

Effect of a novel PACAP-27 analogue on muscarinic airway responsiveness in guinea-pigs *in vivo*

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ABSTRACT: A recent study showed that the novel pituitary adenylate cyclase-activating peptide (PACAP)-27 analogue [Arg^{15,20,21}, Leu¹⁷]-PACAP-27-Gly-Lys-Arg-NH₂ causes sustained airway smooth muscle relaxation *in vitro*. This study examined whether this analogue also has bronchoprotective effects, by inhibiting muscarinic airway responsiveness *in vivo*.

Total lung resistance was measured in anaesthetized, tracheostomized and ventilated guinea-pigs. Increasing doses of acetylcholine were given *i.v.* once before and thereafter repeatedly each hour after intratracheal instillation of either the PACAP-27 analogue or the clinical β_2 -agonist bronchodilator salbutamol. Mean arterial blood pressure (MAP) was monitored to detect cardiovascular side-effects.

Both the PACAP-27 analogue and salbutamol significantly attenuated the airway responsiveness to acetylcholine. The total inhibitory effect of the PACAP-27 analogue (350 nmol) corresponded to that of salbutamol (35 nmol). The inhibitory effect of salbutamol (35 nmol) peaked during the second hour and disappeared prior to 5 h after administration. In contrast, the corresponding effect of the analogue (350 nmol) gradually increased and peaked during the fifth hour after administration, whereas it did not fade during the observation period. Both the PACAP-27 analogue (350 nmol) and salbutamol (35 nmol) produced a transient decrease in MAP within 6 min after administration.

In conclusion, the novel pituitary adenylate cyclase-activating peptide-27 analogue has bronchoprotective properties, by decreasing muscarinic airway responsiveness in guinea pigs *in vivo*. The time course of its effect is compatible with a more sustained duration of action compared with salbutamol.

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The pituitary adenylate cyclase-activating peptides (PACAPs) are vasoactive intestinal peptide (VIP)-like neuropeptides existing in a 38 and a 27-amino acid residue form [1, 2], named PACAP-38 and PACAP-27, respectively, with an identical 1–27 amino acid sequence. Immunoreactivity for PACAPs has been demonstrated close to smooth muscle in airways from humans and other mammals such as the guinea-pig [3–5]. It has also been shown that PACAP-38 or PACAP-27 relaxes guinea-pig and rabbit airway smooth muscle, at least in part by increasing adenosine 3',5'-cyclic monophosphate (cAMP) *in vitro* [6, 7]. *In vivo*, inhaled PACAP-27 or PACAP-38 causes significant bronchodilation with a rapid onset of action without severe cardiovascular side-effects in guinea-pigs [8, 9]. Recently, it has been reported that a novel amino and carboxy-terminally modified PACAP-27 molecule, [Arg^{15,20,21}, Leu¹⁷]-PACAP-27-Gly-Lys-Arg-NH₂, relaxes primate and guinea-pig airway smooth muscle *in vitro* with a more sustained duration of action than unmodified PACAP-27 [10]. Until now, this novel PACAP-27 analogue has not been tested *in vivo*.

The aim of the present study was to determine whether airway administration of this novel PACAP-27 analogue in-

hibits muscarinic airway responsiveness *in vivo* and if so, what the time course of this bronchoprotective effect is compared with the bronchodilating β_2 -agonist salbutamol. The present study also examined whether or not the PACAP-27 analogue causes significant cardiovascular side-effects.

Materials and methods

Animal preparation

This study was approved by the Animal Study Ethics Committee at Göteborg University. Male Dunkin-Hartley guinea-pigs (400–600 g) were used. The animals were anaesthetized with 1 mL·kg⁻¹ of a 3:2 mixture of ketamine (50 mg·mL⁻¹) and xylazine (20 mg·mL⁻¹) by intramuscular injection. Additional doses were given as required to maintain an adequate level of anaesthesia, as demonstrated by no withdrawal response to foot-pad pinching. The animals were placed on a heated blanket (Harvard model 50-7061; Harvard Apparatus, Edenbridge, UK) to maintain body temperature at approximately 38°C.

