

## Opioids modulate the cholinergic contraction but not the nonadrenergic relaxation in guinea-pig airways *in vitro*

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*Opioids modulate the cholinergic contraction but not the nonadrenergic relaxation in guinea-pig airways in vitro.* J.L. Pype, L.J. Dupont, M.G. Demedts, G.M. Verleden. ©ERS Journals Ltd 1996.

**ABSTRACT:** (D-ALa<sup>2</sup>, NMePhe<sup>4</sup>, Gly-ol<sup>5</sup>) enkephalin (DAMGO), a selective  $\mu$ -opioid receptor agonist, has previously been demonstrated to inhibit the cholinergic and the noncholinergic contraction in guinea-pig airways. In contrast, opioids had no inhibitory effect on cholinergic neurotransmission in the upper trachea when stimulated at 8 Hz.

We investigated whether DAMGO, a selective  $\mu$ -opioid receptor agonist, [D-Pen<sup>2,5</sup>] enkephalin (DPDPE), a selective  $\delta$ -opioid receptor agonist, and U-69593, a selective  $\kappa$ -opioid receptor agonist could modulate the cholinergic contraction in the upper trachea at different frequencies of stimulation. Moreover, we have investigated whether DAMGO, DPDPE and U-69593 could also modulate the iNANC relaxation.

DAMGO (1–100  $\mu$ M) inhibited the cholinergic contraction in the upper trachea with a maximum inhibition of 57±15% at 1 Hz (n=4; p<0.05). On the other hand, DPDPE (10  $\mu$ M) and U-69593 (10  $\mu$ M) did not produce any significant inhibition of the cholinergic contraction. Naloxone, an opioid receptor antagonist (100  $\mu$ M), was able to antagonize the inhibitory effect of DAMGO (n=5; p<0.01) on the cholinergic contraction at a frequency of 2 Hz. DAMGO (10  $\mu$ M) did not displace the cumulative concentration-response relationship to acetylcholine (10 nM–10 mM), (n=4; NS). This provides evidence that prejunctional  $\mu$ -opioid receptors (and not  $\delta$ -opioid or  $\kappa$ -opioid receptors) modulate cholinergic contraction in the upper trachea. In contrast, DAMGO (10  $\mu$ M) had no significant inhibitory effect on the nonadrenergic relaxation (n=4; NS) in the upper trachea. Neither DPDPE nor U-69593 had any effect on the nonadrenergic relaxation.

These findings suggest that DAMGO directly inhibits the cholinergic contraction and that the opioid receptor involved in the inhibition of the cholinergic contraction in the upper trachea is of the  $\mu$ -opioid type. The finding that opioids inhibit cholinergic contraction without altering NANC relaxations suggests that distinct populations of nerves mediate these two effects.

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Opioid receptors have been localized to capsaicin-sensitive neurons [1], and have been demonstrated to inhibit neurotransmitter release. Indeed, BARTHÓ *et al.* [2] described a concentration-dependent inhibition of the noncholinergic contraction of the guinea-pig main bronchus by opioid agonists. This is in agreement with FROSSARD and BARNES [3], who suggested that the *in vitro* inhibition of the noncholinergic contraction by opioids was probably mediated through  $\mu$ -opioid receptors.

In guinea-pig airways *in vivo*, opioid agonists have also been shown to inhibit the noncholinergic bronchoconstrictor response to vagal stimulation, which is mediated *via*  $\mu$ -opioid receptors localized to sensory nerve endings in the airways [4]. Moreover, morphine was able to inhibit neurogenic plasma extravasation in guinea-pig airways by an action on opioid receptors on sensory nerves [5]. In addition to the inhibition of neuropeptide release from capsaicin-sensitive sensory nerves,

