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The epithelium and the pharmacology of guinea-pig tracheal tone *in vitro*

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1 Epithelium removal did not influence the development of spontaneous tone in guinea-pig tracheal smooth muscle mounted as open ring preparations with two adjoining cartilaginous rings *in vitro*.

2 Epithelium removal did not change the potency of carbachol but tended to reduce the maximal contraction. In the presence of epithelium the EC_{50} of carbachol was not different in tracheal open ring compared with intact tube preparations (comprising four cartilaginous rings), suggesting that the size of continuous epithelium *in vitro* was not critical for the potency of carbachol.

3 Substance P produced the same response in intact and rubbed tracheae. The enkephalinase inhibitor thiorphan (0.1 mM) by itself contracted the trachea and appeared to potentiate the substance P response five times more in the absence than in the presence of epithelium. Capsaicin (1 μM)-induced contractions did not differ between intact and rubbed preparations.

4 Arachidonic acid, 22 μM, variably produced small relaxations and contractions in intact as well as in rubbed tracheae. The mean effects of arachidonic acid were not significantly altered by epithelium removal.

5 Adenosine produced small contractions and dose-dependent relaxations in the presence and absence of epithelium.

6 Epithelium removal had no effect on the potency of the relaxant agonists theophylline and enprofylline. The isoprenaline curve was shifted 2 fold to the left and the terbutaline curve 1.5 fold to the right. The maximal relaxations were generally reduced in epithelium-free tissue. The reduction reached statistical significance with theophylline.

7 The present results suggest that epithelium removal is of little consequence for the pharmacology of the guinea-pig tracheal open ring preparation *in vitro*.

Introduction

In 1978, Whalley studied the role of the endometrium for drug actions on rat isolated myometrium. Despite removal of a large sheet of endometrial epithelial tissue, including a small part of the uterine smooth muscle, this latter tissue responded normally to acetylcholine and prostaglandin $F_{2\alpha}$. Effects of bradykinin and oxytocin were only slightly reduced in that their maxima were lower in endometrium-deprived preparations (Whalley, 1978). In 1979, Furchgott & Zawadski observed that the vascular endothelial lining was essential for relaxant effects of acetylcholine *in vitro*. Vascular preparations without endothelium responded with contraction to acetylcholine. This exciting observation has been extended to a variety of agents and to *in vivo* conditions (Furchgott, 1981). It has had an impact on work with isolated airway preparations where the focus has been on a role for the epithelium in regulation of airway smooth muscle tone. Despite the fact that many of the accumulated observations are negative or contradictory, it has generally been maintained that airway epithelium importantly affects tracheobronchial smooth muscle reactivity (Barnes *et al.*, 1985; Flavahan *et al.*, 1985; Farmer *et al.*, 1986; Finnen *et al.*, 1986; Frossard & Muller, 1986; Goldie *et al.*, 1986; Hay *et al.*, 1986a,b; 1987; Holroyde, 1986; Reaburn *et al.*, 1986a,b; Thompson *et al.*, 1986; Tschirhart & Landry, 1986; Nijkamp & Folkerts, 1987). It is understandable that positive results

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have been searched for and related to the pathology and pharmacology of asthma. As early as 1900 Fraenkel had suggested that epithelial shedding is a characteristic of asthmatic airways: '. . . ein gemeinsames, die Fälle verknupfendes Band: das ist die reichliche Epitheldesquamation' (Fraenkel, 1900). Fraenkel's proposal has been repeatedly substantiated (Laitinen *et al.*, 1985), and epithelial shedding is a mechanism that is considered in various attempts to explain airway hyperresponsiveness in asthma.

Clearly, airway epithelial cells may produce and release a variety of potent mediators (Hunter *et al.*, 1985). However, it seems less likely that epithelial factors would directly affect airway smooth muscle that, similar to the uterine preparation discussed above (Whalley, 1978), is separated from the epithelium by a layer of tissue that is both thicker than the internal elastic lamina, separating endothelial cells from vascular smooth muscle, and contains a very profuse network of microvessels (Sobin *et al.*, 1963; Laitinen *et al.*, 1987). Hence, the airway epithelium may not be comparable to the vascular endothelium as regards possible influence on smooth muscle tone.