The left carotid artery was cannulated and the catheter filled with heparin-saline (10 U·mL⁻¹) and connected to a pressure transducer (model P23XL; Viggo-Spectramed, Helsingborg, Sweden) to monitor the mean arterial blood pressure (MAP) throughout the experiments. Another polyethylene catheter was inserted into the right external jugular vein for the administration of drugs. A tracheal cannula (10 mm length and 2.7 mm outer diameter) was inserted into the upper cervical trachea through a tracheostomy, s-ecured with a suture and connected to a constant volume mechanical ventilator (Harvard model 50-1718; Harvard Apparatus). Animals were placed in a supine position at an angle of 20°, with the head at an elevated level to allow the instilled drug to reach the lower airways. A tidal volume of 10 mL·kg⁻¹ and a respiratory frequency of 60 breaths·min⁻¹ were used. The ventilatory circuit had a total volume of 18 mL.

Transpulmonary pressure was measured using a differential pressure transducer ($\pm 1,000$ mmH₂O, Model FCO44; Furness Controls, Bexhill, UK), with one side attached to a catheter connected to a side port of the intratracheal cannula and the other side attached to a catheter into the right pleural cavity. Airflow was measured with a pneumotachograph (Model F1L; Mercury Electronics, Glasgow, UK) connected to a transducer (± 20 mmH₂O, Model FCO40; Furness Controls). The signals from the transducers were amplified with an analogue preamplifier (Kungsbacka Mät- & Reglerteknik, Kungsbacka, Sweden), digitized using a 12-bit analogue-digital board (National Instruments, Austin, TX, USA) connected to a Macintosh II computer (Apple Computer, Cupertino, CA, USA) and monitored with customized software (LabView; National Instruments) programmed to calculate lung resistance (RL) instantaneously according to the method of VON NEERGAARD and WIRZ [11].

Study design

Each animal received intratracheal (*i.t.*) instillation of 100 µL of drug or vehicle solution, followed by 1 mL of air through a needle inserted directly into the tracheal lumen *via* the tracheal cannula as described previously [12]. Three different treatment groups were included: the vehicle-treated group (instillation of sterile phosphate-buffered saline (PBS); $n=7$), the PACAP-27 analogue-treated group (instillation of PACAP-27 analogue 350 nmol; $n=4$) and the salbutamol-treated group (instillation of salbutamol hemisulphate 35 nmol; $n=5$). The doses of the PACAP-27 analogue and salbutamol were chosen based on separate dose-response experiments, indicating a significant protective effect, *i.e.* the area under the curve (AUC) for the within-animal shift in the provocative dose of acetylcholine causing a 400% increase in RL above baseline from 1–5 h (AUC_{1–5 h} of $\Delta \log_{10} \text{PD}_{400} \text{RL}$) at these doses (data not shown). Each instillation into the trachea was conducted 30 min after the initial acetylcholine challenge described below.

Muscarinic airway responsiveness

Thirty minutes of equilibration followed the animal preparation described above. An initial acetylcholine challenge was then conducted to determine the airway res-

ponsiveness before drug instillation. Exactly the same procedure was followed for acetylcholine challenge at 1, 2, 3, 4 and 5 h after drug instillation. After measuring baseline RL, four successive *i.v.* bolus injections of acetylcholine (6.25, 12.5, 25 and 50 µg·kg⁻¹) were performed at 5 min intervals. After each injection, RL and MAP were recorded every 15 s over 1 min and subsequently every 30 s for another 2 min. The maximum increase in RL induced by each dose of acetylcholine was measured and this maximum occurred within 30 s after the administration of acetylcholine. In exceptional cases, when transpulmonary pressure exceeded 50 cmH₂O, the challenge was stopped to prevent animal mortality. At 3 min after each acetylcholine injection, lungs were hyperinflated once as described below, then RL returned towards the baseline. When necessary, the tracheal cannula was cleared of secretions by suction with a 10 mL syringe through a polyethylene catheter, positioned inside the tracheal cannula, between the acetylcholine challenges. After drug instillation, RL and MAP were recorded every 15 s over 1 min and subsequently every 30 s for another 5 min. Six min after instillation, the lungs were hyperinflated once with twice the tidal volume, by manually blocking the outflow of the ventilator.