several studies have demonstrated that opioids are able to inhibit the cholinergic neurotransmission. RUSSELL and SIMONS [6] described an inhibition of acetylcholine release by enkephalins, mediated through opioid receptors on cholinergic nerve terminals in dog airways *in vitro*. In guinea-pig airways *in vitro*, opioids reduced cholinergic neural responses [7]. However, according to BELVISI *et al.* [8], the inhibition of the cholinergic contraction would be mediated partly *via* an inhibitory action on excitatory nonadrenergic, noncholinergic (eNANC) nerves and partly by a direct effect on cholinergic neurotransmission. This could explain why opioids produce no significant inhibition of the cholinergic contraction in the upper trachea, which lacks a functional eNANC innervation. In human airways *in vitro*, opioid agonists also inhibit the cholinergic neurotransmission *via* a prejunctional  $\mu$ -opioid receptor [9]. Finally, selective  $\mu$ -opioids appear to inhibit stimulus-evoked release of

acetylcholine from guinea-pig and human trachea [10], confirming previous studies on inhibition of neurotransmission.

Electrical field stimulation (EFS) in the guinea-pig upper trachea results in a rapid and transient cholinergic contraction. In the lower trachea, on the other hand, this cholinergic contraction is followed by a long-lasting noncholinergic contraction, due to the release of neuropeptides from airway sensory nerves. This has already been demonstrated in previous studies in our laboratory [11]. In the presence of atropine and propranolol, EFS of guinea-pig upper trachea results in a nonadrenergic relaxation, which is probably due to the release of vasoactive intestinal peptide (VIP) and nitric oxide (NO) [12]. It is suggested that these neurotransmitters of the inhibitory nonadrenergic noncholinergic (iNANC) system coexist with acetylcholine in airway cholinergic nerves [13], and that VIP and NO may be co-released from cholinergic nerves to act as functional antagonists of cholinergic bronchoconstriction [14]. We have designed this study to investigate whether [D-Ala<sup>2</sup>, NMePhe<sup>4</sup>, Gly-ol<sup>5</sup>] enkephalin (DAMGO), a selective  $\mu$ -receptor agonist, [D-Pen<sup>2,5</sup>] enkephalin (DPDPE), a selective  $\delta$ -opioid receptor agonist, and U-69593, a selective  $\kappa$ -opioid receptor agonist could modulate the cholinergic neurotransmission in the guinea-pig upper trachea. Moreover, since VIP and NO may coexist and may be co-released from cholinergic nerves, we have investigated whether DAMGO, DPDPE and U-69593 could also modulate the iNANC relaxation.

## Materials and methods

### *Tissue preparation*

Dunkin-Hartley guinea-pigs of either sex (250–500g) were killed by cervical dislocation. The lungs and trachea were rapidly removed and placed in Krebs-Henseleit (KH) solution of the following composition (mM): NaCl 118, KCl 5.9, MgSO<sub>4</sub> 1.2, CaCl<sub>2</sub> 2.5, NaH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25.5 and glucose 5.05. The trachea was stripped of connective tissue and opened longitudinally opposite to the smooth muscle layer. Tracheal strips were prepared by cutting the trachea into sections containing 3–4 cartilaginous rings, with the 9–12 tracheal rings adjacent to the larynx considered as upper trachea. The 9–12 tracheal rings adjacent to the carina were defined as lower trachea. Each tracheal strip was mounted between two platinum wire electrodes in a 10 mL organ bath containing KH solution, maintained at 37°C (pH 7.4) and continuously aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Indomethacin (10  $\mu$ M) was present in the organ bath throughout the experiment to inhibit the formation of prostaglandins, which may interfere with neuropeptide release [15].

When nonadrenergic relaxations were studied, the KH solution contained atropine (1  $\mu$ M) and propranolol (1  $\mu$ M), in addition to indomethacin (10  $\mu$ M), to inhibit, respectively, cholinergic and adrenergic involvement in responses to EFS. A resting tone of 1 g was applied in all tissues and histamine (10  $\mu$ M) was added to raise the tone of the smooth muscle [12], when nonadrenergic

relaxations were studied. All tissues were allowed to equilibrate for 40 min, during which time they were washed with fresh KH solution every 10 min. After each washing, histamine (10  $\mu$ M) was added to the organ bath to maintain the active tone (in case nonadrenergic relaxation was studied).

