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Differential responsiveness of proximal and distal parts of isolated guinea pig trachea

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Abstract

This study addressed the question whether proximal and distal guinea pig tracheal segments respond differently to contractile agents. Using a perfused trachea set-up, histamine, KCl or the cyclo-oxygenase inhibitor, indomethacin, could be administered selectively to the mucosa (at the inside) or the serosa (at the outside) of the tracheal segments. Proximal parts contracted significantly more (40–60%) than distal parts when 1 mM histamine was administered to the mucosal or serosal side or when KCl (50 mM) was added to the serosal side. When histamine was administered to the mucosal side of epithelium-denuded segments, the contractions were twice as high in proximal than in distal parts (3057 vs. 1526 mg). Inhibition of tracheal cyclo-oxygenase with indomethacin at the mucosal side increased proximal and distal reactivity to mucosally administered histamine to the same extent. Serosal administration of indomethacin, however, increased histamine reactivity only in proximal segments (from 2690 to 5180 mg). In the latter segments, subsequent administration of histamine to the serosal side further increased the contraction, while serosal histamine in the absence of serosal indomethacin produced a relaxation (net difference of 4672 mg). In conclusion, the higher intrinsic contractility of proximal tracheal segments is counteracted by serosal cyclo-oxygenase products.

Keywords: Tracheal reactivity; Smooth muscle; Nitric oxide (NO) synthase; Cyclo-oxygenase; Epithelium

1. Introduction

One of the hallmarks of asthma is increased responsiveness of the airways to contractile mediators such as histamine (American Thoracic Society, 1987). Airway contractions are widely studied in isolated parts of the airways. There is abundant evidence for contractile reactivity differences between upper and lower airways, but such differences within the tracheal tube are less well documented. However, *in vitro* experiments are often performed on multiple pieces of tissue from the same trachea. Hence, for practical reasons, it is important to know whether there are reactivity differences between different parts of the trachea. In addition, investigation of possible differences may shed new light on the relative role of factors defining tracheal responsiveness.

In our laboratory, an organ bath system with perfused isolated guinea pig tracheas is used. To reduce the use of experimental animals, we generally separate each trachea into a proximal and a distal part. Trachea segments without epithelium are frequently used as a model to mimic epithelial sloughing (Folkerts et al., 1989) as seen in asthmatics (Hogg and Eggleston, 1984). Using this model with histamine as a contractile agent, we observed contractility differences between proximal and distal parts of guinea pig tracheas. This was further investigated by using tracheas with and without epithelium. Since the contractility differences were especially pronounced in the absence of the epithelium, the role of two histamine-induced, epithelium-derived relaxing factors, nitric oxide (NO) (Yan et al., 1994) and prostaglandin E_2 (Braunstein et al., 1988), was studied.

2. Materials and methods

2.1. Animals

Male specified pathogen-free Dunkin Hartley guinea pigs weighing 350–400 g (Harlan Nederland, Horst, The Nether-

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lands) were housed under controlled conditions (humidity 50–70%, temperature 21–23 °C, 12 h day/night rhythm). Water and commercial chow were allowed ad libitum.

2.2. Isolation of tracheal segments

Guinea pigs were sacrificed with an overdose of pentobarbitone–sodium (Nembutal®, 0.6 g kg^{-1} body weight, i.p.). Tracheas were dissected free of connective tissue and blood vessels, isolated, and divided in a proximal and distal part as follows: the part of the trachea that contained the first 14 ± 1 cartilage rings from the larynx was designated proximal, and designated the distal part containing the following 14 ± 1 rings was designated distal parts of the trachea. Typically, three or four rings separated the latter part from the bifurcation, but were not included because of practical reasons relating to the isolation procedure. Two steel hooks were inserted through opposite sites of the tracheal wall with the smooth muscle between them as described before (Folkerts et al., 1989). The hooks comprised three cartilage rings; therefore, the sites of proximal and distal parts where force development was actually recorded were separated by 11 ± 2 cartilage rings. Where indicated, the epithelial layer was removed from the tracheal segments by gently rubbing with a cotton swab as described earlier (Folkerts et al., 1989).