It is, nevertheless, of methodological interest to learn whether or not an easily damaged part of isolated airway preparations, such as the epithelial lining, is of importance for effects *in vitro* of bronchoconstrictors and bronchodilators. Most previous studies have involved guinea-pig tracheal preparations. Characteristic of guinea-pig tracheae is spontaneous development of significant tone *in vitro*, the degree of which may affect drug responses (Persson & Karlsson, 1987). We have now examined the effects of epithelium removal on this spontaneous tone and on responses to adenosine, arachidonic acid, capsaicin, carbachol and substance P. In addition, the effects of enprofylline, theophylline, isoprenaline and terbutaline on the spontaneous tone in normal preparations and those denuded of epithelium have been studied.

Methods

Guinea-pigs of either sex, weighing 200–400 g, were killed by a blow to the neck and the trachea was removed and dissected free from adjoining tissue. From the mid part of each trachea 2 rings were cut, each ring comprising 2 cartilaginous rings. Two threads were then tied to each ring opposite the trachealis and the rings were cut open between the knots. In half of the rings the epithelial lining was removed by gently rubbing the luminal surface with a scalpel. This was carried out under a preparation microscope. During the preparation the tissues were soaked in pregassed Krebs solution (composition as below).

The tissues were suspended in 10 ml baths containing a Krebs solution of the following composition (mM): NaCl 118, KCl 4.6, $CaCl_2$ 2.5, $MgSO_4$ 1.16, $NaHCO_3$ 24.9, KH_2PO_4 1.2 and glucose 5.5. The solution was gassed with 95% O_2 and 5% CO_2 giving a pH of 7.4. Isometric tension changes were recorded by means of Grass FTO 3C force transducers and a Grass 5D or 7D polygraph. Tissues were mounted under an initial tension of 6 mN and allowed to equilibrate for approximately 1 h with washing approximately every 20 min. During the equilibration period the muscle tension increased spontaneously and was allowed to stabilize. Concentration-response curves were obtained by adding drugs cumulatively, in threefold increments, to the organ baths.

Tracheal tube preparations

Tracheal tubes comprising 4 cartilaginous rings each were cut out of the tracheae. The tubes were suspended in the normal 10 ml baths between two metal prongs inserted into the lumen of the tracheal tube. One prong was rigid and the other was attached to a Grass FTO3C transducer. Care was taken to position the prongs on both sides of the trachealis muscle. The tracheal tubes were mounted under an initial tension of 6 mN. For a comparison, normal open ring preparations (as described above) were obtained from the same animals as the intact tubes.

Histology

After completion of organ bath studies, the tissues were removed from the baths and fixed in phosphate buffered 10% formalin (pH 7). Microscopic slides were then prepared, stained with haematoxylin and eosin and examined microscopically for the presence of epithelium.

Calculations

Concentration-response curves of relaxant or contractile agonists were expressed as a percentage of the maximal response for each substance and/or as absolute tension changes (mN). Data are presented as means ± s.e. mean. To obtain EC_{50} values, the equation of the linear part of each concentration-response curve was determined and the concentration corresponding to 50% of the maximal response was calculated. Geometric mean EC_{50} values were obtained from the individual log EC_{50} values. The computations were done on logged values and then converted back to original scale by use of Gauss approximation formula. The effect on E_{max} of epithe-

Table 1 Spontaneous tone and EC_{50} values of drugs in tracheal open ring preparations with (controls) or without (rubbed) epithelium

Initial tone (mN)	*Theophylline*	*Enprofylline*	*Terbutaline*	*Isoprenaline*	*Carbachol*
Controls	14.4 ± 1.6	14.6 ± 1.9	10.7 ± 1.1	11.3 ± 1.1	15.0 ± 2.0
Rubbed	11.0 ± 1.0	12.4 ± 2.4	11.7 ± 1.3	14.1 ± 1.8	17.2 ± 2.4
EC_{50} values					
Controls	35.8 ± 7.9 μM	8.3 ± 0.81 μM	37.4 ± 5.1 μM	4.6 ± 1.3 μM	42.3 ± 3.4 μM
Rubbed	39.6 ± 9.9 μM	8.9 ± 1.1 μM	55.6 ± 5.0 μM	2.4 ± 0.4 μM	44.0 ± 6.5 μM

Values are mean ± s.e. mean; $n = 6$ except carbachol experiments when $n = 8$.

lial removal was assessed by use of Student's t test for paired observations. Shifts in concentration-response (% of maximum) curves were assessed by analysis of covariance. Probability values lower than 0.05 were considered significant (n equals number of animals used).

