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The influence of epithelium on the responsiveness of guinea-pig isolated trachea

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1 This study was designed to investigate the possibility that tracheal epithelium generates a relaxant factor analogous to the endothelial-derived relaxant factor (EDRF) of vascular tissue. The absence of such a factor following epithelial damage in diseases such as asthma might help to explain the airway hyperreactivity characteristic of such diseases.

2 Removal of epithelium by rubbing enhanced the sensitivity of guinea-pig isolated tracheal chains to 5-hydroxytryptamine, histamine, acetylcholine, adenosine, isoprenaline and also minimally to KCl. Responses to $LaCl_3$ and electrical field stimulation were not affected. Low concentrations of adenosine produced contractions only in tissues denuded of epithelium.

3 In the presence of indomethacin 1.4 μM or dithiothreitol (DTT) 1 μM, dose-response curves to histamine were moved to the left in both control and rubbed tissues, and the maximum response was increased. The difference in sensitivity between tissues with and without epithelium was not affected by indomethacin, but was slightly reduced by DTT. Phenidone (0.1 mM) also increased the maximum responses, but increased the sensitivity only of the tissues with intact epithelium, to the same level as that seen in the tissues denuded of epithelium.

4 Superfusion cascade studies provided no evidence for the generation of a relaxant factor from tracheal epithelium.

5 It is suggested that the supersensitivity produced by removal of the epithelium is not due to the removal of a relaxant factor, but rather to the removal of a permeability barrier, allowing a greater concentration of agonist at the level of the underlying smooth muscle.

Introduction

Following the original observation of Furchgott & Zawadzki (1980), several groups have shown that vascular endothelium can generate a humoral factor which relaxes the adjacent vascular smooth muscle. This factor (subsequently named endothelium-derived relaxing factor or EDRF) is released in response to a number of stimuli, including acetylcholine, bradykinin, substance P, 5-hydroxytryptamine and noradrenaline (Furchgott, 1984; Furchgott *et al.*, 1984).

EDRF has a very short half-life, reported as 6.3 s in one study (Griffith *et al.*, 1984). The factor has not been structurally identified but is known not to be a prostaglandin, adenosine or a purine nucleotide. Suggested identities for EDRF include a free radical (Furchgott *et al.*, 1984) or an aldehyde, ketone or lactone (Griffith *et al.*, 1984).

EDRF is capable of modulating the reactivity of vascular smooth muscle to a number of constrictor substances, and consequently may have great clinical relevance. Thus pathological damage to the endothelium would be expected to result in enhanced responses to vasoconstrictor stimuli, leading to exacerbation of the clinical condition. Such suggestions have already been made with regard to pulmonary hypertension and shock lung (Chand & Altura, 1981) and also variant angina (Cocks & Angus, 1983).

The present study was designed to investigate the possibility that airway epithelium might produce a factor comparable with EDRF. The study was prompted by the thought that destruction or functional impairment of airway epithelium, such as occurs in asthma (Laitinen *et al.*, 1985) would then be expected to augment bronchoconstrictor responses to a variety of stimuli. Such airway hyperresponsiveness is a characteristic feature of asthma.

Methods

Male Dunkin-Hartley guinea pigs (350–700 g) were killed by cervical dislocation and the tracheae removed. Each trachea was cut into 8 rings (each containing 2–3 cartilaginous rings) which were allocated alternately to provide two paired preparations, each of 4 rings. One preparation in each pair was denuded of epithelium by inserting a moistened wooden applicator stick into the lumen of each ring, and gently rubbing with a corkscrew motion 5–6 times. All the rings were then cut open opposite the trachealis, and sutured together to form chains.

Tissue bath studies

The tissues were suspended in 12 ml baths containing a modified Krebs solution (Denis *et al.*, 1982) of the following composition (mM): NaCl 120, $NaHCO_3$ 25, $MgSO_4$ 0.5, KH_2PO_4 1.2, KCl 4.72, $CaCl_2$ 2.5 and dextrose 11. The solution was maintained at 37°C and gassed with 5% CO_2 in O_2. Tissues were suspended under an initial tension of 1.5 g and allowed to equilibrate for at least 1 h with frequent washing by overflow. The tension was then readjusted until steady at 1 g, when a cumulative dose-response curve was established to one of the following agonists: acetylcholine, histamine, 5-hydroxytryptamine (5-HT), KCl, adenosine, isoprenaline or lanthanum chloride ($LaCl_3$). In some experiments the tissues were suspended between parallel platinum wire electrodes 1 cm apart and subjected to field stimulation. Square wave pulses were delivered for 10 s periods from a Grass S88 stimulator at a pulse width of 2 ms and a supramaximal voltage of 50 V at source (Jones *et al.*, 1980a). A frequency-response curve was constructed using frequencies of 1–128 Hz. The tissue was allowed to return to its resting tension between each period of stimulation.

