Non-adrenergic, non-cholinergic neural activation stabilizes smooth-muscle tone independently of eicosanoid factors in guinea-pig isolated airways

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1 We examined the effect of variations in resting smooth-muscle tone induced by eicosanoid inhibitors on the direction and magnitude of the non-adrenergic, non-cholinergic (NANC) nervous response in guinea-pig isolated airways.

2 NANC responses (in the presence of 1 μ M atropine, 10 μ M guanethidine) to electrical field stimulation (1200 mA; 0.5 ms; 240 s; 3 Hz) were studied in guinea-pig isolated airway preparations ($n = 4-7$) taken from the proximal trachea, the distal trachea and the main bronchus. Tissues were treated with either the cyclo-oxygenase-blocker, indomethacin (10 μ M), or the leukotriene receptor antagonist, FPL 55712 (11.5 μ M), to modulate the resting tone.

3 Depending on the level of resting tone prior to electrical field stimulation, NANC activation induced either a contraction or a relaxation converging towards a similar level of tone, a 'tonus equilibrium'. This tonus equilibrium displayed an increasing level towards the periphery (3 ± 3 (s.e.mean) % for the proximal trachea, $28 \pm 3\%$ for the distal trachea and $54 \pm 4\%$ for the main bronchus, in % of maximum active tension). After NANC activation, the tonus returned to a level similar to the resting tone.

4 We conclude that changes in resting smooth-muscle tone not only affect the magnitude but also the direction of the NANC response. It is suggested that the NANC nervous system is a stabilizing factor in the regulation of airway smooth-muscle tone, independent of eicosanoid factors. An increasing contractile component and a decreasing relaxant component of the NANC system towards the peripheral airways is indicated.

Keywords: FPL 55712; indomethacin; main bronchus; NANC; resting tone; segmental distribution; trachea

Introduction

Both a contractile and a relaxant non-adrenergic, non-cholinergic (NANC) response have been demonstrated in the airways of guinea-pig and man *in vitro* (Coburn *et al.*, 1973; Richardson *et al.*, 1976; Davis *et al.*, 1982; Szolcsanyi *et al.*, 1982; Andersson *et al.*, 1983; Lundberg *et al.*, 1983a; Taylor *et al.*, 1984) and there are data which indicate a neural origin for both NANC components (Davis *et al.*, 1982; Szolcsanyi *et al.*, 1982; Andersson *et al.*, 1983; Lundberg *et al.*, 1983a).

In a recent study on guinea-pig isolated airways, we demonstrated that indomethacin decreased the active component of the tension in the resting preparation, the resting smooth-muscle tone, and increased the contractile as well as decreased the relaxant NANC response (Lindén *et al.*, 1991a). In the distal trachea, relaxations were even changed into contractions. One explanation of this could be that both the magnitude and the direction of the NANC response are related to the resting smooth-muscle tone. If so, the NANC system may be a stabilizing factor in the regulation of airway smooth-muscle tone.

The purpose of the present study was to examine whether both the magnitude and the direction of the NANC response are related to the resting smooth-muscle tone in guinea-pig airways. Different levels of resting tone were obtained by modulating the spontaneous airway tone with eicosanoid inhibitors (Lindén *et al.*, 1991b) and then activating the NANC system at the different levels of resting tone thus obtained. In this way, the importance of eicosanoid factors during NANC activation was also evaluated.

Methods

Preparation

Twenty-four, specific pathogen-free, Dunkin-Hartley guinea-pigs (350–500 g) were killed by cervical dislocation and exsanguinated. The thoracic contents were removed and placed in a 200 ml oxygenated (94% O₂; 6% CO₂) dissection bath filled with Krebs-Ringer solution (KR composition, mM: NaCl 118, KCl 5.9, CaCl₂ 2.5, MgSO₄ 1.2, NaH₂PO₄ 1.2, NaHCO₃ 25.5 and glucose 5.6) at room temperature. The extrapulmonary airways were dissected free and airway rings were cut transversally. Each of these airway rings contained four to five cartilaginous rings. Two airway rings from the proximal (cervical) trachea, the distal (intrathoracic) trachea and the main bronchi from each animal were used. The airway rings were opened longitudinally along the anterior, cartilaginous part and mounted on steel hooks as isolated strips. These 'frontally-opened airway rings' were mounted in custom-made, temperature-controlled (37°C) and oxygenated 8 ml organ baths. These baths were continuously flushed with fresh KR (0.4 ml min⁻¹). Histological evaluation of the mounted airway rings showed that epithelial and smooth muscle cells were intact (Lindén *et al.*, 1991a).