2.3. Perfused organ baths

Trachea segments were mounted in perfused organ baths according to a modified method of Pavlovic et al. (1989). The organ baths contained Krebs buffer of the following composition (mM): NaCl (118.1), KCl (4.7), $CaCl_2$ (2.5), $MgSO_4$ (1.2), $NaHCO_3$ (25.0), K_2HPO_4 (1.2), and glucose (8.3). The mucosal side of the trachea was perfused with Krebs solution independently from the serosal side by means of a peristaltic pump delivering a flow rate of 2 ml min^{-1}. The Krebs solution was continuously gassed with 5% CO_2 in O_2 and kept at 37 °C. One hook was fixed to the bottom of the organ bath; the other hook was attached to an isometric force transducer (Harvard Bioscience, Kent, UK). Transducers were connected to an analogue–digital converter, delivering digital signals to a computerized set-up. The set-up allowed continuous sampling, on-line equilibrium detection, and real-time display of the responses on a computer screen.

The tracheal tension was set at an optimum counter weight of 2.0 g. The tissues were allowed to reach a stable tone for 60 min, during which the buffer was refreshed every 15 min. If necessary, tissues were allowed additional time to equilibrate without the buffer solution being changed.

2.4. Contractile stimulation; pharmacological modulation of contractions

Non-specific trachea smooth muscle responses were evoked by adding KCl at a final concentration of 50 mM to the buffer at the outside, i.e. the serosal side of the tracheal segments. For pharmacologically specific contractile responses, tracheas were stimulated at the inside, i.e. the mucosal side, with cumulatively increasing concentrations of histamine (10^{-8} to 10^{-3} M), or with a single concentration (10^{-3} M) of histamine at the serosal side of the tracheal tube.

Nitric oxide synthase (NOS) was inhibited by N^{ω}-nitro-L-arginine methyl ester (L-NAME, 150 μM, final concentration), which was administered after the equilibration period, but 20 min before starting the histamine series. Tracheas with epithelium received the NOS-inhibitor in the mucosal buffer only, whereas epithelium-denuded tracheas were treated both serosally and mucosally. Controls received the same concentration of D-NAME; the compounds remained in the buffer throughout the experiment. The cyclo-oxygenase inhibitor, indomethacin (1.0 μM, final concentration), was administered in a similar fashion; controls received vehicle (Tris buffer, 2.5 mM, final concentration).

2.5. Statistics

If concentration–response curves showed a clear plateau, E_{max} values were compared with Student's two-tailed *t*-test. If no clear plateau was reached, the curves were analyzed with a repeated measure test. Where applicable, individual data points were tested with Student's two-tailed *t*-test. *P*-values < 0.05 were considered to reflect significant differences.

3. Results

3.1. Proximal and distal parts of the trachea

Histamine, up to a concentration of 3×10^{-4} M, administered to the inside of epithelium-intact tracheal tubes induced a similar contraction in proximal and distal segments. However, at a concentration of 1 mM histamine, the

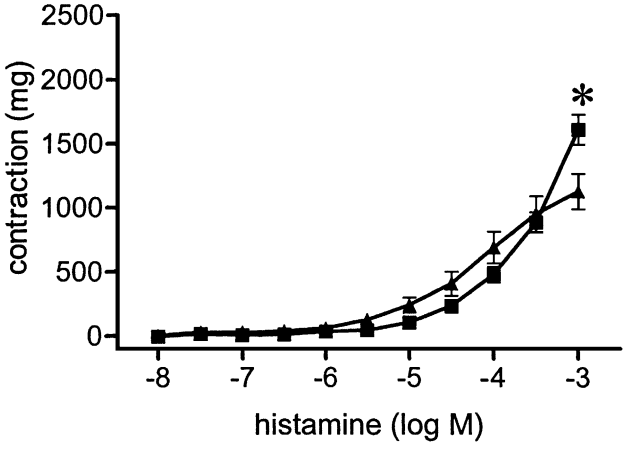

Fig. 1. Contractions of proximal (squares) and distal (triangles) guinea pig trachea segments with intact epithelium following exposure to increasing histamine concentrations administered at the mucosal side. Proximal and distal segments respond differently to the highest concentration of histamine only ($*P < 0.02$). $N = 9$ trachea segments per group.