In another series of experiments a cumulative dose-response curve to histamine was established before and after incubating the tissue for 1 h with 0.1 mM phenidone, 1 mM dithiothreitol (DTT) or 1.4 μM indomethacin. The resting tension was readjusted to 1 g before establishing the second dose-response curve. These concentrations of DTT and phenidone have been shown previously to be maximally effective against EDRF (Griffith *et al.*, 1984).

In all experiments, responses were recorded isometrically by means of Grass FT03 transducers.

Superfusion studies

To detect the release of a relaxant factor from tracheal epithelium, some tissues were subjected to cascade superfusion. Paired tissues (numbered 1 and 2) from one guinea-pig were suspended in jacketed tissue baths in such a way that the Krebs solution superfusing them subsequently passed over a pair of tracheal preparations (numbered 3 and 4) from a second guinea-pig. Epithelium was removed from all the tissues except tissue number 1. Krebs solution (composition as described above) was warmed to 37°C, gassed with 5% CO_2 in O_2 and passed over the tissues at 10 ml min^{-1}.

A resting tension of 1 g was imposed on the tissues throughout. After a 1 h equilibration period, a non-cumulative dose-response curve was established to histamine by injecting aliquots of histamine solutions into the perfusion lines immediately proximal to the upper two tissues. Histamine solutions of various concentrations were prepared in Krebs solution, and were injected in 0.2 ml volumes over a period of 2–3 s.

By this technique, any relaxant factor released from the epithelium of tissue 1 would be expected to suppress responses of tissue 3 immediately below it, in comparison with the responses of tissue 4. The transit time between upper and lower tissues was approximately 1 s.

Histology

After completion of dose-response studies tissues were removed from the organ baths and fixed in 10% phosphate buffered formalin (pH7). Sections, 4 μm thick were then prepared, stained with haematoxylin and eosin, and examined microscopically for the presence of epithelium.

Drugs and solutions

The following drugs were used: acetylcholine chloride, 5-hydroxytryptamine creatinine sulphate, adenosine hemisulphate, indomethacin, phenidone, D,L-dithiothreitol (Sigma); potassium chloride, lanthanum chloride heptahydrate (Fisons); histamine acid phosphate (BDH); isoprenaline hydrochloride (Pharmax). Stock solutions were prepared in Krebs solution except for indomethacin which was dissolved in 0.1 M sodium carbonate and phenidone which was dissolved in saline. KCl was prepared as a 20% w/v solution in distilled water, and aliquots of this added directly to the tissue bath. Stated concentrations of KCl exclude that provided by the formulation of the Krebs solution.

When isoprenaline was used, ascorbic acid was included in the Krebs solution at a concentration of 200 μg ml^{-1}.

Analysis of results

Contractile and relaxant responses were expressed as absolute changes in tension, and then transformed to a percentage of the maximal response for each tissue. The effect of epithelial removal on the peak tension

Table 1 Responses of control and rubbed guinea-pig tracheal chains to several stimuli

Stimulus	*Response*	*Peak change in tension* (mg) *Control*	*Rubbed*	*CR*
Histamine	C	869 ± 133	1273 ± 207	8.2 (5.7–11.8)
Acetylcholine	C	1458 ± 70	1375 ± 187	12.5 (6.7–20.0)
5-Hydroxytryptamine	C	469 ± 106	510 ± 148	5.9 (4.5– 7.1)
KCl	C	1217 ± 152	992 ± 142	1.3 (1.1– 1.6)
Adenosine	C	98 ± 68	323 ± 96*	–
	R	842 ± 57	846 ± 63	3.2 (2.2– 5.0)
Field stimulation	C	383 ± 71	433 ± 105	1.6 (1.0– 2.9)
	R	700 ± 48	700 ± 53	1.0 (0.8– 1.2)
Isoprenaline	R	696 ± 55	817 ± 36	3.6 (3.1– 4.0)
$LaCl_3$	R	800 ± 34	871 ± 15	1.1 (0.8– 1.5)

Results are mean ± s.e.mean ($n = 6$). Note that adenosine and field stimulation can produce both contraction (C) and relaxation (R). CR = concentration-ratio (see Methods). Numbers in parentheses indicate 95% confidence limits. *$P < 0.05$ compared with control.

developed to each stimulus was assessed using Student's t test for paired data. Probability values smaller than 0.05 were considered significant.

