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Factors that determine acetylcholine responsiveness of guinea pig tracheal tubes

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Abstract

Acetylcholine administered to the inside of epithelium-denuded tracheal tubes did cause a potent contraction (2486 ± 120 mg). In contrast, a response was hardly observed in tissues with an intact epithelial layer (674 ± 81 mg), which was due to both the synthesis of nitric oxide and the activity of acetylcholinesterase, since the contractions to acetylcholine were significantly enhanced after preincubation with N^{ω}-nitro-L-arginine methyl ester (L-NAME) or physostigmine (1374 ± 65 and 1120 ± 65 mg, respectively). In addition, the suppressive effect was caused by the barrier function of the epithelial layer, since preincubation of epithelium-denuded tissues with physostigmine significantly increased the pD_2 value for acetylcholine (7.48 ± 0.04) compared to intact tissues preincubated with physostigmine (6.32 ± 0.10) and epithelium-denuded preparations without physostigmine (6.37 ± 0.06). Increasing concentrations of physostigmine administered to the inside of tissues *with* epithelium did induce a potent spontaneous contraction (1440 ± 350 mg) that was prevented by atropine. In contrast to what was expected, the contractile response was diminished in tracheal tubes *without* epithelium (665 ± 221 mg). It is concluded that contractions of epithelium-denuded tissues are more pronounced to exogenous than to endogenous acetylcholine, and that the production and breakdown of this neurotransmitter is very rapid in intact guinea pig airways. Moreover, the release of nitric oxide and the barrier function of the epithelium did suppress the responsiveness to acetylcholine.

Keywords: (Guinea pig); Tracheal reactivity; Acetylcholinesterase; Nitric Oxide (NO); Epithelium

1. Introduction

Efferent activity in the pulmonary vagal nerves plays an important role in the regulation of airway diameter, mucus secretion and ion transport (Skoogh and Ullmann, 1991; Barnes, 1992). Parasympathetic nerves are abundantly present beneath the airway mucosa and between epithelial cells (Jeffery, 1994; Canning and Fischer, 1997). Apart from parasympathetic nerves, airway epithelial cells can be a source of acetylcholine as well (Reinheimer et al., 1996). Investigations on the role of airways epithelium in modulating the sensitivity and responsiveness of the underlying smooth muscle to drugs and neurotransmitters have recently been reviewed (Sparrow et al., 1995; Folkerts and Nijkamp, 1998). It seems that epithelium removal can potentiate some, though not all, spasmogenic drugs acting on tracheal smooth muscle. Removing the epithelium from isolated guinea-pig trachea increases the response to acetylcholine (Flavahan et al., 1985; Holroyde, 1986; Murlas, 1986; Fine et al., 1989). Interestingly, in contrast to the response to acetylcholine, contractions due to methacholine, carbamylcholine and bethanechol are more pronounced and only slightly, if any at all, enhanced by epithelium removal (Goldie et al., 1986; Raeburn et al., 1986; Small et al., 1990). This could be explained by the action of acetylcholinesterase. Contrasting findings have been published on the location of this enzyme in the airways (*in* versus *below* the epithelial layer) (Small et al., 1990; Koga et al., 1992; Taisne et al., 1997). Moreover, acetylcholine might be able to release relaxing factors like prostaglandin E_2 and nitric oxide from the epithelial layer

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as has been demonstrated for a number of other agonists (Brunn et al., 1995; Folkerts and Nijkamp, 1998; McParland et al., 2000).

In the present study, the perfused tracheal tube set up was used (Nijkamp et al., 1993; Folkerts et al., 1995a,b,c). This allowed us to selectively apply drugs and neurotransmitters to the mucosal side or serosal side of the trachea by which the functional role of the airway epithelium could be investigated in more detail. Using this model, the involvement of the airway mucosa as a physical barrier for acetylcholine and as a source for epithelium-derived relaxing factors was elucidated. In addition, the modulation of the acetylcholine-induced contractions by acetylcholinesterase was investigated in intact and epithelium-denuded guinea pig tracheas.

