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Pituitary adenylate cyclase activating peptide mediates inhibitory nonadrenergic noncholinergic relaxation

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Abstract

We investigated the contribution of pituitary adenylate cyclase activating peptide (PACAP) to inhibitory nonadrenergic noncholinergic (inhibitory-NANC) relaxation of tracheal smooth muscle in cats. We also investigated the roles of vasoactive intestinal peptide (VIP) and nitric oxide (NO) on this function. Smooth muscle strips prepared from feline trachea were precontracted with 1 μM serotonin, and inhibitory-NANC relaxation was induced by electrical-field stimulation in the presence of atropine and propranolol. PACAP-(6–38) (a selective antagonist of PACAP; 1, 3 and 10 μM), VIP-(10–28) (a selective antagonist of VIP; 1, 3 and 10 μM) and N^{ω}-nitro-L-arginine methyl ester (L-NAME, a selective NO synthase inhibitor; 3, 10 and 30 μM) each partially but significantly attenuated the amplitude of inhibitory-NANC relaxation. The effects of PACAP-(6–38) and VIP-(10–28) were additive. Addition of PACAP-(6–38) and/or VIP-(10–28) further attenuated relaxation in the presence of L-NAME. These results suggest that PACAP, VIP and NO contribute to the relaxation induced by inhibitory-NANC in tracheal smooth muscle in cats, and that they mediate this relaxation via different pathways.

Keywords: PACAP (pituitary adenylate cyclase activating peptide); VIP (vasoactive intestinal peptide); Nitric oxide (NO); Nonadrenergic noncholinergic (NANC) relaxation, inhibitory; Smooth muscle, tracheal; (Cat)

1. Introduction

Pituitary adenylate cyclase activating peptide (PACAP), first isolated from ovine hypothalamus, is regarded as a novel member of the secretin–glucagon–VIP peptide family (Miyata et al., 1989; Christophe, 1993).

In enteric systems, PACAP, like vasoactive intestinal peptide (VIP), is reported to be a transmitter of inhibitory nonadrenergic noncholinergic (inhibitory-NANC) nerves (Miyata et al., 1989; Sundler et al., 1992; Christophe, 1993). The roles of PACAP and VIP have recently been clarified using selective antagonists (Turner et al., 1986; Robberecht et al., 1992; Vandermeers et al., 1992). One indicative finding is that inhibitory transmission by PACAP is sensitive to apamin, a small conductance Ca^{2+}-activated K^+ channel blocker, while that by VIP is not (Schworer et al., 1993; Jin et al., 1994).

PACAP-immunoreactive nerve fibers are reported to occur around airway muscle bundles in guinea pigs, sheep and humans (Cardell et al., 1991; Uddman et al., 1991; Luts et al., 1993). Further, exogenous administration of PACAP caused prolonged relaxation of tracheal smooth muscle in guinea pigs (Araki and Takagi, 1992; Conroy et al., 1995). These previous findings raised the possibility that, in addition to its role in the enteric system, PACAP may also contribute to airway smooth muscle relaxation as a mediator of inhibitory-NANC. To date, however, the role of PACAP in inhibitory-NANC relaxation of airways has not been investigated.

We therefore studied the contribution of PACAP and VIP to inhibitory-NANC relaxation caused by electrical field stimulation of tracheal smooth muscle in cats by a direct method using the selective antagonists PACAP-(6–38)and VIP-(10–28). We also investigated the contribution of nitric oxide (NO) to inhibitory-NANC relaxation in relation to PACAP and VIP.

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2. Materials and methods

2.1. Tissue preparations

Male cats weighing approximately 3 kg were anaesthetized with pentobarbitone sodium (50 mg/kg i.m.) and then killed by bleeding. The trachea was dissected free and trimmed of connective tissues and fat. A tracheal segment was opened longitudinally along the anterior aspect, and a dorsal strip of transverse smooth muscle was separated from the cartilage and endothelial tissue. This smooth muscle tissue was then cut into a strip measuring 4.0 mm in length and 1.0 mm in width.