### *Electrical field stimulation*

EFS was performed using a Harvard Student Stimulator (Harvard App. Ltd, Edenbridge, Kent. UK). Biphasic square-wave pulses were delivered using a supramaximal voltage of 50 V, a pulse duration of 0.5 ms, and a frequency of 1, 2, 8 or 16 Hz during 15 s (cholinergic contraction) or 30 s (nonadrenergic relaxation). Isometric contractile or relaxant responses were measured using a Grass FT 0.3 force-displacement transducer and were visualized on a computer screen after digitalization of the signal using a commercially available software program (Codas; Dataq Instr. Inc., Akron. OH, USA). The responses were recorded and stored on a personal computer for data analysis.

*Cholinergic contraction.* EFS in guinea-pig upper trachea results in a rapid and transient cholinergic contraction, which can be abolished by atropine pretreatment. In the lower trachea, the cholinergic contraction is followed by a long-lasting noncholinergic contraction, which is atropine-resistant and, therefore, due to the release of neuropeptides from airway sensory nerves.

After a 1 h equilibration period, two control stimuli were delivered, which were discarded if they were not consistent (*i.e.* >10% variation). Then DAMGO, DPDPE or U-69593 was added and, after a 12 min incubation period [3], another two stimuli were delivered. In upper trachea, stimuli were delivered every 4 min; however, in lower trachea (since there is a long-lasting contraction) stimuli were delivered when the tissue had returned to its resting tension. Only one concentration of opioid agonist was investigated per tissue, and only one frequency of stimulation was performed in each tissue. In separate experiments, naloxone (100  $\mu$ M), an opioid receptor antagonist, was added and, after a 10 min incubation period, the same protocol was used as described above.

*Nonadrenergic relaxation.* After a 40 min equilibration period, two control stimuli were delivered, which were discarded if they were not consistent (*i.e.* >10% variation). DAMGO, DPDPE or U-69593 (all 10  $\mu$ M) was then applied and, 12 min later, another two stimuli were delivered. Only one frequency of stimulation was used in each tissue.

The responses to EFS in time-matched controls were stable throughout the whole period of the experiment. All responses to EFS were blocked by tetrodotoxin (1  $\mu$ M), confirming that they were neuronal in origin.

*Acetylcholine concentration-response relationship.* A cumulative concentration-response relationship to exogenous acetylcholine (10 nM–10 mM) was performed in the upper trachea in the presence and in the absence of DAMGO (10  $\mu$ M), after a 12 min incubation period.

The results were expressed as a percentage of the maximum contraction to acetylcholine (10 mM), which was determined at the beginning of the experiment.

### Drugs

The drugs used in these experiments, were obtained from the following sources: acetylcholine chloride, atropine sulphate, indomethacin, propranolol, histamine diphosphate salt (Sigma Chemical Co., Filter Service, Eupen, Belgium), tetrodotoxin (Biomol, Sanver Tech, Boechout, Belgium), [D-Ala<sup>2</sup>, NMePhe<sup>4</sup>, Gly-ol<sup>5</sup>] enkephalin (DAMGO), [D-Pen<sup>2,5</sup>] enkephalin (DPDPE), U-69593, naloxone hydrochloride (Research Biochemicals Inc., Sanver Tech, Boechout, Belgium). Indomethacin was dissolved in alkaline phosphate buffer (pH 7.8) of the following composition (mM): KH<sub>2</sub>PO<sub>4</sub> 20, Na<sub>2</sub>HPO<sub>4</sub> 120. All other drugs were dissolved in distilled water. Aliquots of DAMGO were dissolved in distilled water and stored at -20°C. Fresh drug solutions were made up daily. All concentrations refer to the final bath concentration and drug additions did not exceed 1% of the organ bath volume.

