SUSTAINED RELAXANT ACTION OF [Arg^{15,20,21}, Leu¹⁷]-PACAP-27-NH₂ ON CARBACHOL-INDUCED CONTRACTION OF GUINEA-PIG TRACHEAL SMOOTH MUSCLE *IN VITRO*

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ABSTRACT

Duration of relaxant action of an analogue of pituitary adenylate cyclase activating peptide (PACAP)-27, [Arg^{15,20,21}, Leu¹⁷]-PACAP-27-NH₂, was compared with that of PACAP-27 in the smooth muscle isolated from guinea-pig trachea. The relaxant action was examined on the prolonged contracted state of the smooth muscle, which had been stimulated with carbachol (CCh; 0.1 μ M). Addition of the analogue caused concentration-dependent relaxation; both the onset and offset of which were much slower than those with PACAP-27, vasoactive intestinal polypeptide (VIP), and peptide histidine isoleucine (PHI). More than 90% of the maximum relaxation was maintained for 6 h after addition of the analogue, whereas the relaxation induced by PACAP-27, VIP, and PHI reached a maximum by 20 min after the addition and was followed by gradual contraction. Influence of peptidases involved in the smooth muscle preparation on the peptides was examined using 10 μ M captopril and 1 μ M phosphoramidon as peptidase inhibitors. Although the efficacy and duration of the relaxant action with PACAP-27 were significantly potentiated in the presence of peptidase inhibitors, those with the analogue were only slightly affected. A conclusion is drawn that the analogue has sustained relaxant action on CCh-induced contraction of the tracheal smooth muscle, and that this sustained action is, at least in part, due to much lower susceptibility of the analogue to degradation by peptidases, implying an advantage of the analogue in clinical application.

Two endogenous derivatives of pituitary adenylate cyclase activating peptide (PACAP) have been identified, PACAP-27 and PACAP-38 (13, 14). PACAPs has been shown to exist in airways in various species including humans (5, 12). Recent studies in rodents showed that these peptides produce tracheal smooth muscle relaxation *in vitro* (8) and bronchodilation *in vivo* (11), and this relaxation might be mediated by cyclic AMP (1).

PACAP-27 produces weaker cardiovascular effects, when compared with that of PACAP-38 and

vasoactive intestinal polypeptide (VIP) in rats and dogs (7, 15), and of salbutamol in guinea pigs *in vivo* (11). Previously, we have synthesized several PACAP and VIP analogues, including [Arg^{15,20,21}, Leu¹⁷]-PACAP-27-NH₂ (Fig. 1), [Arg^{15,20,21}, Leu¹⁷]-PACAP-27-Gly-Lys-Arg-NH₂, [Arg^{15,20,21}, Leu¹⁷]-VIP and [Arg^{15,20,21}, Leu¹⁷]-VIP-Gly-Lys-Arg-NH₂. Among these analogues, both [Arg^{15,20,21}, Leu¹⁷]-PACAP-27-Gly-Lys-Arg-NH₂ and [Arg^{15,20,21}, Leu¹⁷]-PACAP-27-NH₂ showed significantly sustained relaxant action on the guinea-pig smooth muscle relaxation *in vitro* (9). Subsequently, we reported that [Arg^{15,20,21}, Leu¹⁷]-PACAP-27-Gly-Lys-Arg-NH₂ produced significant concentration-

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Fig. 1 Amino acid sequence of the PACAP-27 and [Arg^{15,20,21}, Leu¹⁷]-PACAP-27-NH₂

dependent and sustained relaxation of tracheal smooth muscle *in vitro* (17, 18). In the present study, we examined the effect of PACAP-27 analogue, [Arg^{15,20,21}, Leu¹⁷]-PACAP-27-NH₂ on the tracheal smooth muscle, and compared them with those of PACAP-27, VIP and peptide histidine isoleucine (PHI) in the guinea-pig tracheal smooth muscle *in vitro*. The effect of peptidases on the duration of action of PACAP-27 analogue was also examined.

MATERIALS AND METHODS

Animals

Male Hartley guinea pigs (Japan SLC) were used. Animals were kept in a temperature-controlled environment with standard food and water freely available. The weight of animals ranged from 400 to 700 g at the time of experiment.

Tissue Preparation

Tracheal tissue preparations were obtained from the guinea pigs after they were anaesthetised by an intraperitoneal injection of sodium pentobarbital (50 mg/ml). Tracheal airway rings were opened longitudinally along the anterior part and connected to steel hooks as strips, as described previously (10). These tracheal preparations were mounted in temperature-controlled (37°C) and oxygenated (95% O₂; 5% CO₂) baths (AMT 100A, Riken Kaihatsu, Tokyo).

Tension Recordings

The tracheal smooth muscle tension was recorded

via an isometric force transducer (RMP 6004, Nihon Koden, Tokyo) connected to a four-channel signal recorder (R-64M, Rikaden, Tokyo). The difference in tension between the pre-contraction induced by CCh (0.1 µM) and the level during a final, maximum theophylline-induced relaxation (1 mM) was regarded as 100% active tension (precontraction; see below).

