurate estimation of the potency (pD_2) of CNP was not possible as the maximal relaxant effect was not reached at the maximal concentration tested (1 μM). The concentrations of peptides required to produce a 20% reduction in tone induced with histamine were determined. ANP was found to be 86-fold more potent than CNP ($P < 0.01$). Cumulative addition of ANP-(4–23) from 1 nM to 1 μM has no relaxant effect on histamine-contracted tracheal preparations (Fig. 1). In addition, ANP, CNP and particularly ANP-(4–23) did not induce contraction of tracheal preparations at basal tone in the presence of phosphoramidon and indomethacin (three to five experiments).

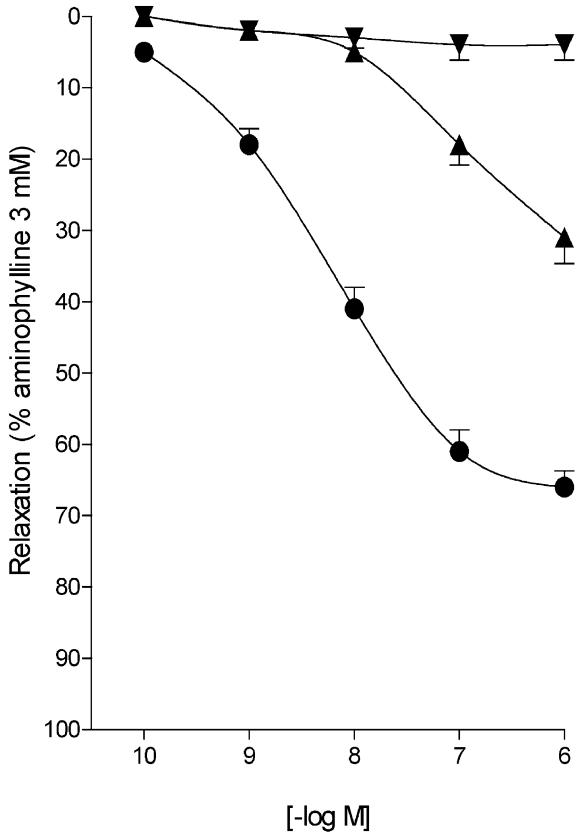

Fig. 1. Concentration–response curves showing relaxations to ANP (●), CNP (▲) and ANP-(4–23) (▼) on histamine-contracted guinea pig tracheas. Symbols represent mean responses expressed as a percentage of the maximum aminophylline (3 mM) relaxation and vertical lines show S.E.M. for 5 to 10 experiments.

3.2. Effects of a selective inhibitor of soluble guanylyl cyclase and of selective phosphodiesterase inhibitors on ANP- and sodium nitroprusside-induced relaxations

The highly selective soluble guanylate cyclase inhibitor, ODQ 10 μM, did not alter the contraction induced by histamine and the relaxant response elicited by ANP (Table 1) In contrast, ODQ extensively prevented the relaxant effect of SNP (Table 1).

Rolipram (3 μM), a selective phosphodiesterase IV inhibitor, and zaprinast (10 μM), a selective phosphodiesterase V inhibitor, did not alter the basal tone of guinea pig tracheal preparations treated with indomethacin or with indomethacin and phosphoramidon and did not influence the magnitude of the contractile response to histamine. However, both inhibitors significantly shifted the ANP and sodium nitroprusside concentration–response curves to the left with significant increases in maximal relaxant effects (Table 1).