2. Materials and methods

2.1. Animals

Specified-pathogen-free guinea-pigs (400–500 g, male Dunkin Hartley, Harlan Olac, England) were housed under controlled conditions. They were allowed water and commercial chow 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. The experiments were approved by the ethical committee of the Faculty of Pharmacy, Utrecht University, The Netherlands.

2.2. Airway responsiveness in vitro

Guinea-pigs were killed with an overdose of pentobarbitone sodium (Euthesate®, 1.0 g/kg body weight, intraperitoneally). Tracheas were dissected free of connective tissue and blood vessels, isolated and perfused in an organ bath according to a modified method of Pavlovic et al. (1993). 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 isometric transducers were connected to an analog-digital converter (Intelligent International PCI System, Burr Brown, Tucson, Arizona, USA), which connected the organ baths to a semi-automatic set-up. This enabled continuous sampling, on-line equilibrium detection, and real-time display of the responses on a computer screen of up to six baths. The tracheal tension was set at an optimal counter weight of 2 g. The inside of the trachea was perfused (2 ml/min) 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 with a 5% CO_2 and 95% O_2 gas mixture as described (Nijkamp et al., 1993). Krebs buffer was refreshed four times on both the mucosal and serosal side with 15-min time intervals; after which a stable tone was reached within 30 min. Only one concentration–response curve was made on a tracheal tube preparation.

2.3. Epithelium-removal

In a separate series of experiments, the epithelial layer was removed with a cotton swab as was described before (Nijkamp et al., 1993; Folkerts et al., 1989, 1995b). To verify that the tissues were denuded of epithelium, histological examinations were performed. 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.

2.4. Experimental protocols

2.4.1. Epithelium as a barrier and / or a source for epithelium-derived relaxing factors

Acetylcholine concentration–response (10^{-8}–10^{-3} M) curves were made on the inside of intact or epithelium-denuded tracheal tubes and on the outside of intact preparations. To investigate the involvement of nitric oxide, intact tissues were incubated at the inside with the nitric oxide synthase inhibitor L-NAME (120 μM), its inactive enantiomer D-NAME (120 μM) or the solvent solution (saline) for 20 min; after which acetylcholine concentration–response curves were made. In additional experiments, epithelium-denuded tissues were incubated at the inside with L-NAME (120 μM) for 20 min; after which acetylcholine concentration–response curves were made.

2.4.2. Activity of acetylcholinesterase

Concentration–response (10^{-8}–10^{-4} M) curves with the acetylcholinesterase inhibitor physostigmine were made on the inside of intact and epithelium-denuded tracheal tubes. To verify the specificity of this agent, concentration–response (10^{-8}–10^{-4} M) curves with neostigmine were made at the inside of intact tissues. Moreover, physostigmine concentration–response curves were made after preincubation of the tissues on the inside with 10^{-7}, 10^{-6} or 10^{-5} M atropine for 10 min. To investigate the involvement of parasympathetic ganglia and prostaglandins, the tissues were preincubated on the inside with the ganglion blocker hexamethonium (10^{-6} M, 10 min) or the cyclooxygenase inhibitor indomethacin (10^{-7} M, 20 min), before concentration–response curves with physostigmine were made.

2.4.3. Effect of acetylcholinesterase inhibition on acetylcholine concentration–response curves

Physostigmine (10^{-5} M, 5–10 min) was incubated at the inside of intact and epithelium-denuded tissues; after which acetylcholine concentration–response curves were made.

2.5. Statistical analysis

The final tracheal contraction in response to 10^{-3} M acetylcholine or 10^{-4} M physostigmine is expressed in mg contraction and the sensitivity of the tissues as pD_2-value, which is the $-\log$ of the EC_{50} value (the concentration provoking a half-maximal response). The pD_2-value was only calculated when a clear plateau was reached after a concentration response curve. Data are presented as mean $\pm$ standard error of the mean (S.E.M.). Student's *t*-test for unpaired observations was used. In all analyses, statistical significance was accepted when P value (two-tailed) was < 0.05.