Experimental Protocol

The inherent tone of the tracheal smooth muscle was initially abolished by adding indomethacin (10 µM) which was added to the Krebs-Ringer solution (mM: NaCl 118; KCl 5.9; CaCl₂ 2.5; MgSO₄ 1.2; NaH₂PO₄ 1.2; NaHCO₃ 25.5 and glucose 5.6). The passive tension was then adjusted to 1.2 g. After a moderate decline during 5 min of equilibration, the tension was readjusted to 1.2 g. Muscarinic precontraction was established after a 45 min equilibration period using Krebs-Ringer solution containing CCh (0.1 µM), causing approximately 50% of the maximum CCh-induced contraction. The induced precontraction was stabilised over 20 min. At the end of the experiment, the remaining active tension was abolished by a maximally effective concentration of theophylline (1 mM) (10, 17).

Non-cumulative concentration response and time course experiments for PACAP-27 and [Arg^{15,20,21}, Leu¹⁷]-PACAP-27-NH₂ served to compare their maximum relaxant effect and potency. The non-cumulative design was chosen because of the relatively slow onset of action of the analogue, when one concentration of drug was used for each tracheal preparation. In the same manner, the relaxant

Fig. 2 Time course of relaxant actions of PACAP-27 (A. upper, $0.03 \mu\text{M}$; middle, $0.1 \mu\text{M}$; lower, $0.3 \mu\text{M}$) and the analogue (B. upper, $0.3 \mu\text{M}$; middle, $1 \mu\text{M}$; lower, $3 \mu\text{M}$) on CCh-induced guinea-pig tracheal smooth muscle contraction. Analysis of the time course of relaxant actions yielded the following values (mean $\pm$ SEM); negative logarithm of the concentration (M) causing 50% of the peak effect ($-\log \text{EC}_{50}$) was 5.68 ± 0.09 for the analogue and 6.46 ± 0.09 for the PACAP-27. The active tension after addition of each bronchodilator concentration is presented as the percentage of the difference between the contraction level induced by preceding stimulation with $0.1 \mu\text{M}$ CCh and the tension in the presence of theophylline (1 mM) (% precontraction). Mean values and SEM ($n = 4-6$) are given. The mean ($\pm$ SD) value of precontraction was $1.58 \pm 0.05 \text{ g}$ and $1.46 \pm 0.09 \text{ g}$, for the analogue and PACAP-27 group, respectively.

effect for the analogue was compared with that of VIP and PHI in equipotent concentrations. The time course of relaxation was recorded during 6 h in each experiment. In addition, 3-h time course experiments in the presence or absence of peptidase inhibitors (captopril, $10 \mu\text{M}$; and phosphoramidon, $1 \mu\text{M}$) were conducted for PACAP-27 and the analogue.

Drugs

Captopril, carbachol, indomethacin, salbutamol (Sigma, St Louis, MO, U.S.A.), sodium pentobarbital (Abbott Laboratories, North Cicago, U.S.A.) and theophylline (Sigma) were purchased as indicated. PACAP-27, PHI, VIP and phosphoramidon were purchased from the Peptide Institute, Osaka.

Statistical Analysis

Data were expressed as mean $\pm$ SEM, unless otherwise stated. Concentration response data were evaluated using Spearman's rank correlation (non-parametric). Multiple statistical comparisons were performed using a one way analysis of variance and Dunnett's multiple range test. Single comparisons were conducted using Student's *t*-test (one- or two-tailed, paired or unpaired when appropriate). *P* values less than 0.05 were considered significant.

RESULTS

Time Course of Relaxant Actions of PACAP-27 and PACAP-27 Analogue on CCh-Induced Tracheal Smooth Muscle Contraction

The time course of relaxant actions of PACAP-27 and the analogue was compared after inducing prolonged contraction of the tracheal smooth muscle with $0.1 \mu\text{M}$ CCh. The relaxation induced by PACAP-27 reached a maximum by 20 min after the addition, and thereafter gradually restored the CCh-induced contraction level (Fig. 2A). In contrast, the relaxation caused by the analogue was substantially prolonged: the times to peak relaxation were $46.2 \pm 0.06 \text{ min}$, $94.2 \pm 0.21 \text{ min}$ and $119.4 \pm 0.10 \text{ min}$ after the addition of the analogue at $0.3 \mu\text{M}$, $1 \mu\text{M}$ and $3 \mu\text{M}$, respectively, and the relaxation was still maintained at a level of more than 90% of its peak value 6 h after the addition (Fig. 2B).

