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Differential effects of epithelium removal on the responsiveness of guinea-pig tracheal smooth muscle to bronchoconstrictors

[1]D.W.P. Hay, *,[2] S.G. Farmer, *,[3]D. Raeburn, R.M. Muccitelli, K.A. Wilson & *J.S. Fedan

Department of Pharmacology, Smith Kline & French Laboratories, P.O. Box 7929, Philadelphia, PA 19101, U.S.A. and *Physiology Section, Division of Respiratory Disease Studies, National Institute for Occupational Safety and Health, 944 Chestnut Ridge Road, Morgantown, WV 26505, U.S.A.

1 The influence of the epithelium on contractions produced by the peptidoleukotrienes, 5-hydroxytryptamine (5-HT) and the thromboxane mimetic, U-44069, was examined in trachea from control and ovalbumin-sensitized guinea-pigs.

2 In control tissues removal of the epithelium produced an approximately 2 to 4 fold leftward shift in leukotriene C_4 (LTC_4) and LTD_4 concentration-response curves, but no effect on LTE_4-induced contractions. Similar results were obtained in preparations from ovalbumin-sensitized animals.

3 Responses produced by 5-HT or U-44069 were similar in the presence and absence of the epithelium in control guinea-pigs.

4 Indomethacin produced contrasting effects on leukotriene-induced contractions in control guinea-pigs: an increase in sensitivity to LTC_4 in the presence but not absence of the epithelium, no effect on LTD_4-induced contractions and a decrease in sensitivity to LTE_4 in both epithelium-containing and epithelium-free preparations.

5 These results indicate that there is selectivity in the effects of epithelium removal on agonist-induced contractions of the guinea-pig trachea. This provides further evidence for the modulatory influence of the epithelium on the reactivity of mammalian airway smooth muscle and supports the postulated existence of an epithelium-derived inhibitory factor. The observation that in intact trachea indomethacin mimics the effects of epithelium removal on LTC_4-induced responses, suggests the involvement of a prostanoid(s) in this phenomenon.

Introduction

Two hallmarks of bronchial asthma are non-specific airway hyperreactivity and an associated damage to or loss of epithelial cells (Laitinen *et al.*, 1985). Removal of the epithelial cell layer alters the responsiveness of *in vitro* preparations of airway smooth muscle from several mammalian species, including man. The increased responsiveness to bronchoactive drugs in epithelium-denuded airway smooth muscle from dogs (Flavahan *et al.*, 1985), guinea-pigs (Hay *et al.*, 1986a; Goldie *et al.*, 1986), cattle (Barnes *et al.*, 1985), rabbits (Raeburn *et al.*, 1986a) and man (Raeburn *et al.*, 1986b) was attributed to the loss of an epithelium-derived inhibitory factor(s) which normally modulates the responsiveness of respiratory smooth muscle to contractile and relaxant agents. Therefore, it is feasible that the airway hyperreactivity in asthmatics results, at least in part, from loss of an epithelium-dependent inhibitory influence on smooth muscle responsiveness.

In addition to influencing responsiveness of the underlying smooth muscle to various drugs, epithelium removal also produces a substantial increase in sensitivity to antigen challenge in trachealis from ovalbumin-sensitized guinea-pigs (Hay *et al.*, 1986b). Indeed, epithelium removal causes a greater increase in sensitivity to antigen than to either methacholine or

[1]Author for correspondence at Department of Pharmacology, Smith Kline & French Laboratories.
[2]Present address: Dr S.G. Farmer, Department of Pharmacology, Nova Pharmaceutical Corporation, 5210 Eastern Avenue, Baltimore, MD 21224, U.S.A.
[3]Present address: Dr D. Raeburn, Department of Pharmacology, Louisiana State University Medical School, 1910 Perdido Street, New Orleans, LA 70112, U.S.A.