3.3. Effects of K^+-channel blockers on ANP-induced relaxations

Glibenclamide (10 μM), a selective blocker of ATP-sensitive K^+ channels (K_{ATP}) and apamin (1 μM), a selective blocker of small-conductance Ca^{2+}-activated K^+ channels, did not influence either the baseline tone or the histamine-induced contraction. These two K^+ channel blockers also did not inhibit the relaxation induced by ANP (Table 2). Pretreatment for 20 min with iberiotoxin (100 nM), a selective blocker of large-conductance Ca^{2+}-activated K^+ channels, did not cause contraction of the preparations as previously reported (Corompt et al., 1998). However, iberiotoxin produced a slight (10–15%) potentiation of the response to histamine as previously shown (Ellis and Conanan, 1994). Therefore, for each experiment, the concentration of histamine was adjusted to obtain a level of contraction similar to the level in control preparations in order to avoid some degree of functional antagonism of relaxation (Corompt et al., 1998). In these conditions, iberiotoxin significantly inhibited the relaxation to

Table 1
Effects of the soluble guanylate cyclase inhibitor ODQ and of selective phosphodiesterase inhibitors on atrial natriuretic peptide- and sodium nitroprusside-induced relaxations of histamine-contracted guinea pig trachea. Maximal relaxations (E_{max}) are expressed as percentage of maximal relaxation to aminophylline (3 mM)

Treatment	Atrial natriuretic peptide			Sodium nitroprusside		
	n	E_{max} (%)	pD_2	*n*	E_{max} (%)	pD_2
Control	5	68±6	8.2±0.2	8	91±4	6.2±0.1
ODQ 10 μM	5	71±7	8.1±0.1	8	19±5[a]	NC
Control	5	62±7	8.1±0.2	7	87±3	6.1±0.2
Rolipram 3 μM	5	83±5[a]	8.4±0.1[b]	7	95±3[b]	6.7±0.2[b]
Control	7	64±8	8.1±0.1	7	86±4	6.2±0.2
Zaprinast 10 μM	7	81±6[b]	8.3±0.1[b]	7	94±3[b]	6.6±0.1[b]

Data are means±S.E.M. Comparisons were performed using the Student's *t* test for paired data. NC = not calculated.
[a] $P < 0.01$.
[b] $P < 0.05$.

Table 2
Effects of potassium channel blockers on atrial natriuretic peptide-induced relaxations of histamine-contracted guinea pig trachea. Maximal relaxations (E_{max}) are expressed as percentage of maximal relaxation to aminophylline (3 mM)

Treatment	Atrial natriuretic peptide		
	n	E_{max} (%)	pD_2
Control	6	72±6	8.1±0.2
Glibenclamide 10 μM	6	74±7	8.2±0.1
Control	5	70±4	8.0±0.1
Ibtx 100 nM	5	34±5[a]	7.4±0.2[b]
Control	6	75±6	8.1±0.1
Apamin 1 μM	6	71±5	7.9±0.2

Data are means±S.E.M. Comparisons were performed using the Student's *t* test for paired data.
[a] $P < 0.01$.
[b] $P < 0.05$.

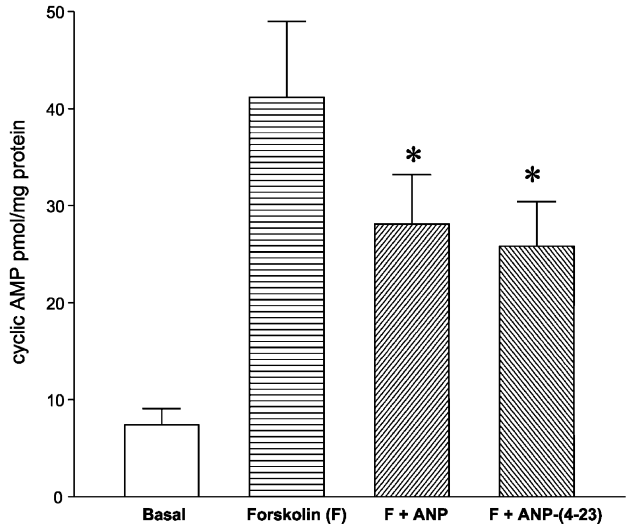

Fig. 2. Effect of 10 nM ANP and 100 nM ANP-(4–23) on forskolin (1 μM)-induced cyclic AMP accumulation in guinea pig tracheal rings. Each bar is the mean ± S.E.M. of 5 to 15 tracheal rings. $^{*}P < 0.05$ for comparisons of accumulation in the presence and absence of ANP or ANP-(4–23). The basal production of cAMP in the absence of forskolin is also shown.