Materials

Acetylcholine chloride and salbutamol hemisulphate were purchased from Sigma Chemical Co. (St Louis, MO, USA), ketamine hydrochloride from Parke-Davis (Barcelona, Spain), xylazine chloride from Bayer Sverige (Göteborg, Sweden) and heparin sodium from Løvens Kemiske Fabrik (Ballerup, Denmark). The PACAP-27 analogue [10] was synthesized by Ito Ham Central Research Institute Co. (Ibaraki, Japan). Its amino acid sequence has been described elsewhere [10]. Both the PACAP-27 analogue and salbutamol were dissolved in sterile PBS. Acetylcholine was dissolved in sterile saline.

Statistical analysis

The provocative dose of acetylcholine causing a 400% increase in RL above baseline (PD₄₀₀ RL), was calculated by linear interpolation of the dose-response curve in each animal. PD₄₀₀ RL values were log₁₀ transformed prior to analysis. To evaluate the change in PD₄₀₀ RL the within-animal shift, $\Delta \log_{10} \text{PD}_{400} \text{RL}$ (the log₁₀PD₄₀₀ RL at each acetylcholine challenge minus the log₁₀PD₄₀₀ RL at the initial acetylcholine challenge) was calculated. Furthermore, the AUC_{1–5 h} of $\Delta \log_{10} \text{PD}_{400} \text{RL}$ during the observation period was calculated to evaluate the total effect of treatment. MAP was analysed in two ways: the peak change within 6 min after instillation *i.t.* of drugs was calculated as per cent of baseline MAP prior to drug instillation *i.t.* (% baseline) and the change in MAP was also calculated as AUC to assess the total effect throughout the observation period. Unless otherwise stated, data are reported as the geometric mean \pm SEM. Analysis of variance (ANOVA) was used to determine any significant variance among groups. If a significant variance was found, the Fischer's prevented least significant difference test (Fischer's PLSD) was subsequently used to determine the significant differences between individual groups. A $p \leq 0.05$ was considered significant.

Results

Lung resistance and muscarinic airway responsiveness at baseline

The (mean \pm SD) baseline RL prior to the initial acetylcholine challenge was 0.18 ± 0.01 cmH₂O·mL⁻¹·s (n=4) in guinea-pigs receiving the PACAP-27 analogue (350 nmol), 0.17 ± 0.04 cmH₂O·mL⁻¹·s (n=5) in guinea-pigs receiving salbutamol (35 nmol) and 0.20 ± 0.02 cmH₂O·mL⁻¹·s (n=7) in guinea-pigs receiving vehicle; these values were not significantly different (ANOVA: $F=1.47$, $p=0.26$). The *i.t.* instillation of bronchodilators and vehicle did not alter this baseline RL (data not shown).

Figure 1 shows the initial response to cumulatively increasing doses of acetylcholine in each respective treatment group. The (mean \pm SEM) initial reactivity to acetylcholine (log₁₀PD₄₀₀ RL) was 1.31 ± 0.14 prior to the PACAP-27 analogue (n=4), 1.19 ± 0.13 prior to salbutamol (n=5) and 1.19 ± 0.07 prior to vehicle (n=7). This parameter did not differ significantly (ANOVA: $F=0.92$, $p=0.42$) between groups.