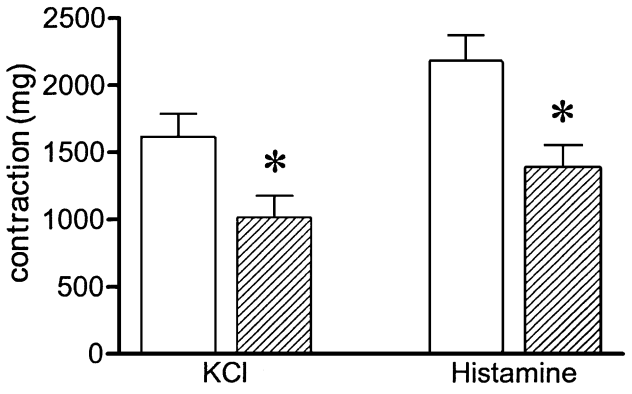

Fig. 2. Contractions of proximal (white bars) and distal (hatched bars) guinea pig trachea segments with intact epithelium upon stimulation with 50 mM KCl or 1 mM histamine administered to the serosal side. Proximal segments contract more than distal segments ($*P<0.05$). $N=9$ trachea segments per group.

contractions of the proximal segments were significantly enhanced (42%, $P<0.02$, Fig. 1) compared to those of the distal ones.

The effects were even more pronounced when KCl (50 mM) or histamine (1 mM) was administered to the outside of intact tracheal segments. The contractions of proximal parts were significantly enhanced as compared to distal parts ($>55\%$, $P<0.05$, Fig. 2).

Interestingly, when histamine was administered to the inside of the tracheal tubes after removal of the epithelium it more than doubled the trachea contractions in the proximal parts as compared to the distal parts ($P<0.0005$, Fig. 3).

3.2. Endogenous-relaxing mediators

The role of endogenous relaxing mediators, like NO and prostaglandins, were investigated to elucidate the differences

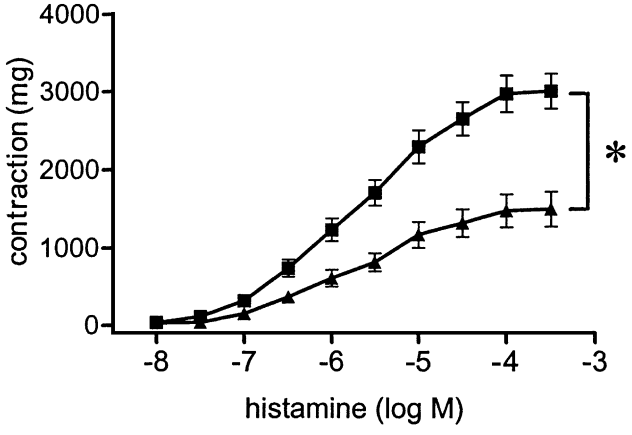

Fig. 3. Contractions of proximal (squares) and distal (triangles) guinea pig trachea segments without epithelium following exposure to increasing histamine concentrations at the mucosal side. Proximal segments are more responsive to histamine than distal segments ($*P<0.0005$). $N=9$ trachea segments per group.