### Analysis of results

Results were expressed as mean±SEM. All contractile or relaxant responses were expressed as absolute changes in tension and then transformed to a mean response for two control stimulations in each tissue. The effect of the opioid agonists on the mean response was then expressed as a percentage inhibition. The effect of exogenous drug addition on EFS-induced contraction or relaxation in each tissue was assessed by use of a Student's t-test for paired data. Cumulative concentration-response relationship to exogenously applied acetylcholine was assessed for significance using Student's t-test for unpaired data. Probability values of less than 0.05 were considered significant.

## Results

In guinea-pig upper trachea, EFS results in a rapid and transient cholinergic contraction (fig. 1a). In the lower trachea, however, this rapid and transient contraction is followed by a long-lasting noncholinergic contraction, which is due to the release of neuropeptides from unmyelinated C-fibres (fig. 1b). In the presence of atropine (1 µM), propranolol (1 µM), indomethacin (10 µM) and histamine (10 µM, to raise the tone of the smooth muscle), EFS (50 V at source, 0.5 ms and 2 Hz for 30 s) in the guinea-pig upper trachea resulted in a nonadrenergic relaxation, which is not inhibited by DAMGO (10 µM). Figure 1c shows the inability of DAMGO (10 µM) to modulate the nonadrenergic relaxation produced by EFS at a frequency of 2 Hz. Naloxone (100 µM) almost completely reversed the inhibitory effect of DAMGO (10 µM) on the cholinergic contraction induced by EFS in the upper trachea (fig. 1d).