ANP in terms of potency and efficacy (Table 2). Pretreatment with iberiotoxin shifted the concentration–response curves 43-fold to the right.

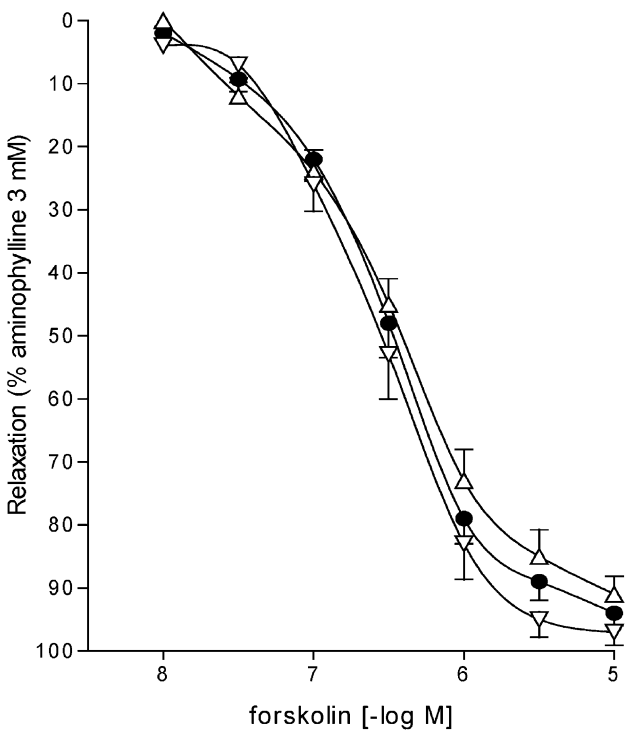

Fig. 3. Effect of 10 nM ANP (△) and 100 nM ANP(4–23) (▽) on the control (●) concentration–response curves for relaxation to forskolin. Symbols represent mean responses expressed as a percentage of the maximum aminophylline (3 mM) relaxation and vertical lines show S.E.M. for five to eight experiments.

Table 3
Effects of atrial natriuretic peptide (ANP) and of the ring-deleted analog of ANP (ANP-(4–23)) on isoproterenol-induced relaxations of histamine-contracted guinea pig trachea. Maximal relaxations (E_{max}) are expressed as percentage of maximal relaxation to aminophylline (3 mM)

Treatment	Isoproterenol		
	n	E_{max} (%)	pD_2
Control	6	94 ± 5	7.5 ± 0.2
ANP 10 nM	7	96 ± 4	7.4 ± 0.1
ANP-(4–23) 100 nM	7	97 ± 4	7.4 ± 0.2

Data are means ± S.E.M.

3.4. Effects of ANP-(4–23) and ANP on cAMP production and on relaxation induced by adenylyl cyclase activators

ANP-(4–23) and ANP, at 100 and 10 nM, respectively, have been previously shown to induce a maximal inhibition of adenylyl cyclase in different cardiovascular tissue preparations (Anand-Srivastava et al., 1984; Anand-Srivastava and Cantin, 1986; Savoie et al., 1995; Palaparti et al., 2000). At these concentrations, ANP-(4–23) and ANP did not alter basal cAMP content in guinea pig trachea (basal: 7 ± 2 pmol/mg protein ($n = 12$); ANP-(4–23): 9 ± 2 pmol/mg protein ($n = 6$) and ANP: 8 ± 2 pmol/mg protein ($n = 7$)). Forskolin, an adenylyl cyclase activator, produced concentration-dependent and complete relaxation of guinea pig trachea. At 1 μM, forskolin induced a near-maximal relaxation and enhanced cAMP accumulation six fold (forskolin: 42 ± 8 pmol/mg protein, $P < 0.01$ vs. basal). ANP-(4–23) and ANP significantly reduced forskolin-stimulated cAMP accumulation by 46% (26 ± 5 pmol/mg protein) and 42% (28 ± 6 pmol/mg protein), respectively (Fig. 2). However, relaxation concentration–response curves to forskolin were not altered in the presence of ANP-(4–23) or ANP (Fig. 3). In addition, concentration–responses curves to isoproterenol, a β-adrenoceptor agonist, were not influenced by the two peptides (Table 3).