Tension recordings

Isometric tension was continuously recorded on a Grass Polygraph (7D) via Grass force transducers (FT03). We used a recently developed method (Lindén *et al.*, 1991b), separating the active component, the tonus, from the passive component of the recorded total tension.

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In order to compensate for the time-decay of the passive tension in the preparations, we used a linear mathematical model (Lindén *et al.*, 1991b) in which the passive tension (g) equals the formula $1.2 - [0.004 \times t]$. t represents time (min after initial adjustment of the applied, passive tension).

There was no significant difference in the level or decay of passive tension in the trachea and the main bronchus (Lindén *et al.*, 1991b) nor was there any difference in the decay of passive tension between control and indomethacin- or FPL 55712-treated preparations (Lindén *et al.*, 1991b).

The active component of the total (active plus passive) tension (g) within each preparation was calculated by subtracting the passive tension. This was done immediately before, at the end of and 25 min after electrical field stimulation of the NANC system, as well as during the maximum 5-hydroxytryptamine (5-HT)-induced contraction. Within each preparation, the levels of active tension were related to the maximum 5-HT-induced active tension (% max). Within each airway segment, the tonus equilibrium was calculated as the mean of the active tension levels at the end of the electrical field stimulation, in all three treatment groups.

Electrical field stimulation

Electrical field stimulation (EFS) from constant current generators was applied via rectangular platinum electrodes (5×30 mm) mounted parallel to the airway preparations (10 mm apart). We used square-wave biphasic impulses of 0.5 ms duration (Lindén *et al.*, 1991a) and a current of 1200 mA, which were found to activate the contractile and the relaxant component of the NANC system to a similar degree (data not shown). The total duration of stimulation for NANC activation was 240 s in order to evoke contractile and relaxant responses of large magnitude (Lindén *et al.*, 1991a). At a frequency of 3 Hz, these stimulation parameters produced submaximal, tetrodotoxin-sensitive contractile and relaxant NANC responses (Lindén *et al.*, 1991a).

Experimental design

The passive component of the total tension in the resting preparation was applied as follows: 25 min of equilibration with the adjustment of the total (active plus passive) tension to 1.4 g was followed by a control contraction (12 Hz; 20 s) to avoid subsequent sliding on the steel hooks connected to the preparations. A wash-out followed. The active component of the total tension was then eliminated with 0.3 mM theophylline which produces a maximum relaxation in guinea-pig airways (Jeppson *et al.*, 1982; Lindén *et al.*, 1991b). In the presence of this drug the remaining, passive tension was adjusted to 1.2 g and the theophylline was then washed out. In preliminary experiments, we ascertained that 1.2 g was an optimum applied, passive tension for contractile responses to EFS (12 Hz; 20 s) in both the trachea and the main bronchus (data not shown).

After adjusting the applied tension, atropine ($1 \mu\text{M}$) and guanethidine ($10 \mu\text{M}$) were added to the KR in all treatment groups in order to obtain cholinergic blockade (Andersson *et al.*, 1981; Lundberg *et al.*, 1983; Taylor *et al.*, 1984) and adrenergic depletion (Kamikawa & Shimo, 1976; Grundström *et al.*, 1981; Brock *et al.*, 1988). In the resting preparations, three different levels of active tension (resting tone) were established by the pharmacological manipulation of the spontaneous airway tone, by use either of the cyclo-oxygenase inhibitor, indomethacin ($10 \mu\text{M}$), or the leukotriene receptor antagonist FPL 55712 ($11.5 \mu\text{M}$) or the vehicle (Lindén *et al.*, 1991b). We have previously demonstrated that the spontaneous airway tone was stable for at least 2 h (Lindén *et al.*, 1991b), which exceeded the total duration of the present experiments. Preparations were incubated with the eicosanoid inhibitors for an equilibration period of 70 min and the NANC system was then activated by EFS (3 Hz, 240 s). After the EFS, a wash-out was performed. The preparations were then flushed with fresh

KR containing the above-mentioned drugs for 25 min. At the end of the experiment $10 \mu\text{M}$ 5-HT was added which produced a maximum serotonin-induced contraction. A maximum 5-HT-induced contraction reached about 80% of a maximum carbachol-induced (0.1 mM) contraction in both the trachea and the main bronchus (data not shown).