Change in muscarinic airway responsiveness during repeated acetylcholine challenge

At the initial acetylcholine challenge in the vehicle-treated guinea-pigs, the mean PD₄₀₀ RL and log₁₀PD₄₀₀ RL were 15.5 and 1.19 ± 0.07 μ g·mL⁻¹, respectively. During the repeated acetylcholine challenge in these guinea-pigs, the muscarinic reactivity was somewhat increased; the log₁₀PD₄₀₀ RL was moderately but significantly reduced (ANOVA: $F=3.6$, $p=0.02$, $n=7$) compared with the initial log₁₀PD₄₀₀ RL to 1.10 ± 0.07 at 1 h (Fischer's PLSD: $p=0.23$, $n=7$), 1.00 ± 0.06 at 2 h (Fischer's PLSD: $p=0.02$, $n=6-7$), 0.92 ± 0.05 at 3 h (Fischer's PLSD: $p=0.002$, $n=6-7$), 0.93 ± 0.02 at 4 h (Fischer's PLSD: $p=0.003$, $n=6-7$) and 0.97 ± 0.06 at 5 h (Fischer's PLSD: $p=0.01$, $n=5-7$).

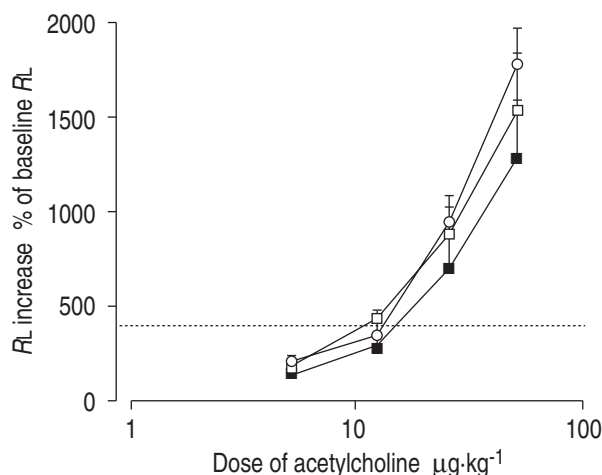


Fig. 1. — Dose-response data presented as increase (%) in lung resistance (RL) above baseline for the initial acetylcholine challenge in each individual treatment group, before the administration of bronchodilators. The intersection with the dashed line indicates provocative dose of acetylcholine causing a 400% increase in RL above baseline (PD₄₀₀ RL). Data for groups before treatment with the pituitary adenylate cyclase-activating peptide (PACAP)-27 analogue (350 nmol, ●), salbutamol (35 nmol, ■) and vehicle (○) are presented as mean with SEM (n=4–7 per group).

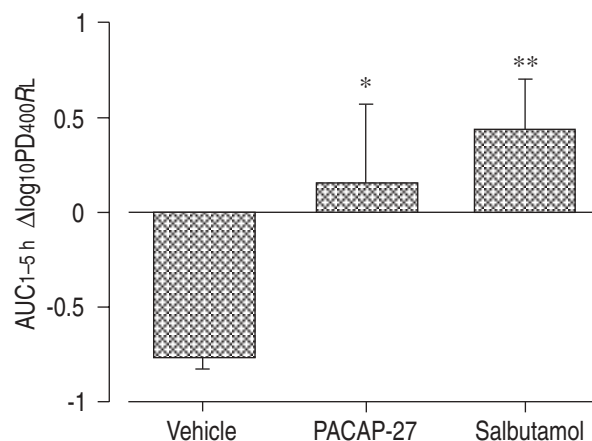


Fig. 2. — Total protective effect over 5 h, assessed as change in the area under the curve (AUC) for the within-animal shift in the provocative dose of acetylcholine causing a 400% increase in lung resistance (RL) above baseline 1–5 h (AUC_{1–5 h} Δlog₁₀PD₄₀₀ RL) for the pituitary adenylate cyclase-activating peptide (PACAP)-27 analogue, salbutamol and vehicle. The AUC_{1–5 h} Δlog₁₀PD₄₀₀ RL differed significantly among groups (analysis of variance: $F=4.47$, $p=0.014$, $n=4-7$). *: $p<0.05$, **: $p<0.01$ versus vehicle, according to Fischer's prevented least significant difference test. Data are presented as mean with SEM (n=4–7 per group).