Dose-response curves were analysed for parallelism (Finney, 1964); this analysis also yielded the concentration-ratio (CR) together with 95% confidence limits, where CR = EC_{50} control tissue/EC_{50} treated tissue.

Results

Histology

Five pairs of tissues were selected at random for histological evaluation. Of the five rubbed tissues, the epithelium was completely removed in 4 and 80% removed in 1. Amongst the control tissues, 50–75% of the epithelium remained intact even after several hours in organ baths. Initial studies had shown that essentially all of the epithelium was intact on the control tissues prior to suspension in organ baths.

Tissue bath studies

Histamine, acetylcholine, 5-HT and KCl produced concentration-dependent contractions of the tracheal preparations. For each agonist, removal of epithelium did not affect the maximum response of the tissue (Table 1) confirming that the technique used to remove the epithelium had not damaged the underlying smooth muscle. However, removal of the epithelium produced significant leftward parallel shifts of the dose-response curves to histamine, acetylcholine, 5-HT and KCl (Figures 1 and 3). In the case of KCl, the shift was so small that its biological significance is doubtful.

Isoprenaline and $LaCl_3$ produced concentration-dependent relaxation of the tissues. Removal of epithelium had no effect on the maximal relaxation achieved or the sensitivity of the tissues to $LaCl_3$, but significantly shifted the dose-response curve to isoprenaline to the left in a parallel fashion (Figure 1, Table 1).

At concentrations greater than 100 μM, adenosine produced concentration-dependent relaxation. Removal of epithelium produced a significant leftward parallel shift of the dose-response curve without affecting the maximal relaxation achieved. At lower concentrations, adenosine contracted the rubbed tissues but had no significant effect on the control tissues (Figure 2).

Electrical field stimulation produced a frequency-dependent transient contractile response immediately followed by relaxation. Neither of these responses was affected in any way by removal of the epithelium (Figure 2).

The effects of indomethacin, phenidone and DTT on the dose-response curve to histamine were examined (Figure 3; Table 2). Indomethacin (1.4 μM) significantly increased the maximal contractile response (more so in the control than the rubbed tissues) and significantly moved the dose-response curve to the left in a parallel fashion in both control and rubbed tissues. However, the concentration-ratio (CR) remained unaffected. DTT (1 mM) had a similar effect, but in addition reduced the CR. Phenidone (0.1 mM) enhanced the maximal responses of control and rubbed tissues to a similar degree and increased the sensitivity of the control tissues to the same level as that seen in the rubbed tissues. The sensitivity of the rubbed tissues was not altered by phenidone.