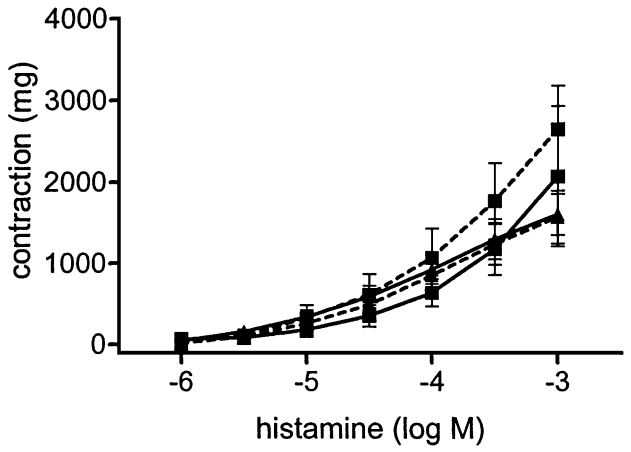

Fig. 4. Effect of mucosal inhibition of NOS in guinea pig trachea segments with intact epithelium on contractile responses to mucosal histamine. The NOS inhibitor, L-NAME (dashed lines), tended to increase histamine-induced contractions in proximal (squares), but not in distal (triangles), segments as compared to its inactive enantiomer, D-NAME (solid lines). $N=6$ trachea segments per group.

in reactivity between proximal and distal tracheal segments. Incubation with the NOS inhibitor, L-NAME, at the inside of the tracheal tubes did not affect the histamine concentration–response curves of the distal parts and tended to increase the responsiveness of the proximal parts ($P>0.05$, Fig. 4). Further, L-NAME administered to both the inside and the outside of epithelium-denuded segments did not change the reactivity of the distal or proximal parts (Fig. 5).

Administration of the cyclo-oxygenase inhibitor, indomethacin, to the inside of the tracheal tubes significantly enhanced the contractions to histamine in both the distal and

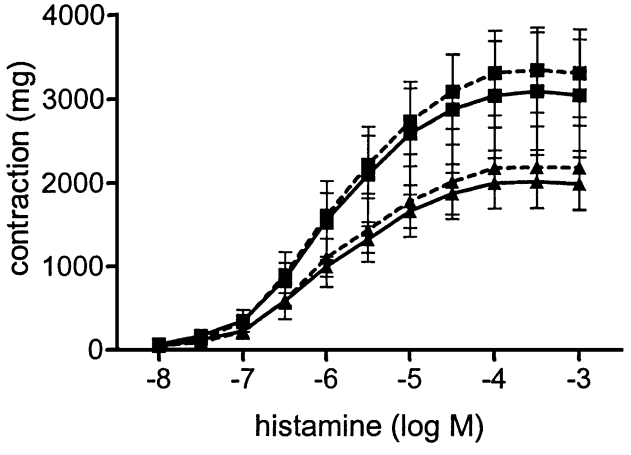

Fig. 5. Effect of concomitant mucosal and serosal inhibition of NOS in guinea pig trachea segments without epithelium on contractile responses to mucosal histamine. The NOS inhibitor, L-NAME (dashed lines), had no effect on histamine-induced contractions in proximal (squares) or distal (triangles) segments as compared to its inactive enantiomer, D-NAME (solid lines). $N=6$ trachea segments per group.

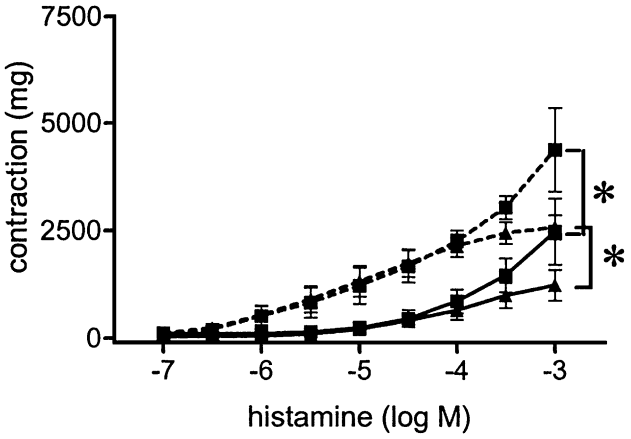

Fig. 6. Effect of mucosal cyclo-oxygenase inhibition on contractile responses of guinea pig trachea segments with intact epithelium. Contractions were induced by histamine at the mucosal side. The cyclo-oxygenase inhibitor, indomethacin (dashed lines), increased histamine-induced contractions both in proximal (squares) and in distal segments (triangles) as compared to its vehicle (solid lines). (* $P<0.05$) $N=6$ trachea segments per group.

proximal segments (Fig. 6). Interestingly, indomethacin administered both to the inside and the outside of epithelium-denuded segments did not affect the responsiveness of the distal segments but significantly enhanced the contractions of proximal segments (>90%, $P<0.05$, Fig. 7).