To measure mechanical changes, the strip was mounted vertically in a 2-ml organ bath and perfused with Krebs solution aerated with 95% O_2 and 5% CO_2 and kept at 37°C. Ionic composition of the Krebs solution was as follows (mM): Na^+, 137.5; K^+, 5.9; Mg^{2+}, 1.2; Ca^{2+}, 2.6; HCO_3^-, 15.5; $H_2PO_4^-$, 1.2; Cl^-, 134.3; glucose, 11.5. pH was maintained at 7.3–7.4. One end of the strip was tied by fine silk thread to a mechanotransducer (Model TB-612T, Nihon Koden, Tokyo, Japan) and the other was connected to a hook at the bottom of the bath. Isometric tension was then measured and recorded continuously by pen recorder (Model LR4120, Yokogawa, Japan). A resting force of 0.5 g was chosen to obtain maximal contraction with 128 mM K^+.

2.2. Study design

The smooth muscle strip was precontracted with 1 μM serotonin in the presence of 10 μM atropine, 3 μM propranolol and 1μM indomethacin. Inhibitory-NANC relaxation was induced by electrical field stimulation. Four series of electrical field stimulation (1 ms, 30 V, 20 Hz) were applied every 15 min, each composed of three stimuli (10, 30 and 50 pulses) in 5-min intervals.

2.3. Drugs

Drugs used were serotonin, N^{ω}-nitro-L-arginine methyl ester (L-NAME), atropine and indomethacin (Sigma, St. Louis, MO, USA); VIP-(10–28) and PACAP-(6–38) (Peptide Institute, Osaka, Japan); and propranolol (Nacalai Tesque, Kyoto, Japan).

2.4. Statistics

Data are expressed as arithmetic mean ± S.E. Statistical significance was determined using Student's paired *t* test. Probabilities of less than 5% ($P < 0.05$) were considered significant.

3. Results

3.1. Reproducibility of inhibitory-NANC relaxation induced by repetitive electrical-field stimulation

Fig. 1 shows muscle relaxation on repetitive stimulation of inhibitory-NANC in tracheal smooth muscle in cats. Inhibitory-NANC relaxation did not change significantly throughout the four series of stimulations, indicating that the amplitude of relaxation obtained with this protocol was reproducible.

3.2. Effect of PACAP-(6–38) on inhibitory-NANC relaxation

To investigate the possible role of PACAP on inhibitory-NANC relaxation, we next observed the effect of PACAP-(6–38), a selective antagonist of PACAP, on exposure 15 min prior to and throughout the second to fourth series of electrical field stimulation. Addition of PACAP-(6–38) by itself did not alter muscle tension even with the highest concentration (10 μM).

As shown in Fig. 2, 1, 3 and 10 μM PACAP-(6–38) partially but significantly attenuated inhibitory-NANC relaxation, respectively, suggesting the partial contribution of PACAP on inhibitory transmission in this tissue.

3.3. Effect of VIP-(10–28) on inhibitory-NANC relaxation

We also examined the possible role of VIP on inhibitory-NANC transmission in feline tracheal preparation