Drugs and solutions

The following drugs were used: anhydrous theophylline (Knoll), enprofylline and terbutaline sulphate (Draco), arachidonic acid sodium salt, isoprenaline chloride, carbamyl choline (carbachol) and capsaicin (Sigma), substance P and thiorphan (Peninsula Laboratories). Theophylline and enprofylline were dissolved in 1 eq. 0.5 M NaOH and then diluted in Krebs solution. Capsaicin and thiorphan were first dissolved in 95% ethanol (1 : 10 of the final volume) and then diluted in Krebs solution. Isoprenaline was dissolved and diluted in Krebs solution including 1 mg ml^{-1} of ascorbic acid as an antioxidant. Terbutaline, carbachol, substance P and arachidonic acid were all dissolved and diluted in Krebs solution.

Results

Histology

All preparations were evaluated histologically for the presence or absence of epithelium. In all of the rubbed tissues the epithelium was absent over the tracheal smooth muscle, which appeared intact. Over the cartilage, epithelial cells were occasionally detected, but never covering more than 10% of the luminal surface. The epithelium remained intact in the control tissues after being used in the organ baths.

Spontaneous tone

All preparations developed a spontaneous tone after being suspended in organ baths. The spontaneous tone in preparations with epithelium ($n = 40$) was 10.9 ± 0.6 mN and 12.5 ± 0.7 mN, 15 and 30 min respectively, after mounting. Corresponding values in epithelium deprived preparations ($n = 40$) were 9.8 ± 0.6 mN and 10.8 ± 0.6 mN, respectively. There was no significant difference in muscle tone between control tissues and denuded tissues ($P > 0.05$) (see also Tables 1 and 2).

Contractile agonists

Carbachol produced concentration-dependent contractions of the tracheal muscle. Removal of the epithelium had no effect on the potency (Table 1) but tended to reduce the maximal contractile effect of the drug (Figure 1). Initial experiments suggested a significant reduction in maximal effect of carbachol in rubbed tracheae (not shown), but this finding could not be repeated in this study. The potency of carbachol was not different in tracheal ring and tube preparations (Figure 2) suggesting that the size of the area of intact epithelium was of no consequence for this contractile effect. However, the maximal contractile effect of the drug appeared larger in the tube preparation compared to the open ring preparations, 26.8 ± 7.2 mN and 15.5 ± 2.7 mN, respectively ($n = 5$, $P > 0.05$).

Capsaicin (1 μM) produced a weak contraction in control as well as in rubbed tissue, 0.75 ± 0.43 mN and 1.4 ± 1.2 mN ($n = 4$) respectively. Two of the controls and two of the rubbed preparations (from the same animal) did not respond to capsaicin (1 μM) whereas they responded normally to carbachol.

Substance P produced dose-dependent contractions in tracheal rings with or without epithelium. There was no difference in potency or maximal contractile effect between denuded and intact preparations (Figure 3a and b).

Thiorphan is an inhibitor of the enzyme enkephalinase, which inactivates substance P (Borson *et al.*, 1987). Thiorphan (0.1 mM) itself increased the tone. This effect was similar in both intact and denuded tracheal preparations (25.0 ± 8.4 mN and 22.6 ± 2.5 mN, respectively, $n = 3$). Following pretreatment with thiorphan, the dose-response