Superfusion studies

No evidence was found for a humoral relaxant factor

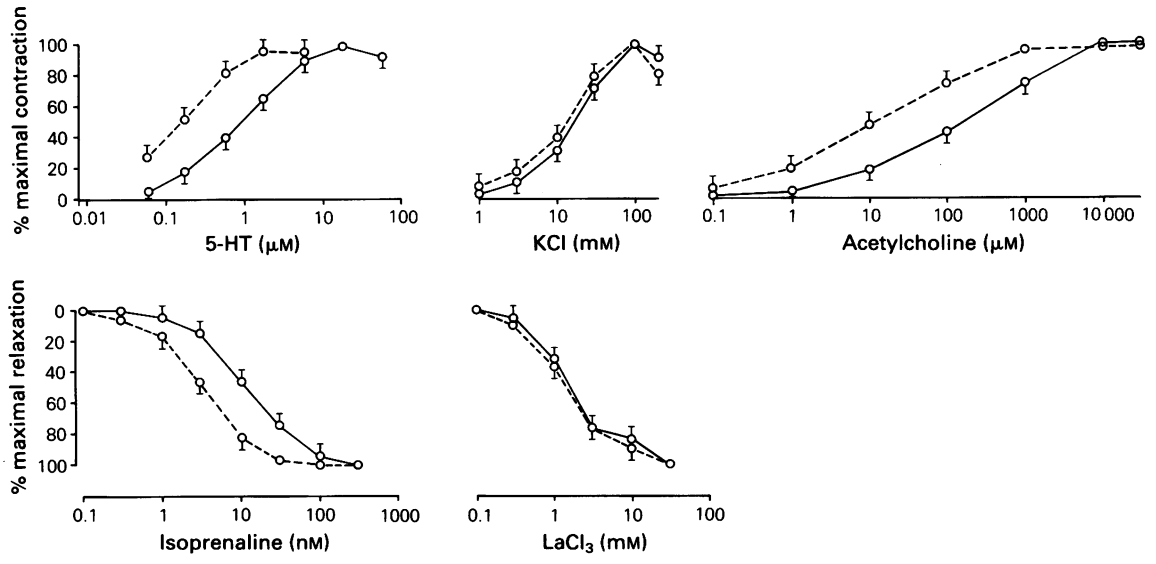

Figure 1 Responses of control (continuous line) and rubbed (broken line) guinea-pig tracheal chains to 5-hydroxytryptamine (5-HT), KCl, acetylcholine, isoprenaline and $LaCl_3$. Results are mean from 6 pairs of tissues with s.e.mean shown by vertical lines. Where s.e.mean is not shown, it falls within the symbol.

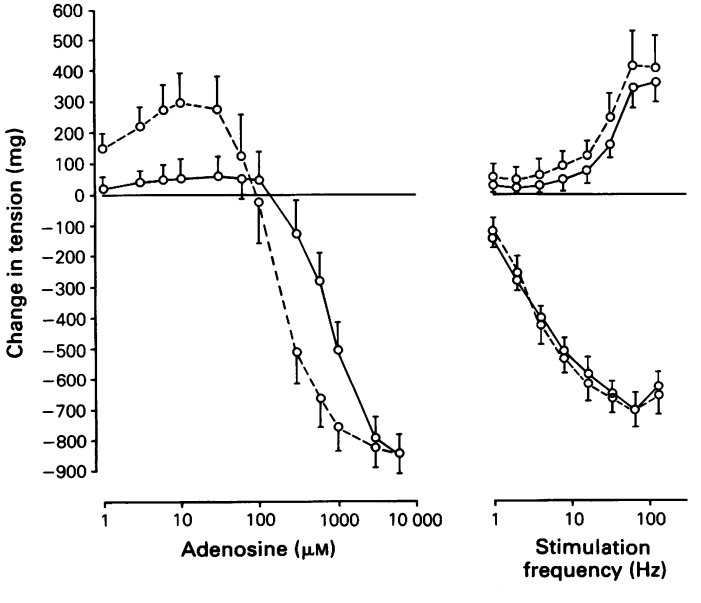

Figure 2 Responses of control (continuous line) and rubbed (broken line) guinea-pig tracheal chains to adenosine and field stimulation. Results are mean from 6 pairs of tissues with s.e.mean shown by vertical lines.
Field stimulation elicits transient contraction followed by relaxation: both responses are shown.