Data analysis

The results were expressed as the mean ($\pm$ s.e. mean). The statistical evaluation of data was performed by use of Student's *t* test (two-tail) for unpaired data with 95% confidence intervals (Table 2) and by means of a parametric correlation coefficient determined at 95% confidence intervals (Colton, 1974); n equals the number of guinea-pigs (independent observations).

Drugs

Atropine (Sigma), guanethidine (Ciba-Geigy), 5-HT (Sigma) and theophylline (Draco) were dissolved in KR-solution. Indomethacin (Merck) and FPL 55712 (7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid, Fisons) were dissolved in ethanol (70%), producing a final ethanol concentration of $<0.35 \text{ ml}^{-1}$ in the organ baths (vehicle). All the drug solutions were made up immediately before use.

Results

Characteristics of NANC responses

Depending on the airway segment and the level of active tension prior to stimulation, either contractile or relaxant NANC responses with one dominant phase were observed, as demonstrated by some original tracings in Figure 1. This pattern was observed in all preparations.

Ability of the NANC system to stabilize airway smooth-muscle tone

In terms of relative active tension (% max), FPL 55712 decreased the resting tone and indomethacin abolished it, thereby producing different levels of active tension prior to stimulation in the three treatment groups of each airway segment (Figure 2). The levels of maximum active tension were of a similar range in all treatment groups (Table 1).

Figure 1 Original tracings of NANC responses of guinea-pig isolated airways: (a) proximal trachea, (b) distal trachea and (c) main bronchus after treatment with vehicle, the leukotriene receptor antagonist, FPL 55712 ($11.5 \mu\text{M}$) or the cyclo-oxygenase inhibitor, indomethacin ($10 \mu\text{M}$). NANC responses were evoked by electrical field stimulation (EFS; 1200 mA; 0.5 ms; 240 s; 3 Hz).

Table 1 Maximum 5-hydroxytryptamine-induced (10 μM) active* tension in guinea-pig isolated airways with and without eicosanoid inhibitors

	Proximal trachea (n = 6)	Distal trachea (n = 6)	Main bronchus (n = 4–7)
Control (vehicle)	2.52 (0.35)	1.86 (0.20)	2.06 (0.11)
FPL 55712 (11.5 μM)	2.01 (0.20)	1.31 (0.12)	1.86 (0.18)
Indomethacin (10 μM)	1.95 (0.23)	1.60 (0.18)	2.26 (0.20)

* After excluding the passive component of the recorded isometric tension, see Methods.
S.e.means are shown in parentheses.

Depending on the level of active tension prior to stimulation in the distal trachea, NANC activation by EFS induced either contraction or relaxation, converging towards a similar level of active tension, a ‘tonus equilibrium’ (EFS in Figure 2). Before NANC activation by EFS, there was a pronounced difference in the level of active tension between the control and the indomethacin-treated group. At the end of EFS, this difference was significantly reduced. The 95% confidence interval for the NANC-induced reduction of the difference between the control and the indomethacin group is presented in a separate table (Table 2). Prior to EFS, there was also a pronounced difference in the level of active tension between the control and the FPL 55712-treated group which was significantly reduced at the end of EFS (Table 2).

NANC responses converging towards a tonus equilibrium were also observed in the other two airway segments, although a very small contractile response was observed in the proximal trachea and no relaxant response was observed in the main bronchus. These NANC responses significantly decreased the difference in active tension noted prior to EFS (Table 2) except in the case of the difference between the control and the FPL 55712 group in the main bronchus

(Table 2). A ‘converging trend’ could, however, also be identified in that case. After NANC activation, the tension returned towards a level similar to the active tension prior to stimulation in the majority of treatment groups (Figure 2). The converging pattern induced by NANC activation was also observed in all segments when the tension was expressed in terms of total tension (active plus passive component, data not shown).

The contractile NANC response was most pronounced in preparations of the main bronchus following indomethacin or FPL 55712 treatment, where the active tension was abolished or decreased prior to stimulation. The pattern in the distal trachea was similar. The relaxant response was pronounced in the control preparations of the proximal and the distal trachea, where the active tension prior to stimulation was higher than in the indomethacin and FPL 55712 groups.

The tonus equilibrium, towards which responses converged during NANC activation, was at a higher level in the more peripheral airways (3 ± 3% and 28 ± 3% and 54 ± 4%, as a percentage of maximum active tension for the proximal trachea, the distal trachea and the main bronchus respectively; n = 15–18). The correlation between the level of the tonus equilibrium and the airway segment was high (r = 0.84) and significant (r = 0.74–0.91 in terms of the 95% confidence interval, n = 15–18).