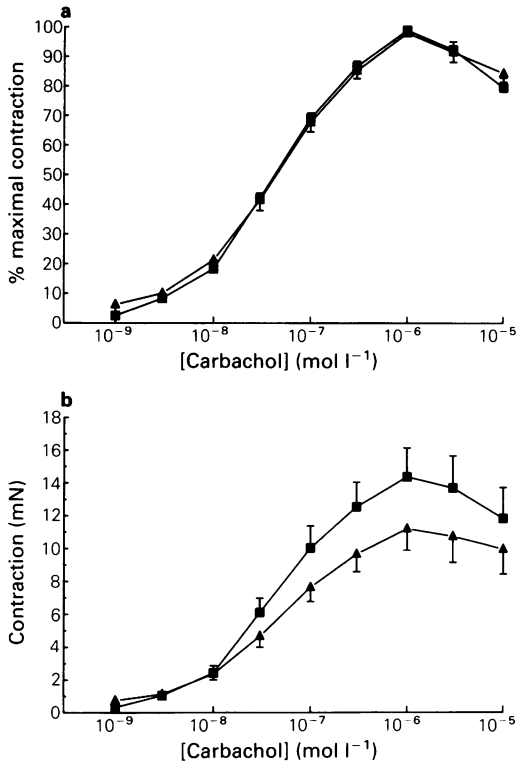

Figure 1 Concentration-response curves for cabachol in intact (■) and epithelium-free (▲) guinea-pig tracheal open ring preparations ($n = 8$). Vertical lines represent s.e. mean.

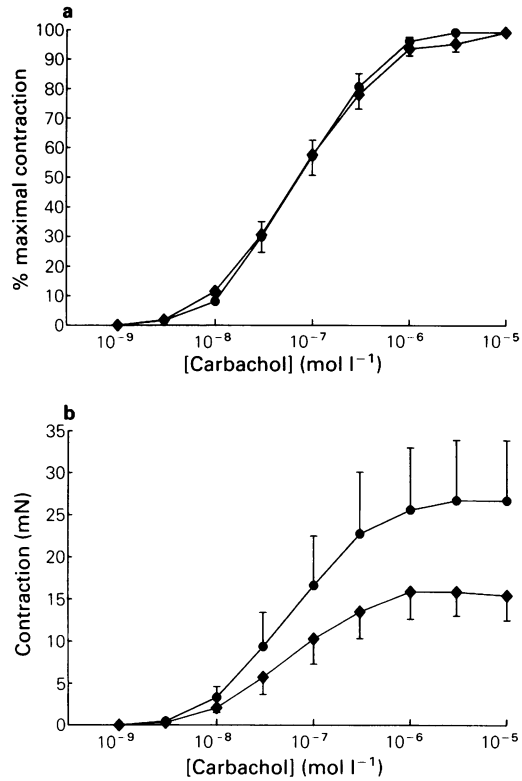

Figure 2 Concentration-response curves for carbachol in guinea-pig tracheal tube preparations (●) and open ring preparations (◆), both having intact epithelium ($n = 5$). Vertical lines represent s.e. mean.

Table 2 Changes in tension induced by arachidonic acid (22 μM) and potassium (20 mM) in tracheal open ring preparations with (controls) or without (rubbed) epithelium

	Controls (mN)	*Rubbed* (mN)
Spontaneous tone	14.2 ± 2.3	13.4 ± 2.0
Arachidonic acid	+3.3 ± 1.6	+5.6 ± 3.7
No. of preparations contracting/relaxing	5/1	5/1
Spontaneous tone	15.5 ± 2.7	11.9 ± 1.9
KCl	+3.2 ± 1.1	+1.2 ± 0.7
* Arachidonic acid	−3.3 ± 2.1	+0.4 ± 0.9
No. of preparations contracting/relaxing	2/4	4/2

Values represent mean ± s.e. mean; $n = 6$.
* In the presence of 20 mM KCl.