4. Discussion

The lung is not only the first target organ for ANP released by the heart's right atrium but it also synthesizes and releases ANP (Perreault and Gutkowska, 1995). Two types of ANP receptors have been characterized in bovine lung: particulate membrane guanylyl cyclases (ANP_A and ANP_B receptors) represent a minor portion of total receptors and the remaining are "clearance receptors" (ANP_C) which are negatively coupled to adenylyl cyclase (Perreault and Gutkowska, 1995). In guinea pig trachea, ANP binding sites have been detected over both smooth muscle and epithelium (Fernandes et al., 1992). The aim of the present study was to investigate the effects of ANP receptor agonists in terms of relaxation and cAMP inhibition in guinea pig tracheal smooth muscle.

4.1. Relaxant effects of ANP and CNP

We and others have shown that the isolated guinea pig trachea is one of the most responsive airway preparation to the relaxant effect of ANP (Candenas et al., 1991; Fernandes et al., 1992; Thomson, 1995). In this preparation, epithelium removal and inhibition of endopeptidase 24-11 with phosphoramidon or thiorphan potentiated the relaxant activity of ANP (Candenas et al., 1991; Fernandes et al., 1992). Phosphoramidon increased the potency of ANP in epithelium-denuded preparations suggesting that the endopeptidase 24-11 metabolizing this peptide is also located in the tracheal wall (Fernandes et al., 1992). In order to study the direct airway smooth muscle effects of ANP and CNP, we have used epithelium-denuded guinea pig tracheal preparations treated with phosphoramidon. Such a pretreatment was essential to compare the potency of these two peptides since the inactivation rates of ANP and CNP as well as of other related peptides by endopeptidase 24-11 are different (Flüge et al., 1997). In addition, indomethacin was present throughout the experiments in order to ensure optimal and reproducible responses to histamine and to prevent the release of prostanoids and the increase in cAMP content caused by histamine in this tissue (Creese and Denborough, 1980; Brink et al., 1981).

ANP_A and ANP_B receptors are membrane (particulate) forms of guanylyl cyclase. ANP is much more potent than CNP at the ANP_A receptors whereas CNP is much more potent than ANP at the ANP_B receptors (Yandle, 1994). In the present study, ANP was more potent than CNP suggesting that ANP_A receptors are involved in the relaxation of guinea pig tracheal smooth muscle.

4.2. Effect of selective inhibition of soluble guanylyl cyclase on ANP-induced relaxation

The quinolinedione, LY 83583 (6-anilino-5,8-quinolinedione), has been reported to be a specific inhibitor of soluble guanylyl cyclase (Mülsch et al., 1988) and to inhibit the relaxation by ANP of bovine trachea smooth muscle contracted with histamine (Ijioma et al., 1995). This suggests that in bovine airways, ANP works, at least in part, through soluble guanylyl cyclase. However, LY 83583 can also inhibit cGMP accumulation by other mechanisms, including the generation of superoxide ions (Furchgott and Jothianandan, 1991). In the meantime, ODQ has been reported to be a specific and potent inhibitor of soluble guanylyl cyclase in a variety of tissues including guinea pig trachea (Ellis, 1997; Hwang et al., 1998). ODQ has nanomolar sensitivity against soluble guanylate cyclase and is ineffective at concentrations up to 100 μM against particulate guanylyl cyclase or adenylyl cyclase (Garthwaite et al., 1995). This compound is therefore a powerful pharmacological tool to distinguish between cGMP accumulation induced by soluble and particulate guanylyl cyclases. As expected, ODQ extensively inhibited the relaxant effects of sodium nitroprusside in guinea pig trachea. In contrast, the effect of ANP was not altered by ODQ demonstrating that the relaxant action of ANP in guinea pig trachea is not mediated by soluble guanylyl cyclase activation. This result is in agreement with a report in rat vascular smooth muscle showing that ODQ did not affect the increase in cGMP or the vasodilation induced by ANP (Moro et al., 1996).