Figure 2 NANC responses at different levels of resting tone in guinea-pig isolated airways: (a) proximal trachea, (b) distal trachea and (c) main bronchus. Different levels of active tension prior to NANC activation (resting tone) were established by pretreatment with vehicle (○—○), the leukotriene receptor antagonist FPL 55712 (11.5 μM, ▲—▲) or the cyclo-oxygenase inhibitor indomethacin (10 μM, ●—●). NANC activation was evoked by electrical field stimulation (1200 mA; 0.5 ms; 240 s; 3 Hz). The active tension is presented as mean percentage (with bars indicating the s.e.mean) of maximum 5-hydroxytryptamine-induced (10 μM) active tension (% max) prior to (Pre-EFS), during (EFS) and after (Post-EFS) electrical field stimulation. n = 4–7 guinea-pigs. Depending on the level of resting tone, NANC activation induced either a contraction or a relaxation, converging towards a similar level of active tension, a ‘tonus equilibrium’.

Discussion

In this study, we confirmed the previously reported effect of eicosanoid inhibitors on spontaneous airway tone in guinea-pig airways (Lindén *et al.*, 1991b). We obtained evidence that these changes in resting smooth muscle tone affect not only the magnitude but also the direction of the response to NANC stimulation. In the distal trachea, a high level of resting tone led to a relaxation, whereas a low level of resting tone led to a contraction during NANC activation. The NANC responses converged, with and without different eicosanoid inhibitors. Thus the NANC responses adjusted the tone towards a similar level, a tonus equilibrium. These findings suggest that the NANC nervous system is a stabilizing factor in the regulation of airway smooth muscle tone, independent of eicosanoid factors.

A converging adjustment of tone induced by NANC activation is a new aspect of the role played by the NANC system in the airways. It has previously been reported that there is a relationship between the magnitude of the relaxant NANC response and the level of total tension (active plus passive component) prior to stimulation in guinea-pig isolated

Table 2 Statistical analysis of the converging effect on airway smooth-muscle tone induced by NANC activation

	NANC-induced reduction of difference in active tension (95% confidence intervals*, in % of maximum active tension)		
	Proximal trachea (n = 6)	Distal trachea (n = 6)	Main bronchus (n = 4–7)
Control minus Indo	8–52%	30–79%	9–56%
Control minus FPL 55712	5–52%	24–54%	–43–66%

* Student’s *t* distribution (two-tailed).
Indo = indomethacin.

airways (Souhrada *et al.*, 1980; Karlsson *et al.*, 1984; Thompson *et al.*, 1988) and similar results have been obtained in human isolated airways (Davis *et al.*, 1982; de Jongste *et al.*, 1987). Kamikawa & Shimo (1989) demonstrated that the magnitude of the contractile NANC response depends on the level of passive tension prior to stimulation in guinea-pig isolated bronchi. We add to this previous data the finding that the level of active tension prior to stimulation, the resting tone, not only determines the magnitude of the NANC response but also appears to determine whether NANC neural activation leads to a relaxation or a contraction. Furthermore, this connection between the resting tone and the NANC response produces a converging adjustment of tone during NANC activation. Apart from the possible, physiological importance of these findings, they also highlight the need for caution when examining contractile and relaxant NANC responses against a varying resting tone.

The present study, as well as previous studies, suggest that the relaxant NANC component dominates in the proximal guinea-pig trachea (Coburn *et al.*, 1973; Kamikawa & Shimo, 1976). However, when the resting tone was reduced in the distal trachea, we also observed a moderate contractile NANC component: this confirms the results of two recent studies (Belvisi *et al.*, 1990; Lindén *et al.*, 1991a). In the guinea-pig main bronchus, on the other hand, the contractile NANC component appeared to dominate: this agrees with most previous studies (Andersson *et al.*, 1983; Lundberg *et al.*, 1983b; Frossard *et al.*, 1987; Kamikawa & Shimo, 1989). In contrast, Grundström *et al.* (1981) found no response to electrical field stimulation in the atropinized main bronchus, whereas Souhrada *et al.* (1980) registered relaxant NANC response in this segment. These discrepancies might be attributable to differences in resting tone prior to stimulation. It is, therefore, possible that a relaxant NANC response could be produced in the guinea-pig main bronchus, as in human bronchi (Richardson *et al.*, 1976; Davis *et al.*, 1982), if the resting tone is increased prior to NANC activation.