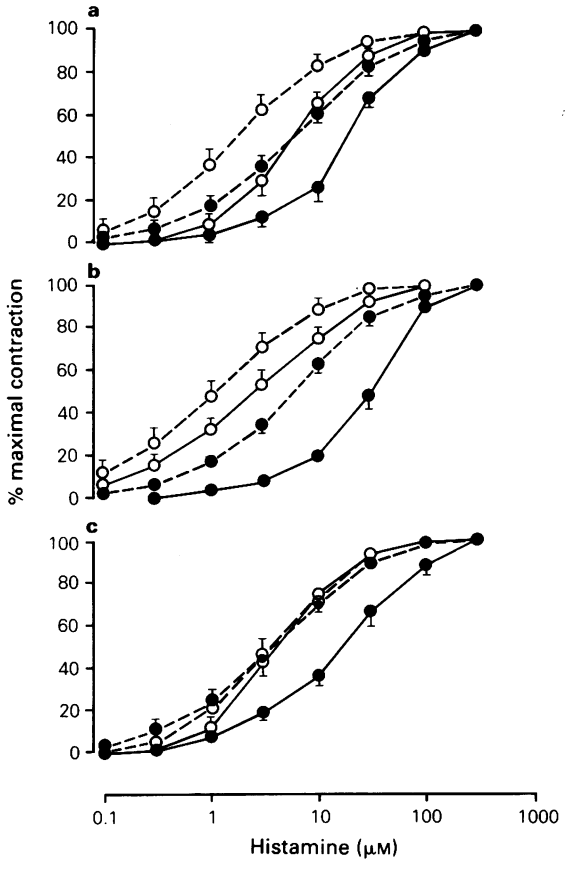

Figure 3 Responses of control (continuous line) and rubbed (broken line) guinea-pig tracheal chains to histamine before (●) and after (○) incubation with (a) indomethacin (1.4 μM), (b) dithiothreitol (1 mM) or (c) phenidone (0.1 mM). Results are mean ± s.e.mean from 6 pairs of tissues. Where s.e.mean is not shown, it falls within the symbol.

released from tracheal epithelium. Thus, although tissues 1 and 2 (see Figure 4) differed in sensitivity to histamine (CR 12.7; 95% confidence limits 6.8–24.1) as expected from the organ bath studies, tissues 3 and 4 displayed identical sensitivity (CR 1.1; 95% confidence limits 0.5–2.4). Similarly, there was no difference between any of the tissues in maximum tension developed (data not shown). Analysis of the two pairs of dose-response curves revealed no significant deviations from parallelism.

Discussion

Removal of epithelium clearly enhanced the sensitivity of guinea-pig tracheal preparations to some, but not all, stimuli. Thus, supersensitivity was seen to the contractile effects of 5-HT, acetylcholine and histamine (but not electrical field stimulation and minimally in the case of KCl), and to the relaxant effects of isoprenaline and adenosine (but not $LaCl_3$ or electrical field stimulation). Maximal responses were not affected, although in the case of adenosine, contractile responses were seen only in tissues denuded of epithelium.

The modulating influence of epithelium on tissue sensitivity could be due to the release of a relaxant factor (such as prostaglandins or a factor similar to EDRF), or to the epithelium functioning as a permeability barrier. It is considered highly unlikely that a relaxant factor was generated from the epithelium for the following reasons. Firstly, although guinea-pig trachea is known to generate PGE_2 when contracted by histamine (Gryglewski *et al.*, 1976), indomethacin did not abolish the sensitivity difference between the rubbed and normal tissues, even though dose-response curves to histamine on both tissues were shifted

Table 2 Responses of control and rubbed guinea-pig tracheal chains to histamine before and after incubation with phenidone (0.1 mM), indomethacin (1.4 μM), or dithiothreitol (1 mM)

	Before treatment			*After treatment*				
	Control (a)	*Rubbed* (b)	*CR*	*Control* (c)	*Rubbed* (d)	*CR*	P *(a-c)*	P *(b-d)*
Phenidone	667± 87	892† ±108	4.2 (3.2–5.6)	1896 ± 112	1604† ± 200	1.1 (0.9–1.4)	<0.001	<0.02
Indomethacin	971 ± 77	871† ± 79	3.2 (2.5–4.2)	3317 ± 331	2304** ± 191	3.1 (2.3–4.2)	<0.001	<0.001
Dithiothreitol	813 ± 54	838† ± 45	5.0 (4.0–6.2)	2129 ± 342	1404* ± 261	2.2 (1.7–3.0)	<0.01	>0.05

Responses are peak changes in mg tension (mean ± s.e.mean, $n = 6$).
CR = concentration-ratio (see Methods). Numbers in parentheses indicate 95% confidence limits.
*$P < 0.05$; **$P < 0.01$; †not significant, for comparison with control tissue.