4.3. Effect of phosphodiesterase inhibitors on ANP-induced relaxation

ANP and sodium nitroprusside potently relaxed histamine-contracted guinea pig trachea. The relaxant activity of these compounds was enhanced in the presence of the phosphodiesterase V inhibitor, zaprinast, supporting the involvement of cGMP accumulation in this effect. In addition, we have also found that the relaxant activity of both ANP and sodium nitroprusside was enhanced in the presence of the phosphodiesterase IV inhibitor, rolipram, suggesting the involvement of cAMP accumulation in this effect. Rolipram (3 μM) is a highly selective inhibitor of the phosphodiesterase IV and it is unlikely, therefore, that the enhancement of the relaxant activity of ANP and sodium nitroprusside was due to non-specific inhibition of phosphodiesterase V activity. In guinea pig trachea, intracellular concentrations of cAMP and cGMP are also determined by their rate of hydrolysis catalyzed by phosphodiesterase III. In this airway preparation, the spasmolytic activity of sodium nitroprusside has been previously shown to be enhanced by rolipram (Turner et al., 1994). This effect of rolipram has been explained by the inhibitory action of cGMP on the hydrolysis of cAMP by phosphodiesterase III. Phosphodiesterase III activity is inhibited by low concentrations of cGMP (Beavo, 1988). The accumulation of intracellular cGMP stimulated by sodium nitroprusside can therefore inhibit phosphodiesterase III and the addition of rolipram causes a more complete inhibition of total cAMP hydrolysis leading to an increase in cAMP content and, therefore, to the enhancement of the relaxant activity of sodium nitroprusside. Since ANP also induces an increase in intracellular cGMP through stimulation of particulate guanylyl cyclase, the enhancement of the relaxant effect of ANP by rolipram in guinea pig trachea may similarly be explained by the additive inhibitory effect on cAMP hydrolysis of the inhibition of phosphodiesterase III activity by cGMP and of inhibition of phosphodiesterase IV by rolipram.

4.4. Effect of selective K^+ channel blockers on ANP-induced relaxation

In guinea pig trachea, the relaxant action of ANP is attenuated in preparations precontracted by highly elevated

extracellular K^+ suggesting that part of the relaxation could be mediated by the opening of K^+ channels (Watanabe et al., 1990; Thirstrup et al., 1997).

We and others have previously reported that the relaxation of guinea pig trachea induced by activators of the soluble form of guanylate cyclase (sodium nitroprusside, SIN-1 (3-morpholinosydnonimine-N-ethylcarbamide)) is inhibited by charybdotoxin and iberiotoxin, two selective blockers of large-conductance Ca^{2+}-activated K^+ channels (Jones et al., 1990, 1993; Corompt et al., 1998). These results suggest that the opening of large-conductance Ca^{2+}-activated K^+ channels by the elevation of intracellular cGMP is involved in the relaxant effects of these activators. In this study, iberiotoxin (100 nM) markedly inhibited the relaxant effect of ANP suggesting also that the opening of large-conductance Ca^{2+}-activated K^+ channels was involved in the relaxant action of this peptide. Our results are in accordance with earlier findings on the inhibitory effect of iberiotoxin on ANP-induced relaxation in guinea pig trachea contracted with histamine (without phosphoramidon) (Thirstrup et al., 1997) or at spontaneous tone (without phosphoramidon) (Mikawa et al., 1997).