Total and peak effect on muscarinic airway responsiveness

The total protective effect throughout the observation period, reflected by the AUC_{1–5 h} of Δlog₁₀PD₄₀₀ RL, is shown for each treatment in figure 2. The PACAP-27 analogue (350 nmol) increased the AUC_{1–5 h} of Δlog₁₀PD₄₀₀ RL significantly compared with vehicle, as did salbutamol (35 nmol). Compared with the vehicle group, the increase in AUC_{1–5 h} of Δlog₁₀PD₄₀₀ RL caused by the PACAP-27 analogue was approximately 75% of that caused by salbutamol.

The peak effect, reflected by the peak log₁₀PD₄₀₀ RL during the five-hour observation period, was 0.18 ± 0.08 for the PACAP-27 analogue (Fischer's PLSD: $p=0.02$, $n=4$) and 0.37 ± 0.07 for salbutamol (Fischer's PLSD: $p=0.001$, $n=5$), which was significantly different (ANOVA: $F=13.0$, $p=0.001$) from the -0.08 ± 0.05 observed in the vehicle group (n=7).

Time course of the effect on muscarinic airway responsiveness

The PACAP-27 analogue (350 nmol) displayed a slow onset of action; it increased the Δlog₁₀PD₄₀₀ RL significantly from 4 h onwards after *i.t.* instillation, as shown in figure 3. In contrast, salbutamol (35 nmol) displayed a more rapid onset of action; it increased Δlog₁₀PD₄₀₀ RL significantly from 1 h until 4 h after *i.t.* instillation. Salbutamol caused no significant effect at 5 h after *i.t.* instillation.

The different treatment groups differed significantly in time until the peak Δlog₁₀PD₄₀₀ RL (ANOVA: $F=7.1$, $p=0.01$, $n=4-7$). This time was significantly longer for the PACAP-27 analogue (4.5 ± 0.3 h) than for either salbutamol (1.6 ± 0.7 h; Fischer's PLSD: $p=0.005$, $n=4-5$) or vehicle (1.8 ± 0.6 h; Fischer's PLSD: $p=0.006$, $n=4-7$).

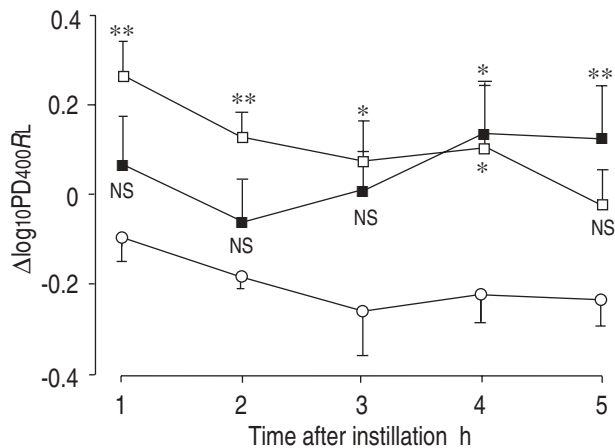


Fig. 3. – Time course of the within-animal shift in the provocative dose of acetylcholine causing a 400% increase in lung resistance above baseline ($\Delta\log_{10}PD_{400} RL$) after administration of the pituitary adenylate cyclase-activating peptide (PACAP)-27 analogue (350 nmol, ■), salbutamol (35 nmol, □) and vehicle (○). The $\Delta\log_{10}PD_{400} RL$ differed significantly among groups over time (analysis of variance: $F=4.6-8.0$, $p \leq 0.03$, $n=4-7$). *: $p \leq 0.05$, **: $p < 0.01$, NS: $p > 0.05$ versus vehicle, according to Fischer's prevented least significant difference test. Data are presented as mean with SEM ($n=4-7$ per group).