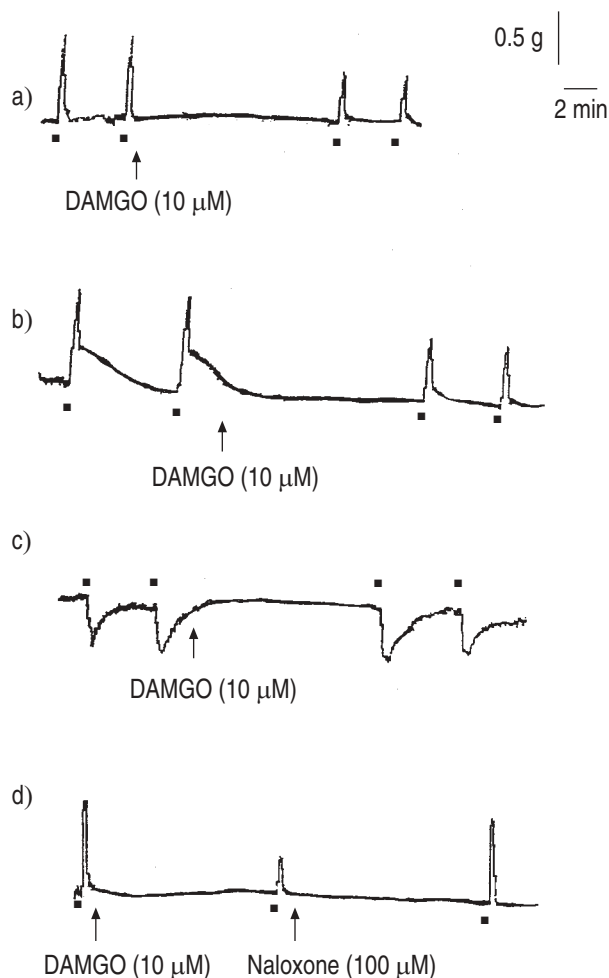


Fig. 1. — In the presence of indomethacin (10 µM), EFS (50 V at source, 0.5 ms and 2 Hz for 15 s, closed squares) in: a) the guinea-pig upper trachea; and b) the guinea-pig lower trachea, results in: a) a cholinergic contraction; or b) a cholinergic contraction followed by a noncholinergic contraction. DAMGO (10 µM) produced a clear inhibition of the EFS-induced cholinergic contraction in the upper (±34%) as well as in the lower (±37%) trachea. c) In the presence of atropine (1 µM), propranolol (1 µM), indomethacin (10 µM) and histamine (10 µM, to raise the tone of the smooth muscle), EFS (50 V at source, 0.5 ms and 2 Hz for 30 s) in the guinea-pig upper trachea resulted in a nonadrenergic relaxation, which is not inhibited by DAMGO (10 µM). d) Naloxone (100 µM) was able to reverse the inhibitory effect of DAMGO (10 µM) on the cholinergic contraction in the upper trachea. EFS: electrical field stimulation; DAMGO: [D-Ala<sup>2</sup>, NMPhe<sup>4</sup>, Gly-ol<sup>5</sup>] enkephalin.

### Effect of opioid agonists on the cholinergic contraction in the guinea-pig upper trachea

DAMGO produced a concentration- and frequency-dependent inhibition of the cholinergic contraction, with a maximum inhibition of 57±15% at a frequency of 1 Hz and at a concentration of 100 µM (n=4; p<0.05) (fig. 2).

U-69593 (10 µM) and DPDPE (10 µM) had no inhibitory effect at all on the cholinergic contraction in the upper trachea at any frequency tested (n=4–5; NS); whereas, DAMGO (10 µM) produced a significant inhibition of the cholinergic contraction, with a maximum inhibition of 34±4% at a frequency of 2 Hz (n=5; p<0.001) (fig. 3).

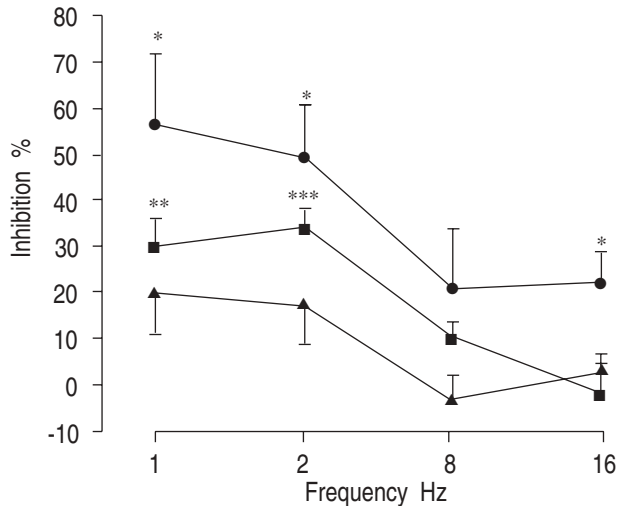


Fig. 2. – The effect of DAMGO 100  $\mu\text{M}$  (●), 10  $\mu\text{M}$  (■) and 1  $\mu\text{M}$  (▲) on the EFS-induced cholinergic contraction in the guinea-pig upper trachea. Values are presented as mean $\pm$ SEM of 4–5 animals. \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ , significance of inhibition. For definitions see legend to figure 1.

#### Effect of naloxone on the DAMGO-induced inhibition of the cholinergic contraction

Naloxone (100  $\mu\text{M}$ ) was able to antagonize the inhibitory effect of DAMGO (10  $\mu\text{M}$ ) on the EFS-induced cholinergic contraction in the upper trachea at a frequency of 2 Hz (10 $\pm$ 5% inhibition with naloxone versus 34 $\pm$ 4% without naloxone), ( $n=5$ ;  $p < 0.01$ ) (fig. 4). Naloxone (100  $\mu\text{M}$ ) alone had no effect on the resting tone nor on the EFS-induced cholinergic contractions. Naloxone (100  $\mu\text{M}$ ) was also able to reverse the inhibitory effect of DAMGO (10  $\mu\text{M}$ ) which is demonstrated in figure 1d.