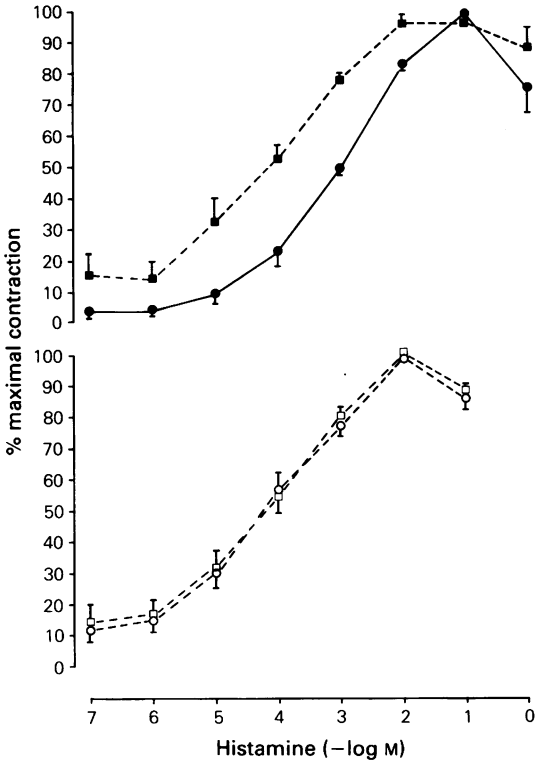

Figure 4 Responses of superfused guinea-pig tracheal chains to histamine. Tissues 1 (●) and 2 (■) were paired from one guinea-pig, tissues 3 (○) and 4 (□) from a second. Only tissue 1 retained epithelium. Superfusate from tissue 1 passed over tissue 3; superfusate from tissue 2 passed over tissue 4. Histamine was administered to the upper tissues as 0.2 ml aliquots of stock solutions of the indicated concentration. Results are mean from 5 pairs of tissues with s.e.mean shown by vertical lines.

No relaxant factor generated by the epithelium of tissue 1 could be detected.

leftwards and upwards as reported previously by others (e.g. Adcock & Garland 1980). Secondly, the removal of epithelium increased the sensitivity of the tissues not only to several contractile stimuli, but also to the relaxant effects of isoprenaline and adenosine. One would expect that if epithelium generated a relaxant factor, its removal would either reduce or have no effect on the sensitivity of the tissue to relaxant stimuli. Thirdly, no relaxant factor could be detected by a cascade superfusion technique.

It would seem more likely that the epithelium represents a permeability barrier whose removal facilitates the access of exogenous substances (both contractile and relaxant) to the underlying smooth muscle. Responses to field stimulation would then not be affected by epithelial removal, due to the intramuscular location of the cholinergic and adrenergic nerves (Jones *et al.*, 1980b). Similarly, responses to $LaCl_3$ and KCl would not be expected to be affected by the presence or absence of epithelium, as inorganic ions such as La^{3+} and K^+ readily pass through the tight junctions of epithelium (Frömter & Diamond, 1972; Machen *et al.*, 1972; Wright, 1983).

The effects of DTT and phenidone on responses to histamine are difficult to interpret. DTT is a reducing agent which has been shown to interfere with the action of vascular EDRF (Förstermann & Neufang, 1984a), possibly by chemical interaction with EDRF itself (Griffith *et al.*, 1984). In the present experiments, DTT resembled indomethacin in that dose-response curves to histamine were shifted leftwards and upwards on both rubbed and normal tissues, but in addition the difference in sensitivity (CR) between the two tissues was partly reduced, from 5.0 to 2.2.

Phenidone is an inhibitor of arachidonic acid cyclooxygenase and lipoxygenase with antioxidant and radical scavenger properties which has been shown to interfere with the action of vascular EDRF (Förstermann & Neufang, 1984b; Griffith *et al.*, 1984). In the present experiments, phenidone 0.1 mM significantly increased the peak tension developed by both rubbed and normal tissues but a leftward shift of the dose-response curve to histamine was seen only in the control tissues. Thus phenidone abolished the sensitivity difference between rubbed and control tissues. The reason for this is not clear, but does not necessarily indicate the presence of a relaxant factor analogous to EDRF. For instance, it is possible that the epithelium of the normal tissues was functionally impaired by the phenidone in such a way that it no longer presented a permeability barrier.

An interesting observation was the contractile response produced by low concentrations of adenosine only in the tissues denuded of epithelium. Although the work of Caparotta *et al.* (1984) suggests that in guinea-pigs this response might be dependent on prostaglandins, it would be interesting to know whether the bronchoconstriction produced by inhaled adenosine in asthmatic but not in normal humans (Cushley *et al.*, 1983; 1984) is in any way dependent on damaged airway epithelium in the asthmatics.

Very recently, studies of the effects of epithelium on the responsiveness of airway smooth muscle have been reported by other groups. Flavahan *et al.* (1985) reported that removal of epithelium from canine bronchial rings enhanced responses to acetylcholine, histamine and 5-HT but attenuated responses to isoprenaline. Similar results were reported by Cuss *et al.* (1985) using bovine tracheal strips, except that responses to histamine were unaffected by epithelium. These reports are not in complete accord with the present study, particularly with regard to isoprenaline-

induced relaxation. As species differences could be responsible for this, it would seem particularly important to repeat this study on human tissue.

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