Effect on arterial blood pressure

The peak change in MAP is shown for each treatment group in table 1. Figure 4 shows the corresponding time course for MAP. Before the administration of the drugs, there was no significant difference in MAP between groups (table 1). After instillation *i.t.*, there was no significant variance in AUC for the five-hour observation period ($AUC_{1-5 h}$ of MAP) for the groups studied, as demonstrated in table 1. However, both the PACAP-27 analogue and salbutamol caused transient, but statistically significant, hypotension, always within 6 min after *i.t.* instillation (table 1).

Discussion

Intratracheal instillation of the novel PACAP-27 analogue inhibited the airway responsiveness to acetylcholine given *i.v.* in guinea-pigs *in vivo*. The onset of action for

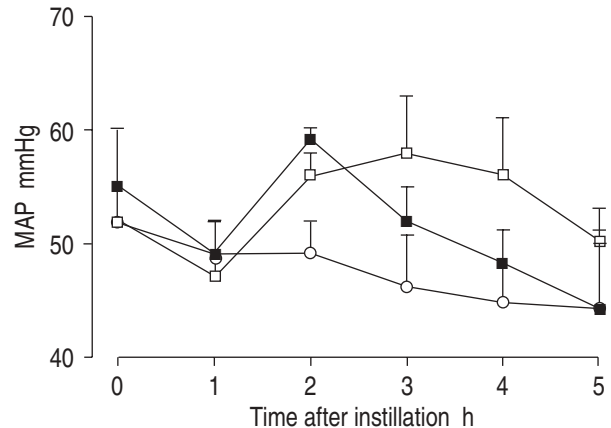


Fig. 4. – Time course of mean arterial blood pressure (MAP) after administration of the pituitary adenylate cyclase-activating peptide (PACAP)-27 analogue (350 nmol, ■), salbutamol (35 nmol, □) and vehicle (○). Data are presented as mean with SEM ($n=4-7$ per group).

this bronchoprotective effect by the PACAP-27 analogue was slower than that of salbutamol given in a dose with a similar total effect over 5 h. The PACAP-27 analogue, but not salbutamol, still caused a significant rightward shift of the dose-response curve to acetylcholine 5 h after administration.

The β_2 -adrenoceptor agonist salbutamol was utilized as a reference bronchodilator. As indicated by the total bronchoprotective effect during 5 h ($AUC_{1-5 h}$ of $\Delta\log_{10}PD_{400} RL$), produced by the PACAP-27 analogue and salbutamol, respectively, the peptide molecule is approximately one-tenth as potent as salbutamol on a molar basis in guinea-pigs. This observation is supported by preliminary experiments (data not shown), which indicated that the total effect produced by 35 nmol of the PACAP-27 analogue corresponds to that of 3.5 nmol of salbutamol, (both instilled *i.t.*). Whereas the original PACAP-27 molecule displays a similar potency to the PACAP-27 analogue in guinea-pig airways *in vitro* [10], it is less potent than salbutamol in this tissue. In contrast, the PACAP-27 analogue is equipotent to salbutamol in primate bronchi *in vitro* [10]. It is, therefore, uncertain whether the PACAP-27 analogue is less potent than salbutamol in primate bronchi.