#### Acetylcholine concentration-response curve

DAMGO (10  $\mu\text{M}$ ) did not displace the concentration-response curve to exogenously applied acetylcholine in the upper trachea (10 nM–10 mM) ( $n=4$ ; NS) (fig. 5).

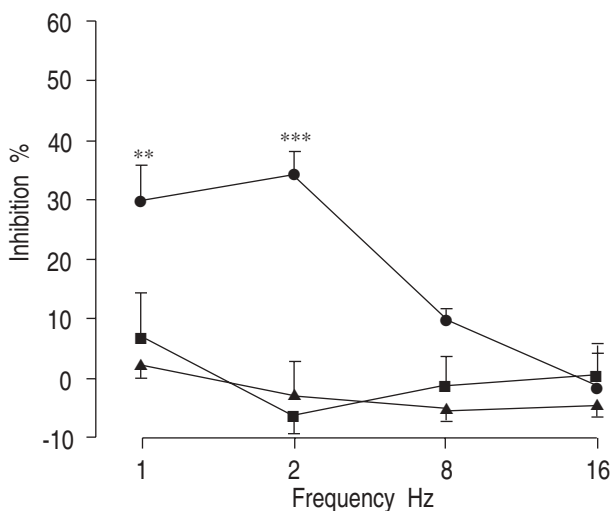


Fig. 3. – Effect of DAMGO (●), [D-Pen<sup>2,5</sup>] enkephalin (▲) and U-69593 (■) (all 10  $\mu\text{M}$ ) on the EFS-induced cholinergic contraction in the guinea-pig upper trachea. Values are presented as mean $\pm$ SEM of 4–5 animals. \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ , significance of inhibition. For definitions see legend to figure 1.

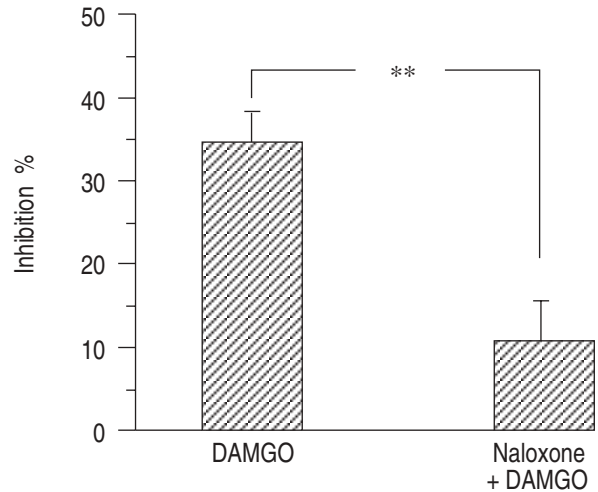


Fig. 4. – The effect of naloxone (100  $\mu\text{M}$ ) on the DAMGO-induced (10  $\mu\text{M}$ ) inhibitory effect on the cholinergic contraction, in the guinea-pig upper trachea (EFS, 50 V at source, 0.5 ms, 2 Hz for 15 s). Values are presented as mean $\pm$ SEM of 4–5 animals. \*\*,  $p < 0.01$ , significance of inhibition. For definitions see legend to figure 1.