Time Course of Relaxant Actions of VIP, PHI and PACAP-27 Analogue

The sustained relaxant action of the analogue was further confirmed by comparing with short-lived relaxant actions with VIP and PHI. In the preliminary study, the relation between the concentrations of the peptides (VIP and PHI) and their relaxant actions was examined according to the protocol used in our previous paper (17, 18). The concentrations of the VIP and PHI which induced 75% of maximum relaxation of CCh-induced contraction

Fig. 3 Time course of relaxant action of VIP ($0.03 \mu\text{M}$, ■), PHI ($0.1 \mu\text{M}$, ○) and PACAP-27 analogue ($1 \mu\text{M}$, ●) on CCh-induced contraction of guinea-pig tracheal smooth muscle. The active tension after addition of each peptide is shown as the percentage (mean $\pm$ SD) of the difference between the contraction level induced by preceding stimulation with $0.1 \mu\text{M}$ CCh and the tension in the presence of theophylline (1 mM) (% precontraction). * $P < 0.05$ (unpaired Student's *t*-test; $n = 4-5$). The mean ($\pm$ SD) value of precontraction was $0.99 \pm 0.20 \text{ g}$, $1.13 \pm 0.12 \text{ g}$ and $1.00 \pm 0.19 \text{ g}$, for the group treated with the PACAP-27 analogue, PHI and VIP, respectively.

in the tracheal smooth muscle were $0.03 \mu\text{M}$ and $0.1 \mu\text{M}$, respectively. The relaxation induced by $0.03 \mu\text{M}$ VIP or $0.1 \mu\text{M}$ PHI reached a maximum by 15 min after the addition, and thereafter gradually restored the CCh-induced contraction level. The relaxation with the analogue ($1 \mu\text{M}$) lasted much longer (Fig. 3).

Influence of Peptidases on the Relaxant Actions of PACAP-27 and the Analogue

A possible mechanism of the long-lasting relaxant action of the analogue was explored using peptidase inhibitors, captopril and phosphoramidon. The relaxant response to the analogue remained almost unchanged in the presence of $10 \mu\text{M}$ captopril and $1 \mu\text{M}$ phosphoramidon (Fig. 4A), whereas the magnitude and duration of the response to PACAP-27 were significantly potentiated (Fig. 4B).

DISCUSSION

The present study demonstrated the long-lasting relaxant action of [Arg^{15,20,21}, Leu¹⁷]-PACAP-27-NH₂ on the CCh-induced contraction of guinea-pig tracheal smooth muscle *in vitro*. The duration of

Fig. 4 Time course of relaxant actions of the analogue (A, $1 \mu\text{M}$) and PACAP-27 (B, $0.1 \mu\text{M}$), with (●) and without (▲) peptidase inhibition with captopril ($10 \mu\text{M}$), and phosphoramidon ($1 \mu\text{M}$) on the CCh-induced guinea-pig tracheal smooth muscle contraction. The active tension after addition of each bronchodilator is presented as the percentage of the difference between the contraction level induced by preceding stimulation with $0.1 \mu\text{M}$ CCh and the tension in the presence of theophylline (1 mM) (% precontraction). * $P < 0.05$, ** $P < 0.01$ (unpaired Student's *t*-test; $n = 5$). The mean ($\pm$ SD) value of precontraction was $1.39 \pm 0.07 \text{ g}$, $1.29 \pm 0.11 \text{ g}$ for the group treated with PACAP-27 and the analogue, respectively.

relaxant action was comparable to that with [Arg^{15,20,21}, Leu¹⁷]-PACAP-27-Gly-Lys-Arg-NH₂, (17, 18), suggesting that the C-terminal Gly-Lys-Arg sequence may not be required for the sustained action. This implies that introduction of Arg residues at positions 15, 20 and 21 in the PACAP-27 molecule may be more important for the duration of the action than the C-terminal extension.

It is well known that VIP and PACAP-27 act via

similar receptors (2, 3). Previous studies showed that PACAP-27 has potency almost identical to that of VIP, indicating that PACAP receptor type II may be similar to or identical with the VIP receptor in the airway smooth muscle (2, 6). However, in contrast to PACAP-27, VIP produces cardiovascular side effects when given systemically in doses causing bronchodilation, and inhaled VIP produces weak bronchodilation with short duration *in vivo* and *in vitro* (4, 16). The present study *in vitro* demonstrated that the relaxation produced by VIP disappeared within less than half an hour after administration, whereas the relaxation induced by the present analogue lasted over 6 h after its administration.

Because we detected the relaxant action of PACAP-27 and the analogue after inducing prolonged contraction of the tracheal smooth muscle with carbachol, known to act postjunctionally on the muscarinic receptor, our data on PACAP-27 and its analogue indicate a direct relaxant effect of these peptides on smooth muscle, presumably via an increase in intracellular cAMP (1).

Our data suggest that the long-lasting action of the analogue may be due in part to greatly lowered susceptibility of this compound to degradation by neutral endopeptidase and/or angiotensin converting enzyme.

In conclusion, this study indicates that the novel PACAP-27 analogue [Arg^{15,20,21}, Leu¹⁷]-PACAP-27-NH₂ produces sustained tracheal smooth muscle relaxation, at least in part, due to insusceptibility to intrinsic peptidases. For these reasons, this novel PACAP-27 analogue deserves further characterization as a long-lasting bronchodilator in human airway *in vivo*.

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