2.6. Chemicals

Acetylcholine chloride, indomethacin, N^{ω}-nitro-L-arginine methyl ester (L-NAME) and N^{ω}-nitro-D-arginine methyl ester (D-NAME) were obtained from Sigma (St. Louis, MO, USA). Hexamethonium was from Fluka (Buchs, Switserland). Neostigmine methylsulphate was from Merck (Amsterdam, The Netherlands). Physostigmine sulphate, atropine sulphate and all other compounds for the Krebs solution were obtained from the Onderlinge Pharmaceutische Groothandel (Utrecht, The Netherlands).

3. Results

3.1. Airway responsiveness in vitro

Guinea-pig tracheal tubes with an intact epithelial layer and stimulated at the inside with increasing concentrations of acetylcholine show a small contraction (Fig. 1A, Table 1). In epithelium-denuded tissues the maximal response was increased by 270% (Fig. 1A). Similar results were obtained when acetylcholine was added to the outside of intact tissues (Fig. 1B, Table 1). Preincubation with the nitric oxide synthase inhibitor L-NAME on the inside of intact tissues doubled ($P < 0.01$) the acetylcholine-induced contractions compared to intact control preparations (Fig. 1B). However, nitric oxide synthase inhibition in intact tissues increased tracheal responsiveness less (Fig. 1B, open squares) than epithelium removal did (Fig. 1A, closed circles). D-NAME did not modulate tracheal responsiveness (Table 1). In epithelium-denuded tissues, L-NAME incubation did not further increase the sensitivity of the tracheal tubes when acetylcholine was administered to the

Table 1
Final contraction and pD_2-value to acetylcholine and physostigmine in guinea-pig tracheal tube
Data are expressed as mean $\pm$ S.E.M. The number of animals is given between parentheses. ND = not detectable, since no clear plateau was reached after the concentration–response curve.

	Final contraction in mg tension	pD_2-value
Acetylcholine (10^{-8}–10^{-3}M)		
Control inside	674 ± 81	ND (6)
Epithelium-denuded inside	2486 ± 120[a]	6.37 ± 0.06 (5)
Intact outside	2330 ± 202[a]	6.22 ± 0.08 (6)
Control inside	587 ± 317	ND (5)
L-NAME inside	1374 ± 65[b]	ND (6)
D-NAME inside	404 ± 96	ND (5)
Physostigmine (10^{-7}–10^{-4} M)		
Control inside	1440 ± 350[b]	6.02 ± 0.08 (9)
Epithelium-denuded inside	665 ± 221	6.15 ± 0.17 (4)
Acetylcholine (10^{-9}–10^{-4}M) after bolus Physostigmine (10^{-5} M)		
Control inside	1120 ± 131[b]	6.32 ± 0.10 (5)
Epithelium denuded	1627 ± 139[c]	7.48 ± 0.04[d,e] (4)

[a] $P < 0.01$ compared to the control-inside group stimulated with acetylcholine.
[b] $P < 0.05$ compared to the control-inside group stimulated with acetylcholine.
[c] $P < 0.05$ compared to control-inside group with physostigmine.
[d] $P < 0.01$ compared to control-inside group with physostigmine.
[e] $P < 0.01$ compared to the epithelium-denuded group stimulated with acetylcholine (Student's unpaired *t*-test).