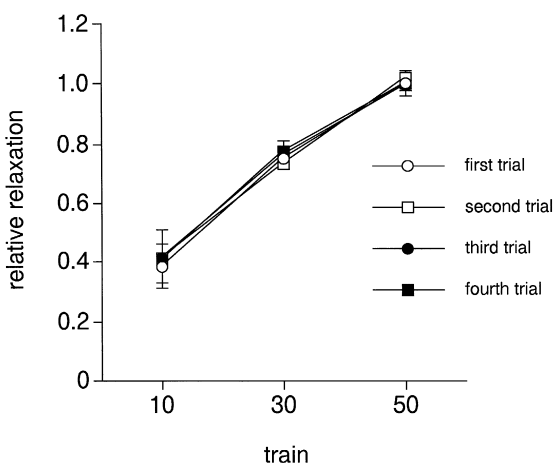

Fig. 1. Reproducibility of inhibitory-NANC relaxation induced by repetitive electrical field stimulation of tracheal smooth muscle in cats. Four series of electrical field stimulation (1 ms, 30 V, 20 Hz) were applied every 15 min. Each was composed of three stimuli (10, 30 and 50 pulses) at 5-min intervals. Vertical axis indicates relative relaxation, in which relaxation induced by the first 50 pulses was taken as 1.0. Open circles, open squares, closed circles and closed squares indicate relative relaxation obtained by the first, second, third and fourth series of stimulations, respectively. Results shown are the mean of five observations with S.E. shown by vertical bars.

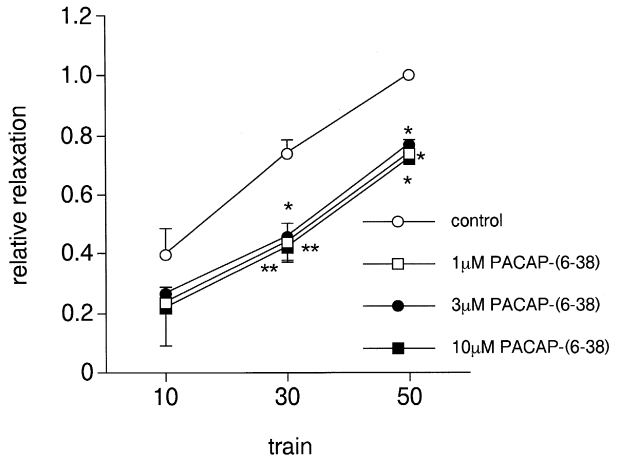

Fig. 2. Effect of PACAP-(6–38) on inhibitory-NANC relaxation of tracheal smooth muscle in cats. Open circles, closed circles, open squares and closed squares indicate relative relaxation observed in the absence and presence of 1, 3 and 10 μM PACAP-(6–38), respectively. Asterisks indicate a significant difference from control (*: $P < 0.05$, **: $P < 0.01$). Results shown are the mean of four observations with S.E. shown by vertical bars.

using the selective antagonist VIP-(10–28) by exposure 15 min prior to and throughout the second to fourth series of electrical field stimulation. Addition of VIP-(10–28) by itself did not alter muscle tension even with the highest concentration (10 μM).

As shown in Fig. 3, the presence of 1, 3 or 10 μM VIP-(10–28) resulted in partial but significant attenuation of inhibitory-NANC relaxation, suggesting that VIP also mediates inhibitory-NANC relaxation in feline tracheal smooth muscle.

3.4. Effect of VIP-(10–28) and PACAP-(6–38) on inhibitory-NANC relaxation

To investigate whether VIP and PACAP mediate muscle relaxation by activating a common pathway, including a common receptor on the muscle cell membrane, we next observed the effect of simultaneous administration of 3 μM VIP-(10–28) and 3 μM PACAP-(6–38) on inhibitory-NANC relaxation.

Fig. 4 shows that addition of PACAP-(6–38) further attenuated the relaxation induced by inhibitory-NANC stimulation, indicating the additive effects of these two antagonists. The result suggests that PACAP and VIP induce muscle relaxation via different mechanisms, at least partially.

3.5. Effect of N^{ω}-nitro-L-arginine methyl ester on inhibitory-NANC relaxation

The possible contribution of NO to inhibitory-NANC relaxation was investigated by observing the effect of N^{ω}-nitro-L-arginine methyl ester (L-NAME), a selective inhibitor of NO synthase. Addition of L-NAME did not alter muscle tension with the concentration lower than 30 μM, while slight increase was observed with 30 μM L-NAME.