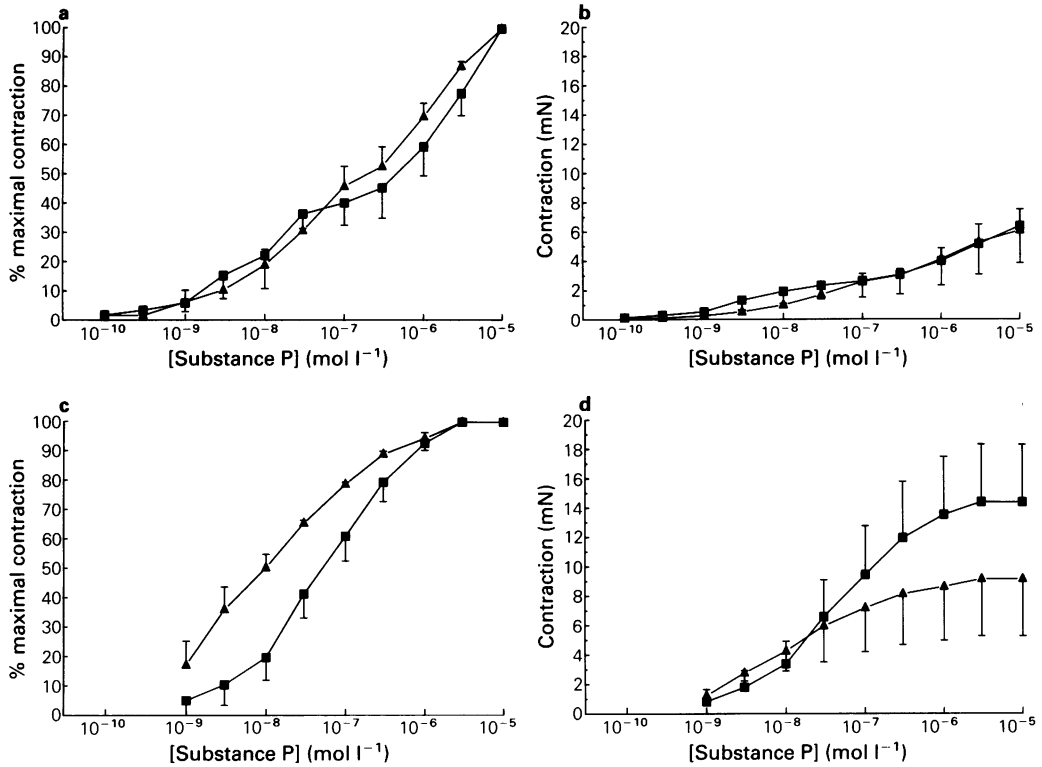

Figure 3 Concentrations-response curves to substance P before (a and b) and after (c and d) incubation with thiorphan (0.1 mM) for 15 min in intact (■) and epithelium-free (▲) guinea-pig tracheal open ring preparations ($n = 3$). Vertical lines represent s.e. mean.

curves to substance P were shifted 5.2 fold to the left ($P < 0.01$) in rubbed as compared with intact tissues (Figure 3c).

Agents producing both contraction and relaxation

Arachidonic acid (22 μM) variably produced small contractions and relaxations in intact and denuded preparations (Table 2). In the presence of KCl, 20 mM, which produced a slight elevation of tone, the number of preparations responding with a relaxation increased (Table 2). The mean effects of arachidonic acid were not significantly ($P > 0.05$) affected by epithelium removal nor by KCl.

Before the concentration-response curve to adenosine was performed the spontaneously gained tension was 20.1 ± 2.9 mN and 18.3 ± 2.3 mN in rubbed and intact preparations, respectively ($P > 0.05$). In concentrations lower than 0.1 mM, adenosine produced slight contraction in tracheae with or without epithelium. At higher concentrations, adenosine induced concentration-dependent relaxations. No significant difference was observed between the rubbed and the control preparations in their ability to produce contractions to adenosine. At 10^{-4} M and 3×10^{-4} M adenosine produced larger relaxations in the absence of epithelium compared to intact preparations. Maximum relaxant effects of adenosine in intact preparations were not readily obtained at the largest soluble drug concentration (10^{-3} M) (Figure 4). Hence, the apparent leftward shift in epithelium-deprived preparations was not analysed statistically.

Relaxant agonists

Theophylline, enprofylline, terbutaline and isoprenaline produced concentration-dependent relaxations of tracheal tissues possessing spontaneous tone. Removal of the epithelium did not significantly affect the potency of theophylline or enprofylline (Figure 5). The concentration-response curve for isoprenaline was shifted about 2 fold to the left in epithelium-

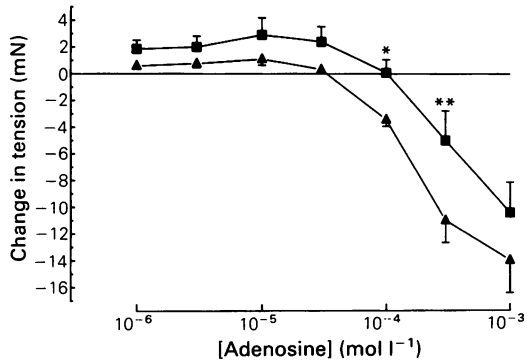

Figure 4 Concentration-response curves for adenosine in intact (■) or epithelium-free (▲) guinea-pig tracheal open ring preparations ($n = 8$). Vertical lines represent s.e. mean. At 10^{-4} M and 3×10^{-4} M the relaxant effect was significantly larger in the epithelium denuded preparations, * $P < 0.05$, ** $P < 0.01$.