The data in this study are expressed in terms of relative active tension in order to detect segmental differences in NANC responses related to maximum contractions. We found this transformation acceptable since the levels of maximum 5-HT-induced active tension were of a similar range in all treatment groups. Furthermore, the converging pattern was also observed when studying the total tension (active plus passive component) expressed in grams.

The level of the tonus equilibrium increased towards the more peripheral airway segments. This suggests segmental differences in the balance between the contractile and the relaxant NANC component in the guinea-pig and agrees with two recent studies of the NANC system (Belvisi *et al.*, 1990; Lindén *et al.*, 1991a). It is possible that these segmental differences reflect differences in the forces applied on the extra- and intrathoracic airways as well as on the extra- and intrapulmonary airways by the surrounding tissue (Mead *et al.*, 1970). The increasing level of the tonus equilibrium towards the periphery also emphasizes the fact that the trachea from guinea-pigs is not necessarily a relevant *in vitro* model for physiological or pathological conditions in the bronchi.

It could be argued that an indomethacin-induced increase in the contractile NANC response follows the loss of relaxant prostanoids released by electrical field stimulation. Another possibility is an indomethacin-induced increase in the release of contractile leukotrienes due to electrical field stimulation, since it has been reported that indomethacin shunts arachidonic acid metabolism towards the synthesis of leukotrienes (Kreutner *et al.*, 1990). In the present study, however, leukotriene receptor antagonism and cyclo-oxygenase inhibition

produced contractile NANC responses of a similar magnitude in the main bronchus. This indicates that the modulation of the NANC responses induced by indomethacin and FPL 55712 is secondary to the reduced resting tone that is present before stimulation. Furthermore, the NANC responses converged towards a similar level of tone both during cyclo-oxygenase inhibition and during leukotriene antagonism. This finding suggests that, although endogenous eicosanoids are potent modulators of airway smooth muscle tone *in vitro* (Ullman *et al.*, 1990; Dahlgren *et al.*, 1983; Lindén *et al.*, 1991b), these agents have a minor effect on tone during NANC activation.

The mechanism controlling the direction of the NANC response is unclear. The present data suggest, however, that this mechanism is non-adrenergic, non-cholinergic and independent of eicosanoids. It is possible that the mechanism connecting the level of resting tone and the direction of the NANC response is neural but it might also involve the actin-myosin system, within the smooth muscle cell.

Nishikawa *et al.* (1983) demonstrated that myosin light chain kinase and protein kinase C may phosphorylate different sites on the myosin molecule. In a subsequent study (Nishikawa *et al.*, 1984), the same group found an inverse relationship between the phosphorylation induced by the two kinases. There was also evidence for an inhibitory effect on the actin-activated MgATPase when myosin was phosphorylated by myosin light chain kinase and protein kinase C compared to phosphorylation of myosin light kinase alone. Based on these findings, Nishikawa *et al.* (1984) suggested that, whereas phosphorylation induced by myosin light chain kinase favours contraction, phosphorylation induced by protein kinase C may favour relaxation. If true, this means that the smooth muscle cell may be protected against severe contraction via activation of protein kinase C.

It was recently reported that one transmitter candidate for the relaxant NANC response, vasoactive intestinal polypeptide (Matzusaki *et al.*, 1980; Palmer *et al.*, 1986; Ellis *et al.*, 1989; Li *et al.*, 1991) is absent in the lung tissue of asthmatics but not in the tissue of normal subjects (Ollerenshaw *et al.*, 1989). In another study, asthmatics also demonstrated an increase in substance P immunoreactive nerve fibres (Ollerenshaw *et al.*, 1990), which probably mediate the major part of the contractile NANC response (Lundberg *et al.*, 1983a,b). It might therefore be important to consider the finding that NANC neural activation stabilizes airway smooth-muscle tone when evaluating the pathophysiological role of the NANC system in obstructive airways disease. It is possible that an imbalance between the contractile and the relaxant components of the NANC response contributes to pathological airway obstruction.

In conclusion, both the direction and the magnitude of the NANC response in guinea-pig isolated airways appeared to be related to the resting tone prior to NANC activation such that this neural activation stabilizes airway smooth muscle tone. The NANC responses converged towards a tonus equilibrium that displayed segmental differences. Endogenous eicosanoids had a minor effect on airway smooth muscle tone during NANC activation. These findings suggest that the NANC nervous system is a stabilizing factor in the regulation of airway smooth muscle tone, independent of eicosanoid factors.

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