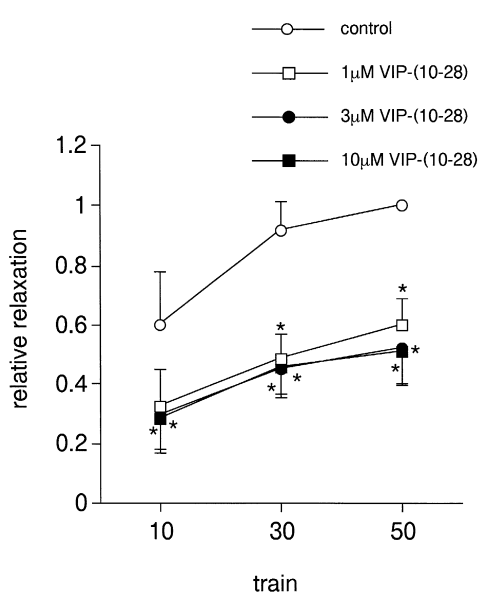

Fig. 3. Effect of VIP-(10–28) on inhibitory-NANC relaxation of tracheal smooth muscle in cats. Open circles, closed circles, open squares and closed squares indicate relative relaxation observed in the absence and presence of 1, 3 and 10 μM VIP-(10–28), respectively. Asterisks indicate a significant difference from control (*: $P < 0.05$). Results shown are the mean of four observations with S.E. shown by vertical bars.

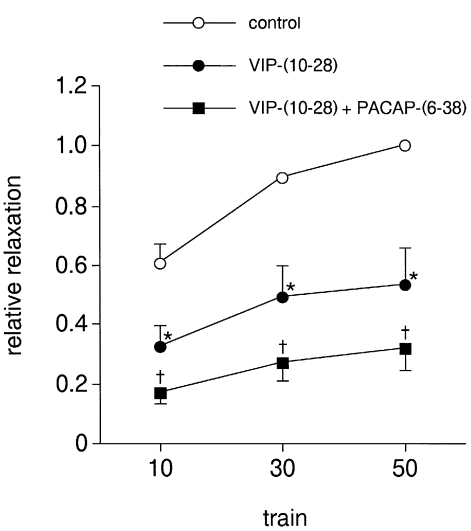

Fig. 4. Effect of VIP-(10–28) and PACAP-(6–38) on inhibitory-NANC relaxation of tracheal smooth muscle in cats. Open and closed circles indicate relative relaxation observed in the absence and presence of 3 μM VIP-(10–28), respectively. Closed squares indicate relaxation observed in the presence of both 3 μM VIP-(10–28) and 3 μM PACAP-(6–38). Asterisks and daggers indicate a significant difference from control (*: $P < 0.05$) and from data obtained in the presence of 3 μM VIP-(10–28) only (†: $P < 0.05$), respectively. Results shown are the mean of four observations with S.E. shown by vertical bars.

As shown in Fig. 5, 3, 10 and 30 μM L-NAME also partially but significantly attenuated inhibitory-NANC relaxation, suggesting that NO also acts as a transmitter in muscle relaxation in feline tracheal smooth muscle.

3.6. Effect of L-NAME and PACAP-(6–38) on inhibitory-NANC relaxation

The inhibitory effect of simultaneous administration of 10 μM L-NAME and 3 μM PACAP-(6–38) was also investigated. As shown in Fig. 6, additional application of PACAP-(6–38) further attenuated inhibitory-NANC relaxation, showing that the effects of these two inhibitors were additive.

3.7. Effect of L-NAME and VIP-(10–28) on inhibitory-NANC relaxation

We next observed the inhibitory effect of simultaneous administration of 10 μM L-NAME and 3 μM VIP-(10–28). Inhibitory effects of L-NAME and VIP-(10–28) on inhibitory-NANC relaxation were also additive (Fig. 7).