In addition, the relaxation induced by urodilatin (a natriuretic peptide closely related to ANP) in bovine bronchi precontracted with methacholine is markedly attenuated by apamin, a selective blocker of small-conductance Ca^{2+}-activated K^+ channels (Nally et al., 1995). Using the same maximal concentration of apamin (1 μM), we did not observe any significant inhibition of ANP-induced relaxation in guinea pig trachea precontracted with histamine. This result extends the previous observation that apamin at a lower concentration (10^{-7} M) did not alter the ANP-induced relaxation in guinea pig trachea at spontaneous tone (Mikawa et al., 1997). This is also in agreement with the absence of effect of apamin (1 μM) on the relaxation to the NO donor SIN-1 in guinea pig trachea contracted with histamine (Ellis and Conanan, 1994). Therefore, at variance with the results in bovine airway smooth muscle preparation, our results do not support the involvement of small-conductance Ca^{2+}-activated K^+ channels in the relaxation of guinea pig trachea in response to ANP.

ANP-induced activation of K_{ATP} has been demonstrated in *Xenopus* oocytes (Sakuta et al., 1994). With respect to airway smooth muscle, blockade of K_{ATP} with glipizide has been reported to increase weakly the apparent affinity of ANP and to decrease the maximal relaxation to ANP in histamine-contracted guinea pig trachea (Thirstrup et al., 1997). This inhibitory effect of glipizide has been ascribed to relaxant properties of this drug (Thirstrup et al., 1997). However, tolbutamide, a K_{ATP} blocker, significantly attenuated the relaxation induced by urodilatin in isolated bovine bronchi (Nally et al., 1995) suggesting that K_{ATP} may be involved in the relaxation to natriuretic peptides. We did not find any alteration of the relaxant effect of ANP in the presence of glibenclamide (10 μM) in histamine-contracted guinea pig trachea as previously reported in this preparation at spontaneous tone (Mikawa et al., 1997). In addition, glibenclamide has been previously shown to have no effect on NO donors-induced relaxation of guinea pig trachea precontracted with histamine (Bialecki and Stinson-Fisher, 1995) or with metacholine (Vaali et al., 1998). As a whole, K_{ATP} channels do not seem to be involved in the relaxation to ANP in guinea pig trachea.

However, the activation of K_{ATP} by ANP has been observed after the blockade of BKCa in cultured vascular smooth muscle cells from rat aortae (Kubo et al., 1994). In isolated human cerebral arteries, it has also been shown that a combination of K^+ channel blockers that possess selectivity for different channels altered the relaxant effect of NO donors whereas no alteration was observed when only one K^+ channel blocker was used, suggesting that K^+ channels may function in a redundant manner and compensate for each other (Hempelmann et al., 2000). In comparison with the inhibitory effect of large-conductance Ca^{2+}-activated K^+ channels, blockade with iberiotoxin, the combination of iberiotoxin and glibenclamide did not, however, inhibit further the relaxation to ANP in guinea pig trachea (data not shown). This latter result does not support a positive interaction between large-conductance Ca^{2+}-activated K^+ channels and K_{ATP} in the relaxation induced by ANP.