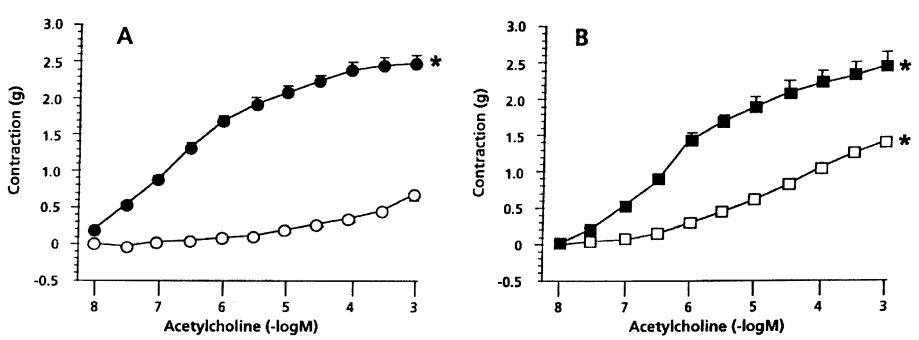

Fig. 1. Acetylcholine concentration–response curves on the inside or outside of isolated guinea-pig tracheal tubes with or without the NO synthase inhibitor L-NAME. (A) Acetylcholine hardly induced a contraction on the inside of intact tracheal tubes (open circles). The acetylcholine concentration–response curves were enhanced in epithelium-denuded trachea tubes (closed circles). (B) When acetylcholine was added to the outside of intact tissues (closed squares) the contractions were similar compared to the acetylcholine concentration–response curve made on the inside of epithelium-denuded preparations (A, closed circles). Incubation of intact tissues with L-NAME resulted in an enhancement of the acetylcholine concentration–response curve made on the inside (open squares) compared to intact controls incubated with saline (data not shown in the figure, see Table 1). * Maximal contraction is increased compared to controls. See Table 1 for details.

inside. The maximal contraction for the control group was 2201 ± 551 mg ($n = 5$) and for the L-NAME group 1812 ± 333 mg ($n = 6$).

3.2. Activity of acetylcholinesterase

Increasing concentrations of the acetycholinesterase inhibitor, physostigmine, added to epithelium-denuded tissues, induced a small contraction (Fig. 2, Table 1). However, in intact tracheal tubes the response to physostigmine was more pronounced and even larger than the contraction observed to exogenous acetylcholine administered to intact tracheal tubes ($P < 0.05$, Table 1). Similar results were obtained when an other acetylcholinesterase inhibitor, neostigmine, was used (1841 ± 321 mg, $pD_2 = 5.63 \pm 0.33$, $n = 6$). Moreover, the physostigmine concentration–response curves were concentration-dependently inhibited by atropine. The maximal responses were significantly reduced by 63%, 80% and 98% with 10^{-7}, 10^{-6} or 10^{-5} M atropine, respectively (Fig. 3). These observations suggest that contractions were induced by endogenously released acetylcholine. In contrast, contractions were not influenced by preincubation with the ganglion blocker hexamethonium (= 1490 ± 316 mg, $n = 5$) or the cyclooxygenase inhibitor indomethacin (1606 ± 268 mg, $n = 8$). Atropine, hexamethonium and indomethacin had no effect on basal tone.

3.3. Effect of acetylcholinesterase inhibition on acetylcholine concentration–response curves

When physostigmine (10^{-5} M) was added as a bolus to the inside of epithelium-denuded tracheal tubes, it induced a contraction of 1028 ± 126 mg (see individual tracing, Fig. 4). In intact preparations this contraction was again enhanced by 67% (1718 ± 202, $P < 0.05$).

When the physostigmine-induced contractions in intact tissues were followed by acetylcholine concentration–response curves, the contraction was significantly increased compared to the tissues not incubated with physostigmine (Table 1). In epithelium-denuded preparations incubated with physostigmine, both the contraction ($P < 0.05$) and pD_2-value ($P < 0.01$) increased compared with intact tissues incubated with the inhibitor (Figs. 4 and 5). Further, it should be noted that in the epithelium-denuded group preincubated *with* physostigmine, the pD_2-value for acetylcholine was significantly ($P < 0.01$) increased compared to the epithelium-denuded group *not* incubated with

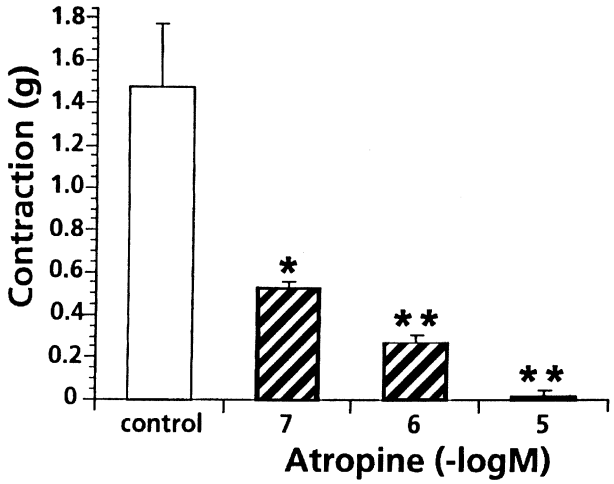

Fig. 3. The maximal contraction induced by administration of physostigmine to the inside of the guinea-pig tracheal tube was concentration-dependently (10^{-7}, 10^{-6}, 10^{-5} M) inhibited by atropine; $^{*}P < 0.05$, $^{**}P < 0.01$.