3.8. Effect of L-NAME, VIP-(10–28) and PACAP-(6–38) on inhibitory-NANC relaxation

We then investigated the inhibitory effect of simultaneous administration of all of three inhibitors used in the present study; i.e. L-NAME, VIP-(10–28) and PACAP-(6–38). Application of 3 μM VIP-(10–28) and 3 μM PACAP-(6–38) in addition to 10 μM L-NAME further attenuated inhibitory-NANC relaxation, indicating that the effects of these three inhibitors were additive. In the

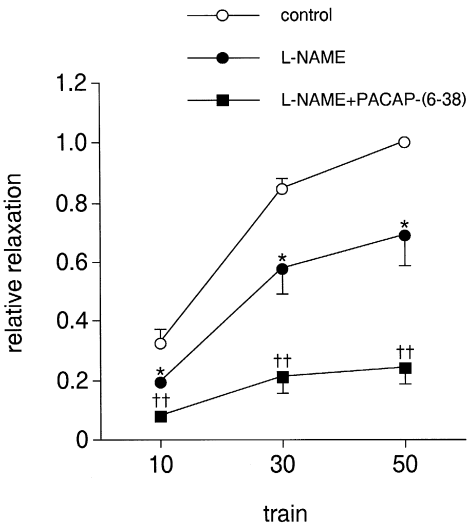

Fig. 6. Effect of L-NAME and PACAP-(6–38) on inhibitory-NANC relaxation of tracheal smooth muscle in cats. Open and closed circles indicate relative relaxation observed in the absence and presence of 10 μM L-NAME, respectively. Closed squares indicate relaxation observed in the presence of both 10 μM L-NAME and 3 μM PACAP-(6–38). Asterisks and daggers indicate significant difference from control (*: $P < 0.05$) and from data obtained in the presence of 10 μM L-NAME only (††: $P < 0.01$), respectively. Results shown are the mean of four observations with S.E. shown by vertical bars.

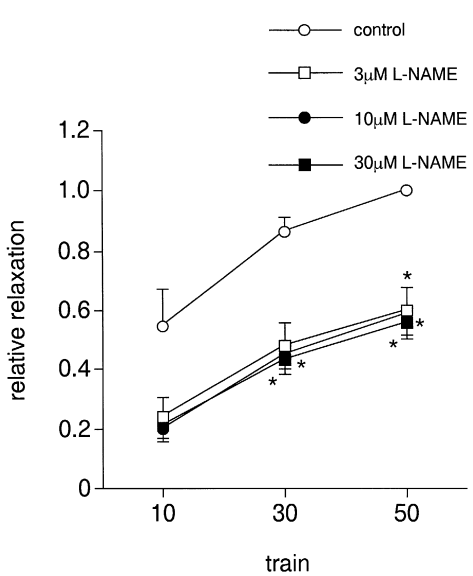

Fig. 5. Effect of N^{ω}-nitro-L-arginine methyl ester (L-NAME) on inhibitory-NANC relaxation of tracheal smooth muscle in cats. Open circles, closed circles, open squares and closed squares indicate relative relaxation observed in the absence and presence of 3, 10 and 30 μM L-NAME, respectively. Asterisks indicate a significant difference from control (*: $P < 0.05$). Results shown are the mean of three observations with S.E. shown by vertical bars.

