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Endogenous nitric oxide modulation of potassium-induced changes in guinea-pig airway tone

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1 An experimental set up is used whereby the serosal (out)side or mucosal (in)side of the guinea-pig isolated tracheal tube can be stimulated selectively with drugs and reactivity measured.

2 Potassium induces a concentration-dependent (5-70 mM) monophasic contraction of tracheal tubes when added on the outside. In contrast, on the inside, potassium induces a concentration-dependent relaxation at low concentrations (5-40 mM) which was reversed into a contraction up to approximately basal tone at higher concentrations (50-70 mM).

3 Epithelium denudation reversed the potassium-induced relaxation into a contraction. Interestingly, in the 'half' epithelium-denuded trachea the contractions were significantly ($P<0.01$) reduced by 46% compared to complete epithelium-denuded tissues.

4 Incubation with the nitric oxide (NO) synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 120 μM) for 30 min on the inside of the tracheal tube completely prevented the relaxation. However, L-NAME did not reverse the potassium-induced relaxation into a contraction. This indicates that potassium does not penetrate through the epithelial layer.

5 It is concluded that depolarization of smooth muscle cells leads to a monophasic contraction and that depolarization of the epithelium leads to a relaxation of tracheal smooth muscle. The epithelial layer has an important barrier function and can release relaxing factors like NO.

Keywords: Guinea-pig airway; nitric oxide; airway epithelium; tracheal responsiveness; potassium

Introduction

Evidence points to an important role for the airway epithelium in modulating the responsiveness of the underlying smooth muscle. Hyperresponsiveness of the airways, which is the main feature of asthma, is associated with damage or loss of the airway epithelium in bronchial asthma (Laitinen *et al.*, 1985; Djukanovic *et al.*, 1990). Removal of the epithelial layer from isolated airways of several mammalian species has been shown to enhance the contractile response to various bronchoconstrictor agents, including histamine, acetylcholine, 5-hydroxytryptamine and leukotrienes C_4 and D_4 (Folkerts *et al.*, 1989; Vanhoutte, 1989). In addition, arachidonic acid induces a relaxation in intact tracheae and a contraction in epithelium-denuded tissues (Nijkamp & Folkerts, 1986). These findings led to the concept that intact epithelium may act as a protective barrier between constrictors and airway smooth muscle (Munakata *et al.*, 1989; 1990; Sparrow & Mitchell, 1991) or it may modulate the airway tone through the release of relaxant substances, which may include prostanoids and epithelium-derived relaxing factor(s). Recently, we provided pharmacological evidence that one of the epithelium-derived relaxing factors might be nitric oxide (NO) (Nijkamp *et al.*, 1993). In a perfused tracheal tube set up according to Pavlovic *et al.* (1989), in which the serosal (out)side or the mucosal (in)side of the trachea can be stimulated selectively with drugs, it was demonstrated that preincubation on the inside with N^{ω}-nitro-L-arginine methylester (L-NAME) enhanced the contractile response to histamine and cholinoceptor agonists. This effect was mimicked by removal of airway epithelium, suggesting that the airway epithelial layer releases nitric oxide which counteracts the bronchoconstrictor effect of spasmogens (Nijkamp *et al.*, 1993; Nijkmap & Folkerts, 1994). Bovine and human cultured airway epithelial cells have the capacity to produce nitric oxide (Robbins *et al.*, 1992; Chee *et al.*, 1993). Immunocytochemical staining has demonstrated the expression of the nitric oxide synthase enzymes in human and rat airway epithelial cells (Kobzik *et al.*, 1993; Springall *et al.*, 1993). From these data it is likely that NO is produced by the epithelium under physiological conditions. In the present study tracheal smooth muscle (outside) or tracheal epithelium (inside) was selectively depolarized with the non-receptor operating drug, potassium.

Methods

Animals

Specified-pathogen-free guinea-pigs (400-500 g, male Dunkin Hartley, Harlan Olac Ltd, England) were housed under controlled conditions. Water and commercial chow were allowed *ad libitum*. The guinea-pigs were free of respiratory airway infections as assessed by the health monitoring quality control report by Harlan Porcellus (England), and by histological examination.