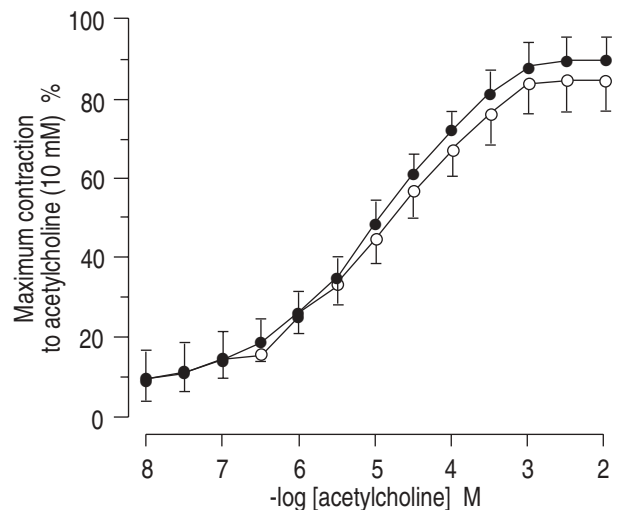


Fig. 5. – Responses to exogenously applied acetylcholine (10 nM–10 mM) as a percentage of the maximum contraction to acetylcholine (10 mM) in guinea-pig upper trachea, in the presence (●) and in the absence (○) of DAMGO (10  $\mu\text{M}$ ). Values are presented as mean $\pm$ SEM of four animals. DAMGO: [D-Ala<sup>2</sup>, NMPhe<sup>4</sup>, Gly-ol<sup>5</sup>] enkephalin.

#### Effect of opioid agonists on the iNANC

DAMGO, DPDPE and U-69593 (all 10  $\mu\text{M}$ ) produced no significant inhibitory effect on the EFS-induced non-adrenergic relaxation in guinea-pig trachea at any frequency tested (1, 2, 8 and 16 Hz) ( $n=3-5$ ; data not shown).

#### Discussion

This study describes the effects of [D-Ala<sup>2</sup>, NMePhe<sup>4</sup>, Gly-ol<sup>5</sup>] enkephalin (DAMGO), [D-Pen<sup>2,5</sup>] enkephalin (DPDPE), U-69593, selective  $\mu$ -opioid,  $\delta$ -opioid and  $\kappa$ -opioid receptor agonists, respectively, on the EFS-induced cholinergic contraction and the lack of effect on the EFS-induced iNANC relaxation in guinea-pig airways *in vitro*. It has been demonstrated [8] that DAMGO significantly inhibited the cholinergic contraction in lower trachea and main bronchi without having a significant

inhibitory effect on the EFS-induced cholinergic contraction in the upper trachea at a frequency of 8 Hz. Moreover, it was suggested that the major component of the cholinergic inhibition by DAMGO is indirect, through an inhibition of the facilitatory action of eNANC nerves on cholinergic neurotransmission, although another component of the inhibition seems to be attributed to a direct action on the cholinergic nerves. We have studied the effect of selective opioid agonists on the upper trachea, since it is demonstrated that there are regional variations in eNANC responses throughout the length of the trachea [16]. Indeed, in the present study, EFS in the lower trachea produced a cholinergic contraction, followed by a long-lasting contraction, which is non-cholinergic and probably due to the release of neuropeptides from airway sensory nerves, since pretreatment of the animals with capsaicin abolished this contraction [11]. In the upper trachea, on the other hand, EFS only induces a rapid and transient contraction, due to the release of acetylcholine from cholinergic motor nerves.

In the present study, using lower frequencies of stimulation, we were able to demonstrate a concentration- and frequency-dependent, DAMGO-mediated inhibition of the EFS-induced cholinergic contraction in guinea-pig upper trachea (demonstrating a direct effect on cholinergic nerves), which was antagonized by naloxone, an opioid receptor antagonist. The  $\delta$ -opioid and  $\kappa$ -opioid receptor agonists, on the other hand, produced no inhibitory effect at all on the cholinergic neurotransmission in the upper trachea, in contrast with DAMGO, indicating that a  $\mu$ -opioid receptor is most likely to be involved. DAMGO did not affect the cumulative concentration-response relationship to acetylcholine, providing evidence for its prejunctional effect.