In additional experiments, proximal segments with epithelium were incubated with indomethacin at the inside or at the inside and the outside to firstly assess the concentration–response curve of histamine administered to the inside. Then a single concentration of histamine (10^{-3} M) was administered to the outside. Intact proximal trachea segments incubated at both sides with indomethacin were more reactive to

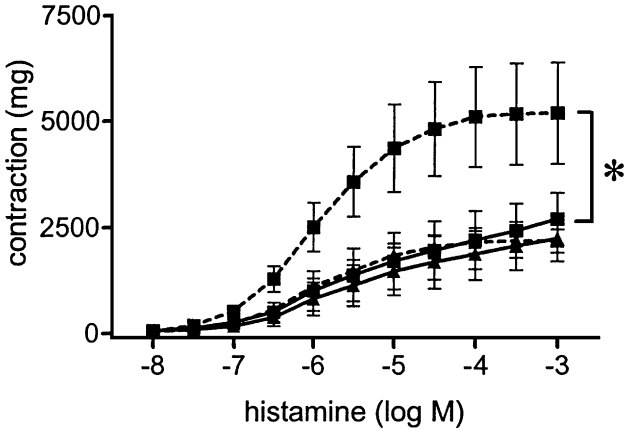

Fig. 7. Effect of concomitant serosal and mucosal cyclo-oxygenase inhibition with indomethacin on contractile responses of guinea pig trachea segments without epithelium. Contractions were induced by histamine at the mucosal side. Indomethacin had no effect on histamine-induced contractions in distal segments (dashed line, triangles) compared to its vehicle (solid line, triangles). In contrast, indomethacin substantially increased responsiveness to histamine of proximal segments (dashed line, squares) as compared to vehicle treatment (solid line, squares). (* $P<0.05$) $N=6$ trachea segments per group.

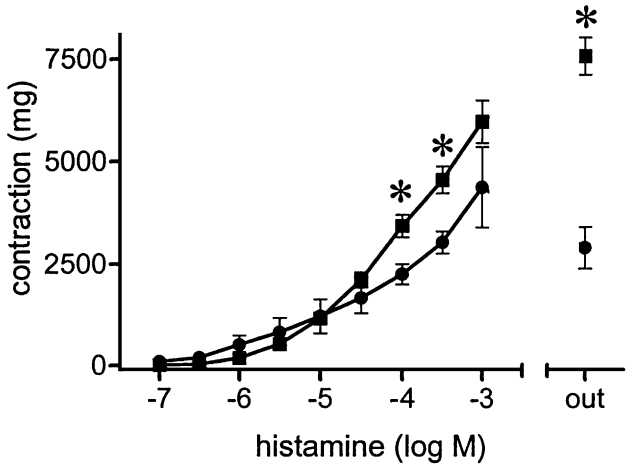

Fig. 8. Comparison of the effects of mucosal cyclo-oxygenase inhibition with concomitant mucosal and serosal cyclo-oxygenase inhibition in proximal trachea segments with intact epithelium. Mucosal histamine induced stronger contractions (* $P<0.05$) when the cyclo-oxygenase inhibitor, indomethacin, was present both in the mucosal and the serosal buffer (squares) than when indomethacin was administered in the mucosal buffer only (circles). Histamine given serosally (out) after the mucosal exposure led to a decrease in tension in the absence of a cyclo-oxygenase inhibitor in the serosal buffer, but to an additional increase in tension when a cyclo-oxygenase inhibitor was present in the serosal buffer. (* $P<0.05$, with vs. without serosal cyclo-oxygenase inhibitor). $N=9$ trachea segments per group.

histamine (>50% at concentrations 10^{-4} and 3×10^{-4} M, $p<0.05$, Fig. 8) than segments exposed to indomethacin at the inside only. Administration of histamine to the outside, induced an additional contraction in segments exposed to indomethacin at both sides, whereas a relaxation was observed in segments exposed to indomethacin at the inside only. This resulted in a net difference of 4672 mg ($P<0.05$, Fig. 8).