Airway responsiveness in vitro

Guinea-pigs (400 to 500 g) were killed with an overdose of pentobarbitone sodium (Nembutal, 0.6 g kg^{-1} body weight, intraperitoneally). Tracheae were dissected free of connective tissue and blood vessels, isolated and perfused in an organ bath according to a modified method of Pavlovic and colleagues (1989). In short, two hooks were inserted through opposite sides of the tracheal wall with the smooth muscle between them. One hook was attached to a fixed point in the organ bath; the other hook was connected to an isometric transducer (Harvard Bioscience, Kent, UK). The tracheal tension was set at an optimal counter weight of 2 g. The inside of the trachea was perfused (1 ml min^{-1}) independently from the outside with Krebs solution of the following composition (mM): NaCl 118.1, KCl 4.7, $CaCl_2$, 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose, 8.3, which was continuously gassed

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with a 5% CO_2 and 95% O_2 gas mixture as described before (Nijkamp *et al.*, 1993). Every 15 min the Krebs buffer was refreshed on both sides until a stable tone was reached (usually within 90 min). A stable tone remained for at least 60 additional min. Thereafter, the inside or the outside of the trachea was perfused for 30 min with Krebs buffer containing 20 μl solvent solution (Krebs buffer) or L-NAME (120 μM). Accordingly, cumulative concentration-response curves were obtained with potassium (5–70 mM) on the inside or the outside of the trachea. Only one concentration-response curve was made from a tissue segment.

In separate series of experiments the epithelial layer was completely removed or 50% was removed with a cotton swab (Folkerts *et al.*, 1989). In the tissues in which the epithelium was removed for 50% the reactivity was measured in the epithelium-denuded part and the Krebs-solution with or without drugs *first* passed along the intact part of the trachea and then passes along the epithelium-denuded part.

Nitric oxide measurements

Nitric oxide was measured in 100 μl organ bath fluid obtained from the tracheal tube before and after stimulation with potassium. These samples were injected into a gas stripping apparatus containing 2 ml of a 1% solution of NaI in glacial acetic acid which was connected to a Sievers 270B NO analyser (Boulder, CO, USA). The sensitivity of the NO analyser is <10 pmol ml^{-1} with a linearity of 4 orders of magnitude. Calibrations were made according to the manufacturer's instructions with standard solutions of sodium or potassium nitrite (Menon *et al.*, 1991).

Histology

To verify that the tissues were partly or completely denuded of epithelium, histological examinations were peformed. The tissues were fixed in neutral buffered formaldehyde (10%) and embedded in paraffin blocks. Sections measuring 5 μm were cut and stained with hematoxylin and eosin for histological evaluation.

Statistical analysis

Data are expressed in g contraction or mM for the EC_{50} value (the concentration provoking a half maximal response) and presented as mean ± standard error of the mean (s.e.mean). When the means of two groups were compared, Student's *t* test for unpaired observations was used. When maximal effects were compared of more than two groups one-way ANOVA was used. When the means of the same group before and after stimulus were compared, a Bonferroni correction was performed. In all analyses, statistical significance was accepted when *P* value (two tailed) was <0.05.

Chemicals

N^{ω}-nitro-L-arginine methyl ester (L-NAME) was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Potassium chloride and all other drugs for the Krebs solution and NO measurements were obtained from the Onderlinge Pharmaceutische Groothandel (Utrecht, The Netherlands).

Results

Airway responsiveness in vitro

Increasing concentrations of potassium added to the outside of the trachea induced a profound monophasic concentration-dependent contraction of the smooth muscle (Figure 1). The maximal response was reached at a concentration of 50 mM and the tissues tended not to relax afterwards. Increasing concentrations of potassium added on the inside of the trachea induced a significant ($P<0.01$, Bonferroni) concentration-dependent relaxation up to a concentration of 40 mM. Reversal of relaxation was observed with higher concentrations of potassium; however, basal tone was not exceeded (Figure 1). After tissues had reached their maximal contraction due to outside stimulation, the preparations were subsequently exposed on the inside to potassium (70 mM) which resulted in a relaxation of approximately 0.52 ± 0.19 g ($n=13$). Similarly, tissues that had been relaxed due to inside exposure, contracted after outside stimulation 1.53 ± 0.19 g ($n=9$).

To compensate for the excess in positive ions after the addition of potassium, an experiment was peformed in which 78 mM NaCl was used in the Krebs-solution instead of 118 mM NaCl. When the tissues were stimulated with one dose of potassium (40 mM), a similar relaxation and contraction after inside and outside stimulation was obtained as was observed in the concentration-response curve with potassium under standard Krebs conditions.

Nitric oxide measurement

The amount of NO in the organ bath samples of the continuously perfused tracheal tubes was below the detection limit (<10 pmol ml^{-1}) of the NO analyzer.