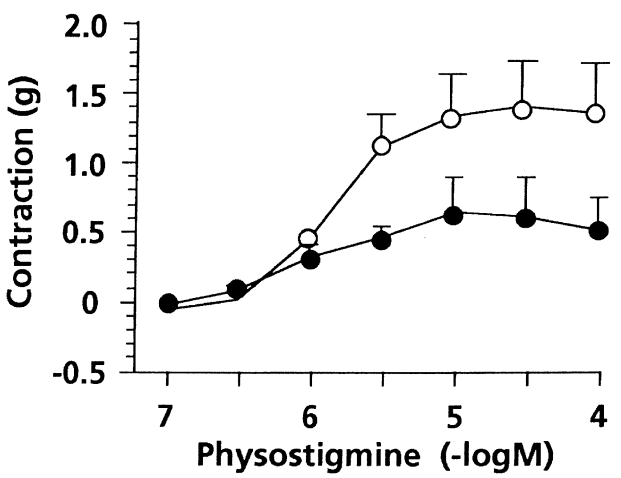

Fig. 2. Physostigmine concentration–response curves on the inside of isolated guinea-pig tracheal tubes. Physostigmine induced a potent concentration-dependent contraction when administered on the inside of the tissues (open circles). The contractions were less pronounced when physostigmine was administered to epithelium-denuded tissues (closed circles). See Table 1 for details.

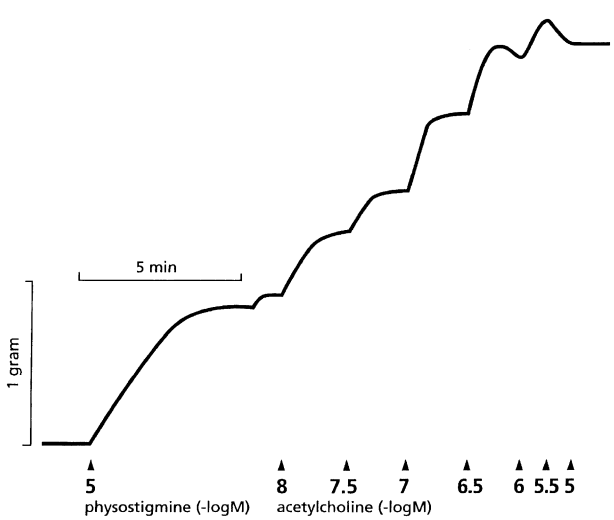

Fig. 4. Individual tracing of a contraction of an epithelium-denuded guinea pig tracheal tube stimulated on the inside with physostigmine followed by an acetylcholine concentration–response curve. Administration of the different concentrations is indicated by arrowheads.

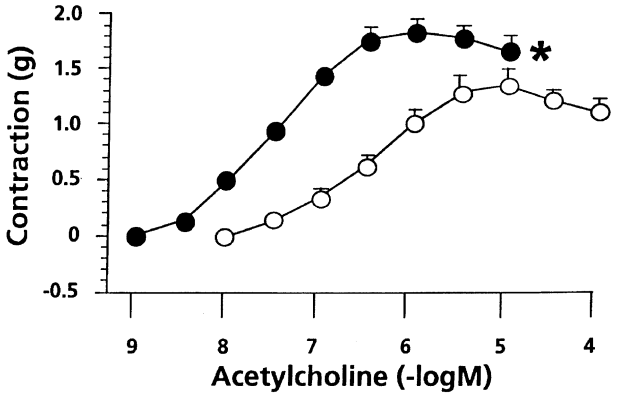

Fig. 5. Acetylcholine concentration–response curve on intact and epithelium-denuded guinea-pig tracheal tubes after preincubation with physostigmine (10^{-5} M, 5–10 min). The acetylcholine concentration–response curve in intact preparations was enhanced (open circles) after physostigmine incubation compared to the contractions obtained in intact tissues not incubated with physostigmine (Table 1). In epithelium-denuded tissues the acetylcholine concentration–response curve was enhanced after physostigmine incubation (closed circles) compared to the intact preparations (open circles). * Maximal contraction and pD_2-value are significantly increased (open circles). See Table 1 for details.

physostigmine (Table 1). Together, these results suggest a barrier function for the epithelial layer.