The time course of the effect of the PACAP-27 analogue on muscarinic airway responsiveness ($\log_{10}PD_{400} RL$) suggests a slower, but more sustained action than that of salbutamol. Because the design of the current study did not allow any assessment of the effect of the PACAP-27 analogue beyond 5 h after administration, it is not possible to make any valid conclusion regarding the time limit of action for this novel peptide molecule in this airway model. It is clear, however, that the effect of the tested dose of salbutamol disappeared after 4 h of administration, confirming the limited duration of action for this bronchodilator. The molecular basis for the sustained effect of the PACAP-27 analogue is not fully understood. In primate bronchi and in the guinea-pig trachea *in vitro*, the long duration of action of the analogue is at least in part due to its resistance to peptidase degradation [10]. The original PACAP-38 molecule, which consists of PACAP-27 plus an additional 11 amino acids, contains basic amino acids at the carboxy-terminal, as does the current PACAP-27 analogue [13]. This basic nature of additional amino acids

Table 1. – Effect of the pituitary adenylate cyclase-activating peptide (PACAP)-27 analogue and salbutamol on mean arterial blood pressure (MAP)

Pretreatment	n	MAP _{baseline} mmHg	Peak change 0–6 min %	AUC _{0–5 h} of MAP mmHg·h ⁻¹
Vehicle	7	53±4 NS ¹	-7±4	250±14 NS ²
PACAP-27 ana- logue 350 nmol	4	56±3 NS ¹	-51±5***	247±90 NS ²
Salbutamol 35 nmol	5	51±3 NS ¹	-34±1***	263±11 NS ²

Data are presented as mean±SEM. Peak change_{0–6 min}: peak change in MAP 0–6 min after intratracheal instillation; AUC_{0–5 h} of MAP: area under the MAP curve 0–5 h after intratracheal instillation. ***: $p < 0.001$ versus vehicle according to Fischer's prevented least significant difference (PLSD) test preceded by analysis of variance (ANOVA): $F=37.6$, $p=0.0001$. NS¹: nonsignificant ANOVA: $F=0.40$, $p=0.68$; NS²: nonsignificant ANOVA: $F=0.46$, $p=0.64$.

may, therefore, contribute to the sustained action of PACAP-38 [9, 14, 15] and the PACAP-27 analogue. Further studies will be required to determine whether or not the size of the novel PACAP-27 analogue (molecular weight 3,600 Da) and its eventual lipophilic properties also contribute to its sustained duration of action.

There were no significant differences in the total effect on blood pressure during 5 h (AUC_{1–5 h} of MAP) after administration of either the PACAP-27 analogue or salbutamol, compared with vehicle during the five-hour observation period. This suggests that the PACAP-27 analogue causes no sustained, severe cardiovascular side-effects at a dose causing significant bronchoprotection. The transient hypotension, which was observed within 6 min after administration of the PACAP-27 analogue or salbutamol, is difficult to attribute to a mechanism involving receptor activation and cAMP elevation, at least for the PACAP-27 analogue. This is because of the substantial difference in time course of the induced hypotension *versus* the bronchoprotective effect of the analogue.

It is likely that the transient hypotension caused by salbutamol and the PACAP-27 analogue is the consequence of a rapid change in airway osmolarity, due to the intratracheal instillation procedure *per se*. This explanation is supported by several observations. Firstly, preliminary experiments showed that at 35 nmol and 350 nmol, respectively (data not shown) the PACAP-27 analogue and salbutamol caused transient hypotension of a similar magnitude, whereas the vehicle, with its low osmolarity, did not. Secondly, a recent comparison of inhaled PACAP-27 and PACAP-38 in guinea-pigs *in vivo* showed no cardiovascular side-effects at doses causing significant bronchodilation [9]; inhalation probably leads to a lower topical concentration than does instillation *i.t.* Third, infusion *i.v.* of the original PACAP-27 does not cause cardiovascular side-effects at doses causing bronchodilation in guinea-pigs *in vivo* [8]. In addition, instillation *i.t.* of allergen causes transient hypotension in unsensitized guinea-pigs, using the same airway model and set-up [16].

In conclusion, this study in guinea-pigs indicates that a novel amino and carboxy-terminal modified pituitary adenylate cyclase-activating peptide-27 analogue produces a significant and sustained inhibitory effect on muscarinic airway responsiveness after airway administration *in vivo*. This novel analogue therefore deserves further evaluation regarding its potential as an inhaled, long-acting bronchodilator.

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