Epithelium-denudation

Inside stimulation with potassium of epithelium-denuded tissues resulted in a reversal of the relaxation into a potent contractile response (max $= 1.92 \pm 0.17$ g) (Figure 2). Outside stimulation with potassium of epithelium-denuded tissues resulted in a similar maximal contraction of the tissue, however the EC_{50} value was significantly decreased compared to inside stimulation (Table 1). In half-denuded preparations, in which the epithelium-containing part was first exposed to potassium and the reactivity was measured in the epithelium-denuded part, the maximal contraction was significantly ($P<0.01$) reduced by 46% to 1.04 ± 0.18 g ($n=6$, Figure 2). The EC_{50} value was 27.4 ± 1.7 mM and did not differ from completely epithelium-denuded tissues (Table 1).

Histological evaluation

Figure 3 shows that, by using the cotton swab technique (Folkerts *et al.*, 1989), the epithelium is partly removed. On the left hand side the epithelium was removed and in this part the reactivity was measured. On the right hand side the epithelium

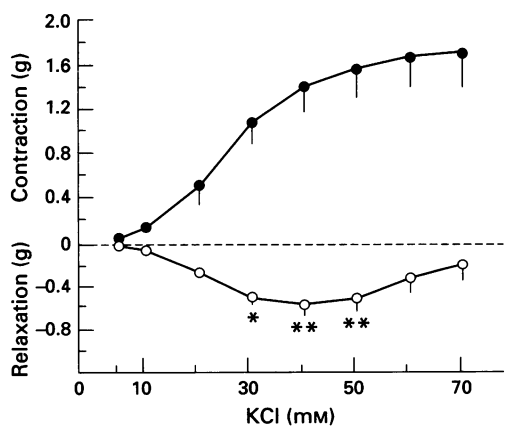

Figure 1 Potassium concentration-response curve obtained on guinea-pig tracheal tubes: (●) outside stimulation ($n=13$); (○) inside stimulation ($n=9$). Outside stimulation resulted in a monophasic concentration-dependent contraction. Inside stimulation caused a relaxation up to 40 mM potassium and a reversal of relaxation at concentrations >40 mM. *$P<0.05$; **$P<0.01$ as compared to basal value (Bonferroni).

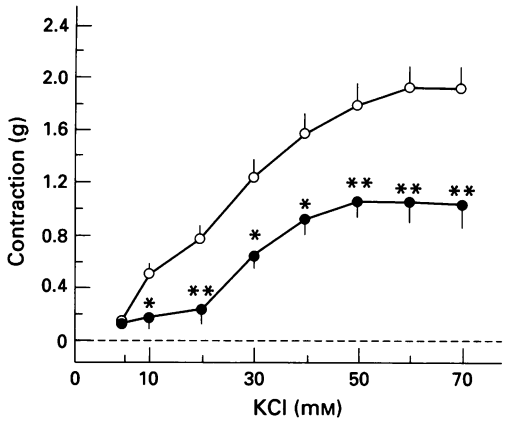

Figure 2 Potassium concentration-response curve obtained on the inside of guinea-pig tracheal tubes: (○) completely epithelium-denuded ($n=8$); (●) half epithelium-denuded ($n=6$). Epithelium denudation reversed the potassium-induced relaxation into a potent contractile response. In 'half' epithelium-denuded tracheae the responses were significantly suppressed compared to completely epithelium-denuded tissues (*$P<0.05$; **$P<0.01$, Student's unpaired t test).

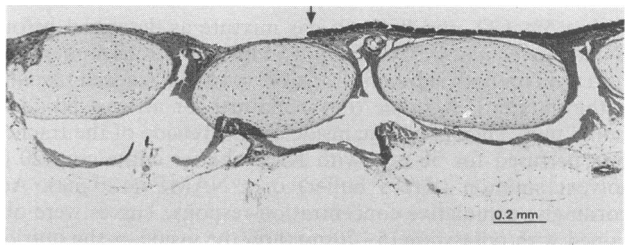

Figure 3 Light micrograph of paraffin-embedded material. Guinea-pig tracheal tube half epithelium-denuded. Left side epithelium-denuded, right side epithelium intact. Arrow indicates the border between denuded and intact epithelium.