deprived preparations ($P < 0.01$), whereas the terbutaline curve was shifted 1.5 fold to the right ($P < 0.01$) (Figure 6). The maximal relaxant effect to the relaxant agonists was generally smaller in the denuded tissues but reached statistical significance only with theophylline, $P < 0.05$ (Figure 5).

Discussion

Contractile effects

Taken together, the published effects of epithelium removal on the pharmacology of airway contractions seem confusing. The initial work by Flavahan *et al.* (1985) in dog bronchi suggested that the response to electrical field stimulation, in particular the duration of the contraction, was increased by epithelium removal. However, later work by Barnes *et al.* (1985) in bovine trachea, Holroyde (1986) and Thompson *et al.* (1986) in cat and guinea-pig trachea, demonstrated no effect of epithelium on the response to electrical field stimulation. Effects of epithelium removal, if any, on other contractile actions also vary. Contractions produced by one or more of histamine, muscarinic agents, 5-hydroxytryptamine,

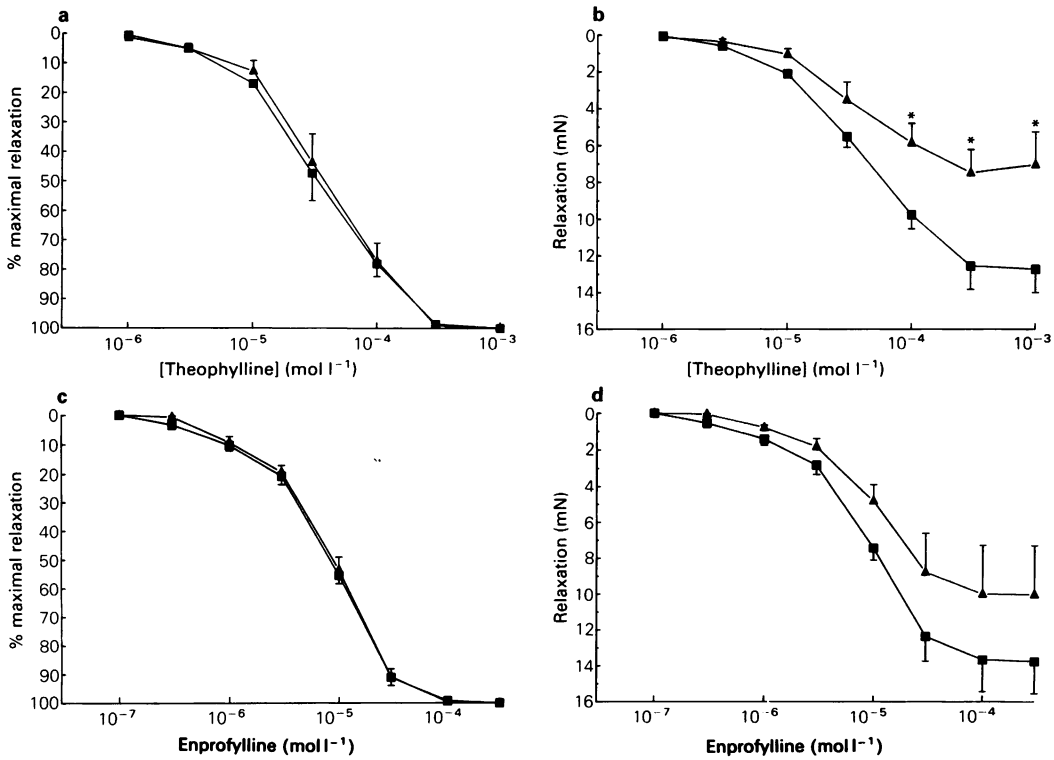

Figure 5 Concentration-response curves for theophylline (a and b) and enprofylline (c and d) in intact (■) and epithelium-free (▲) guinea-pig tracheal open ring preparations ($n = 6$). Vertical lines represent s.e. mean; * denotes significantly different, $P < 0.05$.