4.5. Effect of ANP and ANP-(4–23) on cAMP accumulation- and relaxation-induced by forskolin

In different cell membrane preparations, ANP binding to the ANP_C receptor causes a reduction in adenylyl cyclase activity through an inhibitory guanine-nucleotide binding regulatory protein, leading to a decrease in cAMP levels (Anand-Srivastava et al., 1990; Palaparti et al., 2000). ANP-(4–23) and other ring-deleted analogs of ANP which interact with only ANP_C receptors inhibit cAMP accumulation in membrane preparations from several rat tissues (Anand-Srivastava et al., 1984; Anand-Srivastava and Cantin, 1986; Anand-Srivastava et al., 1990; Savoie et al., 1995; Palaparti et al., 2000). In the present study, neither ANP nor ANP-(4–23) had any effect on basal cAMP content in guinea pig trachea. This result is in keeping with a previous report showing the lack of inhibitory effect of ANP (up to 10^{-5} M) on the cAMP content in unstimulated guinea pig trachea (Watanabe et al., 1990). In contrast, cAMP accumulation in response to forskolin was reduced when tracheal smooth muscle was exposed to ANP or ANP-(4–23). The two peptides inhibited the forskolin-stimulated cAMP production by about 40–45%. This level of inhibition is in keeping with the results in heart and vascular cell preparations (Anand-Srivastava et al., 1984; Anand-Srivastava and Cantin, 1986; Savoie et al., 1995; Palaparti et al., 2000). In most tissues, the inhibitory effect of ANP on cAMP content is explained by the inhibition of adenylyl cyclase. However, in human skin fibroblasts, ANP reduced cAMP content by activating

a phosphodiesterase rather than by inhibiting adenylate cyclase (Lee et al., 1988). In the present study, the inhibition of forskolin-stimulated cAMP accumulation by ANP or ANP-(4–23) in guinea pig trachea was observed in the presence of a phosphodiesterase inhibitor (IBMX). In this airway tissue, unlike the human skin fibroblast, the inhibitory effect of ANP and ANP-(4–23) is therefore not related to the activation of a phosphodiesterase and is related likely to adenylyl cyclase inhibition. This result provides, for the first time, strong evidence that ANP_C receptors are functional in guinea pig airway smooth muscle and extends the preliminary observation in rat lung plasma membranes of ANP-induced inhibition of forskolin-stimulated adenylyl cyclase activity (Resink et al., 1988).

It was important to determine whether the inhibitory effect of ANP and ANP-(4–23) was limited to the biochemical action of forskolin on cAMP or whether it was associated to an inhibitory effect on the mechanical response. We have found that the forskolin-induced relaxation of guinea pig trachea was not inhibited in the presence of ANP and ANP-(4–23). The relationships between elevation in cAMP content, activation of cAMP-dependent protein kinase and relaxation of airway smooth muscle are indeed not simple ones and the relationship between elevation in cAMP content and relaxation of airway smooth muscle may differ with respect to the agonist used to stimulate adenylyl cyclase (Zhou et al., 1992). Forskolin must induce a greater increase in cAMP content compared with isoproterenol to cause the same degree of relaxation of canine trachealis. One explanation for this observation is that a portion of the cAMP generated in response to forskolin is formed in a functional compartment which lacks access to cAMP-dependent protein kinase (Zhou et al., 1992). However, similarly to forskolin-induced relaxation, isoproterenol-induced relaxation of guinea pig trachea was not inhibited in the presence of ANP and ANP-(4–23). The possible explanations are that in the presence of ANP or ANP-(4–23), forskolin and isoproterenol generated a large enough increase in cAMP content to elicit an optimal activation of cAMP-dependent protein kinase or that these compounds can induce relaxation by mechanisms in addition to the activation of adenylyl cyclase.

In conclusion, the results of this study suggest that at least two types of ANP receptors (ANP_A and ANP_C) are functional in the guinea pig airway smooth muscle. ANP-induced relaxation appears to involve the activation of ANP_A receptors, cGMP accumulation through particulate guanylyl cyclases stimulation and the opening of large-conductance Ca^{2+}-activated K^+ channels. In addition, the activation of ANP_C receptors by ANP or ANP-(4–23) inhibits the cAMP accumulation in response to forskolin but this inhibitory effect is not sufficient to alter the relaxation induced by either forskolin or isoproterenol, an adenylyl cyclase activator acting through β-adrenoceptor stimulation.

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