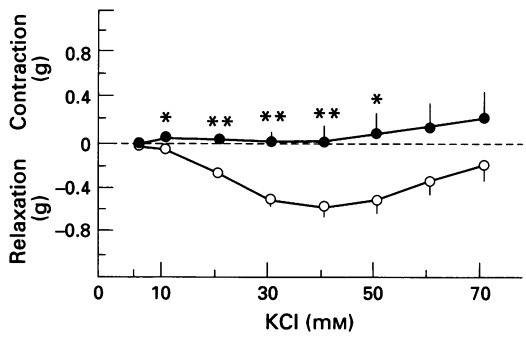

Figure 4 Potassium concentration-response curve obtained on the inside of guinea-pig tracheal tubes. Inside stimulation (○) control ($n=9$); (●) N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME, inside 120 μM, $n=7$). Incubation of L-NAME on the inside completely suppressed the potassium-induced relaxation (*$P<0.05$, **$P<0.01$, Student's unpaired t test).

was still intact and this was part was first exposed to potassium.

Effect of L-NAME incubation

The potassium-induced relaxation (inside stimulation) was completely prevented when L-NAME was incubated for 40 min on the inside (Figure 4). Note, that this treatment did not reverse the relaxation into a contraction. When L-NAME was incubated on the outside, potassium still induced relaxation although the effect was blunted. Incubation of L-NAME on the inside had no effect on the potassium concentration-response curve made on the outside (Table 1).

Incubation of the tissues on the outside with L-NAME did not affect the KCl concentration-response curve made on the outside (Table 1). When L-NAME was added on the outside of epithelium-denuded tissues, the potassium concentration-response curves did not differ whether made on the inside or outside of the trachea (Table 1). EC_{50} values did not differ between the experimental groups (Table 1).

Discussion

In standard organ baths potassium induces an initial contraction followed by a relaxation and a sustained contraction of intact trachea (Nielsen-Kudsk *et al.*, 1986). In the present study a tracheal tube set-up was used that enabled us to apply drugs selectively to the serosal (out)side or the epithelial (in)-side of the trachea. Using this method it was found that potassium induced a monophasic contraction when it was added on the serosal side. In contrast, potassium induced a relaxation when added on the inside. Similar results were obtained if 78 mM NaCl was used in the Krebs solution instead of 118 mM NaCl, indicating that the excess of positive ions was not responsible for this effect. From these results it may be concluded that depolarization of smooth muscle cells leads to a contraction, whereas depolarization of epithelial cells results in a relaxation of tracheal tubes. Epithelium removal caused a reversal of the relaxation into a potent contractile response.

Munakata *et al.* (1988) did not find a relaxation after addition of K(in) in tracheal tissues with spontaneous tone and

Table 1 Effect of N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) on potassium-induced contractions of guinea-pig tracheal tubes with and without epithelium

	Epithelium intact		*Epithelium denuded*	
Treatment	*Max. response* (g tension)	*EC_{50} value* (nM)	*Max. response* (g tension)	*EC_{50} value* (n)
KCl (in)	−0.59 ± 0.10 (9)	22.4 ± 1.6	1.92 ± 0.17 (8)	27.1 ± 1.2
+ L-NAME (in)	−0.05 ± 0.16 (7)*	ND		
+ L-NAME (out)	−0.19 ± 0.24 (5)	20.6 ± 2.1	1.98 ± 0.15 (5)	24.5 ± 1.5
KCl (out)	1.71 ± 0.21 (13)	25.2 ± 1.2	1.86 ± 0.21 (5)	22.0 ± 1.9*
+ L-NAME (in)	1.68 ± 0.38 (7)	24.8 ± 1.0		
+ L-NAME (out)	2.53 ± 0.38 (10)	26.9 ± 2.4	1.89 ± 0.18 (5)	22.8 ± 1.6

Maximal responses (in g) and EC_{50} values (in mM) of concentration-response curves after mucosal (inside) and serosal (outside) stimulation are presented.
*$P<0.05$ as compared to control value (KCl in). The number of experiments is given in parentheses. The contractile responses did not differ significantly from each other (ANOVA). ND = not detectable.

kata *et al.* (1990), demonstrated that depolarization of lial cells did not result in the release of nitric oxide. Three ıs for this discrepancy might be offered. Firstly, in the ıentioned studies guinea-pig perfusion pressure was red which means that 15 to 25 ml Krebs solution min^{-1} l into the tracheal tube. Pressure differences due to iction of the tracheal tube were registered. In our ex- ents, Krebs solution 2 ml min^{-1} was sucked through the a and isometric tension differences were measured ac- g to Pavlovic *et al.* (1989). Further, the total perfusion was only 7 ml whereas Munakata *et al.* (1990) used l. Therefore, the relatively high speed of perfusion and eater volume of perfusion fluid may have masked the of nitric oxide. Furthermore, the differences in the d of perfusion of the tracheal tube and measurement of responses (pressure vs tension) may contribute to the nt results obtained.