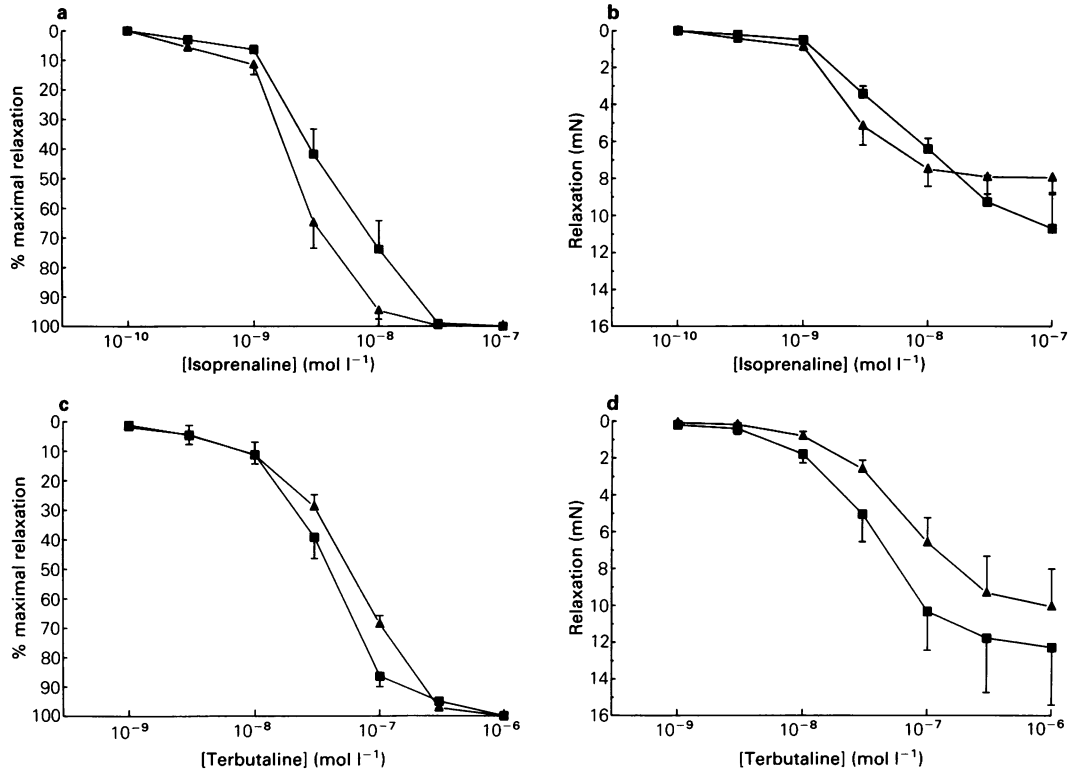

Figure 6 Concentration-response curves for isoprenaline (a and b) and terbutaline (c and d) in intact (■) and epithelium-free (▲) guinea-pig tracheal open ring preparations ($n = 6$). Vertical lines represent s.e. mean.

and K^+ were slightly increased (either pD_2 or E_{max} or both) (Flavahan *et al.*, 1985; Barnes *et al.*, 1985; Hay *et al.*, 1986a,b; Raeburn *et al.*, 1986a,b; Finnen *et al.*, 1986; Holroyde, 1986), or reduced (this study, cf. Whalley, 1978), or were not affected by epithelium removal (Goldie *et al.*, 1986; Raeburn *et al.*, 1986a, Hay *et al.*, 1986a,b; Thompson *et al.*, 1986 and this study). Barnes *et al.* (1985) found that indomethacin did not alter a slightly increased sensitivity to acetylcholine in bovine epithelium-denuded tracheae. In guinea-pig trachea Holroyde (1986) found that indomethacin moved histamine dose-response curves to the left in both control and epithelium-denuded preparations. Hay *et al.* (1986b), also working with guinea-pig trachea, demonstrated that indomethacin was without effect on the sensitivity to histamine but produced the same effect as epithelium removal on the EC_{50} value of methacholine (that was significantly reduced by a factor of about 2). Ovalbumin in sensitized guinea-pig tracheae became more potent after epithelium removal, whereas its maximum effect was either unchanged (Hay *et al.*, 1986a) or increased (Hay *et al.*, 1987). In sensitized rat tracheae Frossard & Muller (1986) found a diminished response to ovalbumin when the epithelium had been removed. Tschirhart & Landry (1986) found that epithelium removal in guinea-pig tracheal preparations increased the potency of substance P by over forty times. However, we found no change in the response to substance P. This result tallies with the observation that contractions induced by the tachykinin-releasing agent capsaicin, were not significantly changed by epithelium removal (this study). In preparations treated with thiorphan to prevent its breakdown, substance P was slightly more potent in the absence of epithelium. It may also be noted that Frossard & Muller (1986) found that substance P relaxed rat tracheal preparations and this response was slightly reduced by epithelium removal.