In the presence of atropine and propranolol, EFS in guinea-pig upper trachea produced a nonadrenergic relaxation, which, at least in guinea-pig airways, is probably due to the release of NO and VIP from iNANC nerves [12, 17]. The iNANC system is very important since it is the only neural bronchodilator mechanism in human airways, and it is accepted that NO acts as the only neuro-transmitter of the iNANC relaxation in human airways [18–20]. The exact source of NO still remains unknown, although there is convincing evidence that NO may be released from nerves [21]. It has been suggested that the neurotransmitters of the nonadrenergic relaxation coexist with acetylcholine in airway cholinergic nerves [13], and that VIP and NO may be co-released from cholinergic nerves to act as functional antagonists of cholinergic bronchoconstriction [14]. As a consequence, if DAMGO inhibits the cholinergic contraction in the upper trachea, we hypothesized that DAMGO would also inhibit the nonadrenergic relaxation in the upper trachea. However, DAMGO (10  $\mu$ M) was unable to produce an inhibition of the EFS-induced non-adrenergic relaxation at a concentration and frequency of stimulation which already produced a significant inhibition of the cholinergic contraction. As on the cholinergic contraction, neither DPDPE nor U-69593 had any effect on the EFS-induced nonadrenergic relaxation (data not shown). These results may suggest that the cholinergic and the iNANC nerves in the guinea-pig trachea represent different entities, or that these results are merely a reflection of the fact that the mechanisms

involved in the release process of the different neurotransmitters (*e.g.* acetylcholine for cholinergic contraction, VIP and NO for iNANC relaxation) may be different.

The first concept has been put forward by CANNING and UNDEM [22]. They have used a preparation in which the extrinsic innervation of an isolated segment of the rostral portion of the guinea-pig trachea can be studied functionally. Surprisingly, they found that removal of the oesophagus selectively abolished vagally-mediated nonadrenergic relaxations of the guinea-pig trachea, while vagally-mediated cholinergic contractions were not effected. These observations led to the hypothesis that the guinea-pig trachealis receives excitatory and inhibitory innervation from distinct vagal parasympathetic pathways, and that neurons mediating nonadrenergic relaxation are associated with the oesophagus, prior to innervating the trachealis. This is also in agreement with the study by WATSON *et al.* [23], in which no iNANC relaxation was observed when a guinea-pig tracheal tube preparation was stimulated by the preganglionic cervical vagus nerve, whereas transmural stimulation (activation of postganglionic intrinsic nerves) did induce an iNANC relaxation. This is further supported by studies on denervated human airways, obtained from heart-lung transplant recipients undergoing a second transplantation. In these tissues, cholinergic neural responses seem to be preserved [24], whereas the iNANC responses were virtually absent [25]. These observations may also argue against the co-localization of NO and acetylcholine in human airway cholinergic nerves.

The present findings may support the hypothesis that the cholinergic and iNANC nerves are different entities, since DAMGO clearly inhibited the EFS-induced cholinergic contraction, whilst having no inhibitory effect at all on the nonadrenergic relaxation. On the other hand, these results may also suggest that these nerves do not necessarily display the same prejunctional modulatory receptors. Recently, it has been demonstrated that loop diuretics (frusemide and bumetanide) are able to inhibit the cholinergic, as well as the noncholinergic contraction [26, 27], and the nonadrenergic relaxation in guinea-pig airways *in vitro* [17]. These results are not in contradiction with the present results, since loop diuretics do not act through stimulation of a prejunctional receptor but probably through inhibition of nerve activation (myelinated cholinergic nerves as well as unmyelinated C-fibres)

The iNANC system might be of particular importance since, in the absence of a functional adrenergic innervation, it is the only inhibitory neural pathway in human airways. It has already been suggested that modulation of NO release could result in exaggerated bronchoconstriction [14], and NO has recently been demonstrated to modulate cholinergic responses by functional antagonism of acetylcholine at the level of the airway smooth muscle [28]. Moreover, NO is also a potent endogenous anti-inflammatory compound [14]. As a consequence, it seems that inhibition of NO release is most undesirable in asthma. Therefore, further research is definitely needed to investigate whether human airways, like guinea-pig trachea, also receive cholinergic excitatory and inhibitory innervation from distinct pathways, providing evidence that acetylcholine and NO do not necessarily function as co-transmitters

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