4. Discussion

In the present study a marked difference in acetylcholine responsiveness was observed between intact and epithelium-denuded tissues. Concentration–response curves obtained when injecting acetylcholine on the inside of epithelium-denuded tissues and on the outside of intact tissues were identical. This suggests that the contraction itself does not release epithelium-derived relaxing factors. In another study, using the same experimental set-up, we made concentration–response curves on the inside of intact guinea-pig tracheal tubes with the cholinergic receptor agonists arecoline, carbamylcholine and methacholine (Nijkamp et al., 1993). These cholinergic agonists were far more potent than acetylcholine, suggesting that somehow the acetylcholine-induced contractions were suppressed. Epithelium-derived nitric oxide can modulate airway responsiveness in vitro and in vivo (Nijkamp et al., 1993; Folkerts et al., 1995a,b; Ricciardolo et al., 1996). Nitric oxide synthesis inhibition did not modulate the responsiveness for arecoline, and slightly enhanced the contractility in response to carbachol and methacholine (to about 2100 mg) (Nijkamp et al., 1993). It has been suggested that the selective potentiation of acetic—as opposed to carbamic acid esters of choline—might be explained in terms of the acetic esters having the ability to liberate epithelium-derived relaxing factors (Goldie et al., 1988). Although smooth muscle contractions in response to acetylcholine indeed increased after L-NAME incubation, it did not reach the levels observed in epithelium-denuded tissues or the responses obtained with the different cholinergic receptor agonists (see above and Nijkamp et al., 1993). Since it was shown that nitric oxide synthesis inhibition in epithelium-denuded tissues did not further enhance tracheal responsiveness, it is likely that nitric oxide is synthesised by the epithelial layer (Nijkamp et al., 1993; Ricciardolo et al., 2000; Tamaoki et al., 2000). Moreover, it was demonstrated in human bronchi that nitric oxide synthesis inhibition increased histamine responsiveness without damaging the integrity of the epithelial layer (Folkerts et al., 1995c).

Acetic esters of choline are susceptible to hydrolysis by cholinesterases, whereas the corresponding carbamic acid esters are resistant to hydrolysis (Koelle, 1965). To investigate the involvement of acetylcholinesterase in this response, concentration–response curves were obtained in the presence of esterase inhibitors. Physostigmine induced a potent contraction when added on the inside of intact tracheal tubes. Similar results were obtained with neostigmine, thus excluding nonspecific actions of physostigmine unrelated to acetylcholinesterase inhibition. In addition, physostigmine-induced contractions were concentration-dependently inhibited by atropine, thus demonstrating involvement of muscarinic receptors and acetylcholine in the response to physostigmine. Since the ganglion blocker hexamethonium did not affect physostigmine-induced contractions, it is possible that acetylcholine originates from the basal turnover at the synaptic cleft of the neuro-effector junction of the parasympathetic nerve. There are indications that prostaglandins can modulate acetylcholine release from parasympathetic nerves (Ullman et al., 1988; Deckers et al., 1989; Wessler et al., 1990). However, indomethacin did not change the contractions induced by physostigmine, which might be explained by the fact that the experiments were done under basal conditions and not after electrical field stimulation (Ullman et al., 1988; Deckers et al., 1989). Physostigmine-induced contractions (10^{-4} M) were more pronounced than those produced by 10^{-3} M acetylcholine (Table 1). This implies a high acetylcholine turnover and a high activity of acetylcholinesterase under basal conditions.