'half' epithelium-denuded tracheal tubes the potassium ıtration-response curve on the inside was suppressed by ompared with tissues completely denuded of epithelium. ium first passes the epithelium-intact part before sti- ng the epithelium-denuded region in which the con- e response was measured. The epithelial layer probably d an epithelium-derived relaxing factor that could in- the contraction in the epithelium-denuded part. This lium-derived relaxing factor could be NO, since L- E completely prevented the relaxation of intact tissues nside stimulation with potassium. Recently, we demon- l that the epithelial layer might be a source of NO since pithelium removal or incubation of intact tracheal tubes itric oxide synthesis inhibitors, induced a similar degree way hyperresponsiveness to histamine. Moreover, in- on of epithelium-denuded tissues with nitric oxide sis inhibitors did not induce an additional increase in responsiveness (Nijkamp *et al.*, 1993). In agreement his it was found, that cultured bovine airway epithelial ıetabolize L-arginine to L-citrulline, an effect blocked by oxide synthesis inhibitors, indicating that airway epi- cells have the capacity to produce nitric oxide (Robbins 1992). A human cultured epithelial cell line produces spontaneously, which can be suppressed by a nitric synthesis inhibitor and restored by L-arginine, suggesting nstitutive production of NO (Chee *et al.*, 1993). The utive nitric oxide synthase is present in rat airway epi- cells but not in human airway epithelium (Kobzik *et al.*, The cause of this discrepancy might be that the speci- of the human airways were taken from diseased lungs the healthy airways of the rat. Indeed, in biopsies of ı airways, immunoreactivity to inducible nitric oxide se was seen in the epithelium in 22 of 23 asthmatic cases, ly 2 of 14 non-asthmatic controls (Springall *et al.*, 1993). the present study, besides the epithelial cells, sensory and/or cholinergic nerves could be responsible for NO synthesis (Lei *et al.*, 1993). Although the parasympathetic pathway remains a viable option for NO production, the sensory nerves can be excluded since potassium still induces a tracheal relaxation in capsaicin-pretreated guinea-pigs (personel observation).

Addition of potassium on the inside of intact tracheae does not stimulate the smooth muscle cells because incubation of L-NAME on the inside only prevented the relaxation. The relaxation did not reverse into a contraction as seen in epithelium-denuded tissues. From the present results it is likely that the epithelial layer acts as a firm barrier, since even such a relatively simple molecule as potassium was not able to penetrate through the epithelial layer. The epithelial barrier exhibits a high transepithelial potential difference and has a high tissue resistance to electrolytes (Welsh, 1987). Gao & Vanhoutte (1994) suggested that Na^+-K+-ATPase plays a role in the barrier function of the epithelium to potassium chloride. Damage, to the epithelial layer may: (a) break this protective barrier and (b) diminish the release of relaxant factors like NO. Interestingly, the degree of epithelial damage in the respiratory tract of asthmatics is associated with the degree of airway hyperresponsiveness (Laitinen *et al.*, 1985). In addition we have demonstrated that the virus-induced airway hyperresponsiveness in the guinea-pig is associated with epithelial damage (Folkerts *et al.*, 1992; 1993). After inhalation of low doses of L-arginine this hyperresponsiveness is completely blocked (Folkerts *et al.*, 1995). Further, L-NAME did not additionally enhance the virus-induced airway hyperresponsiveness. Therefore, a diminished production of NO during airway contractions might contribute to the airway hyperresponsiveness. In contrast, as mentioned above in diseased airways the inducible nitric oxide synthase is present and under basal conditions the NO concentration in exhaled air is high in asthmatic patients (Kharitonov *et al.*, 1994). However, it cannot be excluded that during bronchoconstriction the NO released by the activity of the constitutive enzyme is diminished. Indeed, in healthy human subjects nitric oxide concentrations in exhaled air are increased after breath holding or exercise (Persson *et al.*, 1993), which might be released to relax the airways.

It is concluded that depolarization of tracheal smooth muscle cells leads to a contraction, and depolarization of tracheal epithelial cells leads to a relaxation. This study demonstrates that the epithelial layer acts as a protective barrier and can release relaxing factor(s).

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