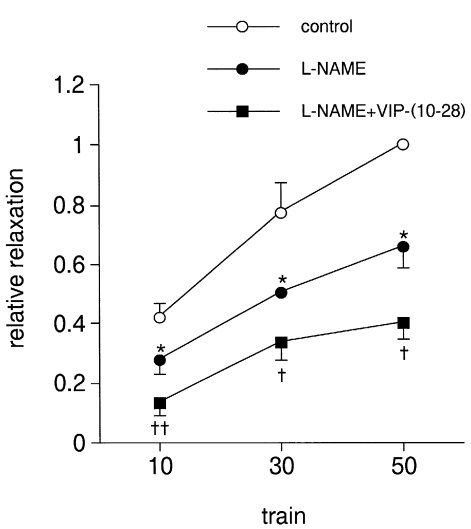

Fig. 7. Effect of L-NAME and VIP-(10–28) on inhibitory-NANC relaxation of tracheal smooth muscle in cats. Open and closed circles indicate relative relaxation observed in the absence and presence of 10 μM L-NAME, respectively. Closed squares indicate relaxation observed in the presence of both 10 μM L-NAME and 3 μM VIP-(10–28). Asterisks and daggers indicate significant difference from control (*: $P < 0.05$) and from data obtained in the presence of 10 μM L-NAME only (†: $P < 0.05$, ††: $P < 0.01$), respectively. Results shown are the mean of four observations with S.E. shown by vertical bars.

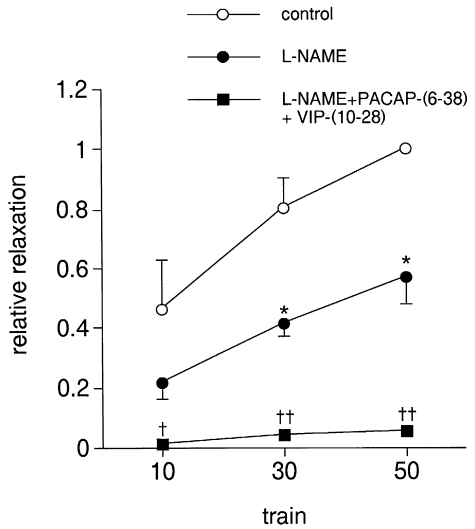

Fig. 8. Effect of L-NAME, PACAP-(6–38) and VIP-(10–28) on inhibitory-NANC relaxation of tracheal smooth muscle in cats. Open and closed circles indicate relative relaxation observed in the absence and presence of 10 μM L-NAME, respectively. Closed squares indicate relaxation observed in the presence of both 10 μM L-NAME, 3 μM PACAP-(6–38) and 3 μM VIP-(10–28). Asterisks and daggers indicate significant difference from control (*: $P < 0.05$) and from data obtained in the presence of 10 μM L-NAME only (†: $P < 0.05$, ††: $P < 0.01$), respectively. Results shown are the mean of four observations with S.E. shown by vertical bars.

presence of three inhibitors, muscle relaxation was almost completely abolished (Fig. 8).

4. Discussion

Our results show that PACAP-(6–38), a selective antagonist of PACAP (Robberecht et al., 1992; Vandermeers et al., 1992), partially but significantly inhibited inhibitory-NANC relaxation of tracheal smooth muscle in cats. This finding, which to date has not been directly documented through the use of selective antagonists in airway systems, suggests the contribution of PACAP on inhibitory-NANC relaxation. The effect on this relaxation of VIP-(10–28) (Turner et al., 1986), a selective VIP receptor antagonist, was similar to that of PACAP-(6–38), suggesting that VIP may also contribute to inhibitory-NANC-induced smooth muscle relaxation, in accordance with the results of previous studies (Jing et al., 1995).