In epithelium-denuded preparations, physostigmine-induced contractions were less pronounced than in intact tissues. Three suggestions may be put forward to explain this result. First, it was recently demonstrated that airway epithelial cells store and release acetylcholine, although this was less in guinea pig airways (Reinheimer et al., 1996). In intact preparations, acetylcholine could theoretically be released from both parasympathetic nerves and epithelial cells. Hence, in epithelium-denuded preparations, acetylcholinesterase inhibition should result in less acetylcholine release and therefore less contraction. Secondly, epithelium removal may damage parasympathetic nerves, an effect that may result in a decreased acetylcholine release. However, this hypothesis is unlikely since Wessler et al. (1990) showed that epithelium removal enhanced

acetylcholine-release from nerve endings both under basal conditions and after electrical stimulation. The most likely explanation is that in intact tissues the released acetylcholine is trapped under the epithelial layer. Removal of the epithelial layer allows acetylcholine to diffuse away from the target organ causing dilution in the organ bath fluid. Hence, inhibition of acetylcholine-breakdown results in less pronounced effects.

When acetylcholine concentration–response curves were made on intact tissues preincubated with physostigmine, a significantly enhanced contraction was observed and the pD_2-value increased up to a level comparable to that obtained in epithelium-denuded tissues without physostigmine (Table 1). It is therefore obvious that acetylcholinesterase plays a crucial role in the suppression of the acetylcholine-induced contractions. However, we cannot exclude the possibility that enzymes, other than acetylcholinesterase, metabolize acetylcholine as well. Fedan and Frazer (1992) also incubated guinea pig tracheal tubes on both sides with a much lower concentration physostigmine (10^{-7} M) for a longer time period (1.5 h), which induced a small contraction. In their study, the sensitivity of intact tissues increased for acetylcholine after mucosal administration, the maximal contraction was hardly effected and the relative role of epithelial acetylcholinesterase could not be defined.

Interestingly, in our study the acetylcholine concentration–response curves were even further increased after physostigmine preincubation in epithelium-denuded tissues compared to intact tissues. This could partly be explained by the fact that the contractions by physostigmine itself were significantly lower in epithelium-denuded tissues (665 mg) compared to the intact group (1440 mg). The lower increase in smooth muscle tone due to physostigmine could permit a higher increase in response to acetylcholine. However, the pD_2-value for acetylcholine was also significantly increased compared to both the intact group incubated with physostigmine and to the epithelium-denuded group not pretreated with physostigmine (Table 1). Therefore, it is likely that the epithelial layer represents a physical barrier for acetylcholine/physostigmine, or that epithelium-derived relaxing factors other than nitric oxide and prostaglandins are involved. It has been demonstrated earlier that in guinea-pig tracheal tube preparations, the epithelium provides a barrier not only for acetylcholine but also for a variety of agonists (Munakata et al., 1989; Fedan and Frazer, 1992).

There is speculation about the location of acetylcholinesterase. Acetylcholine might be metabolized by an epithelium-dependent acetylcholinesterase activity (Koga et al., 1992; Taisne et al., 1997). However, Small et al. (1990) demonstrated by histochemical staining that acetylcholinesterase was not present in the epithelial layer from guinea-pig airways. The underlying tissues contained acetycholinesterase-positive nerve fibers and the trachealis muscle itself was positively stained. Results from the present study support these data since physostigmine induces contractions in epithelium-denuded tissues. In addition, the pD_2-value for acetylcholine was further increased in epithelium-denuded tissues preincubated with physostigmine, compared to the epithelium-denuded tissues not preincubated with this agent (Table 1).

In conclusion, it is clear that there is a remarkable difference in responsiveness to acetylcholine in preparations with or without epithelium. This is partly due to epithelium-derived relaxing factors like nitric oxide (Ricciardolo et al., 2000; Tamaoki et al., 2000). Moreover, there is a high endogenous release of acetylcholine in the guinea pig trachea, but this is quickly hydrolysed by endogenous acetylcholinesterase and therefore does not induce a contraction. The activity of acetylcholinesterase is so high that it even prevents clear contractions to very high concentrations of exogenous administered acetylcholine (10^{-3} M). If the contractions to physostigmine and acetylcholine were summated, the final contraction did not differ between the intact and epithelium-denuded groups (about 2700 mg). However, there is an additional increase in the sensitivity after epithelium removal that is suggestive for a barrier function of the epithelium.

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