Agents producing mixed effects

Adenosine may produce both a slight contraction (at low concentrations) and a relaxation of guinea-pig tracheae *in vitro* (Coleman, 1976). Holroyde (1986)

reported contractions to adenosine only in epithelium-denuded tracheal preparations. However, in this study biphasic responses to adenosine in both types of preparation were recorded. In fact, the contractile response was slightly larger in intact tissue. This finding suggests that epithelium removal does not provide a model for adenosine-induced bronchoconstriction. Nijkamp & Folkerts (1987) found that a relaxant response to arachidonic acid (22 mM) was changed into a contraction by epithelium removal. In the present study we could not confirm this observation.

Spontaneous tone

We thought initially that variations in spontaneous tone, in part, could have contributed to the differing observations related above. Clearly, the potency and even the direction (contraction or relaxation) of the effect of mediators may be dependent on the tone present when the effect of a mediator is evaluated (Persson & Karlsson, 1987). Furthermore, it is difficult to find information in published material about spontaneous tone of the various *in vitro* epithelium-denuded preparations. In the present study it was demonstrated that the spontaneous development of tone was not altered by epithelium removal.

Relaxant effects

Different results have been obtained also concerning relaxant effects in tracheobronchial preparations with and without epithelium. In agreement with the tendency in this study, Flavahan *et al.* (1985) and Goldie *et al.* (1986) demonstrated that the maximum effect of isoprenaline was reduced by epithelium removal, but this was not found by Barnes *et al.* (1985), Hay *et al.* (1986b), Holroyde (1986) or Farmer *et al.* (1986). The latter three and this study demonstrated an increased potency to isoprenaline in epithelium-deprived preparations, which was not seen by the other workers (Flavahan *et al.*, 1985; Goldie *et al.*, 1986). In fact, Barnes *et al.* (1985) found that epithelium removal caused a variable decrease in the potency of isoprenaline.

Farmer *et al.* (1986) observed no significant change in the effects of salbutamol and the present work showed a marginally reduced potency of terbutaline in epithelium-denuded tissues. As shown by Goldie *et al.* (1986), the sensitivity to theophylline may not be affected by the epithelium. The same result has now been obtained with theophylline and the adenosine non-blocking xanthine enprofylline, which is a more potent airway relaxant than theophylline (Persson, 1986a). Hence, the common anti-asthma drugs may show little dependence on intact epithelium for their smooth muscle action. Derangement of the epithelium may reduce the absolute amount of relaxation obtained with bronchodilators in guinea-pig isolated tracheae. This aspect may deserve consideration in drug evaluation *in vitro*.

The present results together with selected data from other studies suggest that epithelium removal generally is of little consequence for the pharmacology of airway tone *in vitro*. It may be argued that epithelium-smooth muscle interactions would be more pronounced and more important *in vivo*. However, *in vivo* any released epithelial factor must pass a well-perfused and copious network of microvessels in the mucosa/submucosa (Sobin *et al.*, 1963; Laitinen *et al.*, 1987) and hence would much less readily reach the deeper-lying muscle *in vivo* than *in vitro*. This reasoning does not exclude the possibility that airway epithelial factors have very important regulatory functions in health and disease. It would seem natural that the primary actions of such factors would involve nearby structures including nerves, secretory cells and the microvasculature. In particular, studies of plasma exudation effects of epithelial factors may be warranted (Persson, 1986b). Hence, through a chain of events *in vivo* tracheobronchial smooth muscle may indirectly be affected by material produced by the epithelium.

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