4. Discussion

Mucosal perfusion with histamine of trachea segments with intact epithelium resulted in concentration-dependent increases in reactivity that were similar for proximal and distal segments at all concentrations, the highest excepted. At the latter concentration, stronger contractions were observed in proximal segments than in distal ones. This could be explained by the possibility that the epithelium of proximal parts is more permeable to histamine than epithelium of distal parts. The epithelium is a physical barrier for agents that are applied to the luminal side of the airways (Sparrow et al., 1995). Recently, we found that the epithelial layer is a physical barrier for acetylcholine in a guinea pig tracheal tube set up (Folkerts et al., 2001) and Fedan et al. (2000) demonstrated a reversible increase in epithelial permeability in guinea pig airways after ozone treatment (Fedan et al., 2000). In two other studies, guinea pigs became hyperreactive to inhaled but not intravenously administered histamine or methacholine suggesting that the epithelial diffusion barrier

to airborne agents had been compromised (Yeadon et al., 1992; Matsubara et al., 1995). In our study however, the proximal segments showed stronger contractions than the distal segments when intact trachea segments were stimulated non-specifically at the serosal side with KCl or histamine which exclude a role for permeability changes. Apparently, the intrinsic contractile capacity of tracheal smooth muscle is higher in proximal than in distal tissue. Combining the results of Figs. 1 and 2, we hypothesized that intact epithelium conceals the higher contractile potential of proximal trachea segments. This hypothesis was substantiated by the observation that epithelium-denuded proximal tubes exhibited substantially higher contractions than similar distal ones throughout the range of concentrations of histamine administered to the mucosal side.

Removal of the epithelium probably not merely accentuates the higher contractile potential of proximal, as compared to distal, trachea segments. Notably, contractions of intact proximal and distal segments up to 1000 mg appeared similar (Fig. 1), whereas differences between proximal and distal segments without epithelium were obvious at contractions far below 1000 mg (Fig. 3). Although the permeability of epithelium to histamine might be different in proximal and distal segments, implication of a relaxing factor in the epithelium of the proximal trachea that partly compensates for the locally higher intrinsic smooth muscle contractility is likely as well.

In the light of these considerations, the role of one of the major epithelium-derived relaxing substances in the airways, NO (Gaston et al., 1993; Ward et al., 1995), was studied. It has been reported that NO is produced by the tracheal epithelium (Figini et al., 1996) and implicated in the functional antagonism of histamine-induced airway contractions (Folkerts et al., 1995; Nijkamp et al., 1993; Yan et al., 1994). If this would be the main case in the proximal trachea, inhibiting epithelial NOS should have a more pronounced effect in proximal than in distal trachea segments. The NOS inhibitor, L-NAME, had no effect in distal segments but tended to increase the response to histamine in the proximal segments throughout the concentration–response curve as compared to the inactive enantiomer, D-NAME. Since NOS inhibition was without any effect in epithelium-denuded tissues, a minor part of the reactivity differences in epithelium-denuded tracheas might be accounted for by a lack of epithelial NO. So another relaxant activity besides NO possibly contributes to the supposed relaxant activity in proximal segments. Therefore, the contribution of cyclo-oxygenase metabolites to the relaxant activity was studied as well. Like NO, prostaglandins have been shown to counteract histamine-induced contractions in the airways (Anderson et al., 1979; Braunstein et al., 1988). In intact tracheas, indomethacin administered to the inside increased the reactivity to histamine in proximal and distal segments to a similar extent. This indicates that, contrary to the findings for NO, relaxing cyclo-oxygenase metabolites are synthesized in both proximal and distal trachea segments. Since indomethacin increased tracheal tension when given mucosally, the epithelium seemingly was the most likely source of cyclo-oxygenase metabolites in these experiments.