Although competitive antagonists of PACAP and VIP both inhibit inhibitory-NANC relaxation (Turner et al., 1986; Robberecht et al., 1992; Vandermeers et al., 1992), it was considered possible that they bind common receptors for PACAP and VIP. We therefore next discuss whether PACAP and VIP receptor antagonists PACAP-(6–38) and VIP-(10–28) act on separate or a common receptor. cDNAs for three distinct PACAP-VIP receptor subtypes have been cloned (Segre and Goldring, 1993; Harme and Lutz, 1994; Rawlings and Hezareh, 1996): PAC_1 receptor has higher affinity to PACAP than VIP (Pisegna and Wank, 1993; Spengler et al., 1993), whereas the affinities of $VPAC_1$ receptor and $VPAC_2$ receptor are similar (Ishihara et al., 1992; Lutz et al., 1993; Inagaki et al., 1994). In addition, pharmacological findings have suggested the existence of a VIP-selective receptor with low affinity to PACAP (Jin et al., 1994; Ekblad and Sunder, 1997). Simultaneous application of 3 μM PACAP-(6–38) and 3 μM VIP-(10–28) demonstrated that these two antagonists had additive effects, suggesting the contribution of different receptors selective for VIP and PACAP, although an additive effect of two antagonists which bind to a common site may occur when doses are insufficient for maximal effect. This possibility is unlikely in the present study because the effect of PACAP-(6–38) or VIP-(10–28) on inhibitory-NANC relaxation did not differ between the doses of 1, 3 and 10 μM, suggesting that these antagonist bind to their receptors maximally in the concentration used in the present study. Further support for this lack of summation at the same site comes from binding assay studies, which showed maximal binding for both at the concentration of 0.3 to 1 μM (Turner et al., 1986; Robberecht et al., 1992; Vandermeers et al., 1992) and is compatible with the pharmacological findings of the present study. These results therefore strongly suggest the contribution of selective receptors for PACAP and VIP. Further study is needed to confirm the existence of a selective receptor for VIP.

A third candidate for inhibitory-NANC neurotransmitter is NO. Supporting previously findings (Belvisi et al., 1992; Ellis and Undem, 1992), L-NAME, a selective NO synthase inhibitor, partially but significantly attenuated inhibitory-NANC relaxation. Although relaxation in the presence of PACAP-(6–38) and VIP-(10–28), which may be mediated by NO, was not proportional to the degree of inhibition of inhibitory-NANC relaxation by L-NAME in the absence of PACAP-(6–38) and VIP-(10–28), this discrepancy in inhibition rate can be explained by interaction between mediators. Namely, in the enteric system, PACAP released from nerve endings activates NO synthase to increase NO production in smooth muscle cells. NO in turn facilitates the release of PACAP from inhibitory-NANC nerve endings (Murthy and Makhlouf, 1994; Murthy et al., 1997; Grider et al., 1994). Similar interaction between VIP and NO has also been reported (Lilly et al., 1993; Murthy and Makhlouf, 1994; Murthy et al., 1997). When such interaction occurs, antagonists of PACAP and VIP can, in addition to their direct effects on the selective receptors, further attenuate relaxation by decreasing NO production. The inhibitory effect of L-NAME may also include an indirect effect through a decrease in the release of PACAP and VIP from nerve endings. These interactions may explain the overlapping of effects of antagonists in the present study.

Although we cannot rule out such interaction between PACAP and NO or interaction between VIP and NO in the

present study, these neurotransmitters may at least in part be released independently of each other, because further attenuation of relaxation was observed by additional administration of PACAP-(6–38) or VIP-(10–28) in the presence of L-NAME. These findings therefore suggest that the mediators in the present study, PACAP, VIP and NO, are released directly by inhibitory-NANC stimulation, and that in addition to their direct effects they each also facilitate release or production of the other two to enhance relaxation.

Although the present results indicate the contribution of PACAP to inhibitory-NANC relaxation only in cats, the reported existence of PACAP in human airway nerve endings also (Cardell et al., 1991; Uddman et al., 1991; Luts et al., 1993) raises the possibility that PACAP, as well as VIP, may contribute to pathophysiological states in human airway smooth muscle tone such as occur in bronchial asthma.

In conclusion, the present study is the first to report the mediation of PACAP in inhibitory-NANC relaxation in an airway smooth muscle system using its selective antagonist. In cats, at least three different transmitters, PACAP, VIP and NO, contribute to tracheal smooth muscle relaxation. The additive effect of inhibitors for these transmitters revealed that all three mediate muscle relaxation via different pathways, at least partially. In addition to their direct effects, each may interact to facilitate release or production of the other two. Further study is needed to elucidate whether interaction between inhibitory transmitters occurs in airway systems, as in enteric systems.

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