Quite surprisingly, however, indomethacin administered concomitantly at the mucosal and serosal sides of epithelium-denuded tracheas led to a marked increase in histamine-induced contractions in proximal, but not in distal, segments as compared to controls. The data suggest that in proximal trachea segments, apart from the contributions of epithelium-derived relaxing factors, one or more non-epithelial cyclo-oxygenase metabolites clearly compensate for the higher intrinsic smooth muscle reactivity in these segments. This notion was confirmed by the observation that histamine induced stronger contractions in proximal segments with intact epithelium when indomethacin was present both at the mucosal and the serosal sides as compared to the mucosal side only. Moreover, serosal stimulation with histamine led to an additional increase in tension when mucosal and serosal cyclo-oxygenase products were inhibited, but to a decrease in tension when the cyclo-oxygenase inhibitor was present at the mucosal side only. Thus, inhibition of cyclo-oxygenase in the serosa of the proximal trachea had a larger effect on smooth muscle contraction than inhibition of cyclo-oxygenase in the mucosa. In contrast, inhibition of cyclo-oxygenase in the serosa of the distal trachea had no additional effect on smooth muscle reactivity compared to cyclo-oxygenase inhibition in the mucosa only. Therefore, we conclude that cyclo-oxygenase products from the serosa of the proximal trachea have a dampening effect on the local muscle reactivity. This finding may be very relevant. Since histamine induces the production of cyclo-oxygenase products (Anderson et al., 1979; Braunstein et al., 1988) and since histamine is mainly produced by serosal mast cells that inhabit the serosa, serosal cyclo-oxygenase products may be important functional antagonists of allergen-induced contractions in proximal trachea segments.

The introduction of the perfused trachea organ bath system made it possible to study the consequences of selective stimulation of the mucosal or serosal tissues. Such studies have identified the tracheal epithelium as a source of relaxing factors (see (Folkerts and Nijkamp, 1998) for a review). However, to our knowledge, this is the first study to demonstrate that the serosa has a distinct role in the modulation of tracheal tone as well and that this is particularly true for the proximal part of the trachea. Further research is needed to identify which cyclo-oxygenase products counteract contraction and which cells produce the products. In addition to the epithelium, constitutive cyclo-oxygenase expression has been demonstrated in cultured human airway fibroblasts and smooth muscle cells (Petkova et al., 1999). Another question that remains to be answered is whether hyperreactivity induced by cyclo-oxygenase inhibition is indeed merely the result of downregulating the synthesis of cyclo-oxygenase products. Since cyclo-oxygenase and lipoxygenase compete for arachidonic acid, cyclo-oxygenase inhib-

ition may increase the synthesis of lipoxygenase products (Babu and Salvi, 2000). This may provide an alternative mechanism for indomethacin-induced hyperreactivity in our experiments. To test this, experiments in the presence of leukotriene receptor antagonists, or the combined presence of cyclo-oxygenase and lipoxygenase inhibitors, should be done.

We conclude that in the guinea pig, intrinsic contractility of tracheal smooth muscle tissue is higher in the proximal than in the distal segment. This higher contractility is clearly compensated for both by mucosal NO and serosal cyclo-oxygenase products. Neglecting these differences can easily lead to larger variances when studying multiple pieces of one trachea. This may lead to the use of more, rather than less experimental animals in order to attain statistically significant results. In addition, false conclusions may be drawn regarding intrinsic smooth muscle contractility, or the importance of NOS or cyclo-oxygenase in the trachea. Conceivably, similar complications may apply to other pathways as well. Thus, it is important to perform pilot experiments to investigate whether there are differences within the trachea regarding the effects on tracheal tone of the particular pathway under study.

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