histamine. The contractile response of guinea-pig trachealis to antigen challenge *in vitro* is mediated by histamine and peptidoleukotrienes, which are responsible, respectively, for the initial rapid phase and the later, sustained phase of the contraction (Adams & Lichtenstein, 1979). Since histamine and leukotrienes act in concert to produce the response to antigen challenge, it is possible that the greater effect of epithelium removal on antigen-induced contractions is due to an enhanced tracheal responsiveness to leukotrienes This postulate was examined in the present study. A further reason for examining the influence of the epithelium on responses to the leukotrienes is their possible role as mediators in the pathophysiology of asthma (Hedqvist & Dahlén, 1983). In view of the evidence, at least in the guinea-pig trachea, that the postulated inhibitory influence may involve a prostanoid (Hay *et al.*, 1986a), the effects of a cyclo-oxygenase inhibitor, indomethacin, on leukotriene-induced contractions were investigated.

5-Hydroxytryptamine (5-HT) and thromboxane A_2 are potent bronchoconstrictors (Creese *et al.*, 1984; Cohen *et al.*, 1985), and the present study was extended therefore to examine the effects of epithelium removal on responses produced by 5-HT and the thromboxane mimetic, U-44069. Preliminary accounts of the findings have been presented previously (Hay *et al.*, 1986c; Farmer *et al.*, 1986a).

Methods

Tissue preparation

Tracheae were removed from adult male Hartley and English short-hair guinea-pigs (Hazelton Research Animals, Denver, PA and Camm Research Institute, Wayne, NJ; 450–600 g body weight) and placed in modified Krebs-Henseleit solution (MKH). Following removal of adherent fat and connective tissue the trachea was cut open along its longitudinal axis, directly opposite the smooth muscle, and strips consisting of two adjacent cartilage rings were prepared. The preparations were then placed in MKH in 10 ml water-jacketed organ baths (maintained at 37°C) and connected via silk suture to force-displacement transducers. Mechanical responses were recorded isometrically using multi-channel polygraphs. Tissues were equilibrated under 1 g resting load for at least 1 h and washed every 15 min with fresh MKH before the start of each experiment. The composition of the MKH, which was gassed with 95% O_2:5% CO_2, was (mM): NaCl 113.0, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, glucose 5.5.

The epithelium was removed mechanically from alternate strips of trachea (permitting paired analysis) by gently rubbing the luminal surface with a cotton-tipped applicator. It has been demonstrated previously that this procedure effectively removes the epithelium from guinea-pig trachea without producing obvious damage to the underlying mucosal and smooth muscle layers, and does not alter the basic mechanical properties of the tissue (Hay *et al.*, 1986a).

Concentration-response curves

Concentration-response curves for the contractile agents were obtained by their cumulative addition to the organ bath in 10 fold increments for ovalbumin and in 3 fold increments for the other agonists. Each drug concentration was left in contact with the preparations until the response reached a plateau. Only one concentration-response curve was obtained from each preparation. Before determination of leukotriene C_4 (LTC_4) and LTD_4 concentration-response curves, tissues were preincubated for 45 min with L-serine borate (45 mM) and L-cysteine (3 mM), respectively, to prevent the metabolism of LTC_4 and LTD_4 (Snyder *et al.*, 1984).

Where appropriate, indomethacin (3 μM) was added 45 min before, and remained present throughout the experiment. Indomethacin was observed to relax baseline tension. Therefore, when calculating the maximum responses to leukotrienes, account was taken of the reduction in resting tone of the tissues following exposure to indomethacin, i.e. the magnitude of the indomethacin-induced relaxation was subtracted from the total developed tension in response to a particular leukotriene to yield the actual maximum developed response (from the original baseline) of each tissue to the leukotriene.

Sensitization procedure

In some experiments guinea-pigs were actively sensitized with ovalbumin (Grade V, crystallized, lyophilized and salt-free; Sigma Chemical Co., St. Louis, MO) as previously described (Hay *et al.*, 1986b). Tissues from guinea-pigs sensitized with ovalbumin are referred to in the text as 'sensitized tissues', whereas preparations from non-sensitized guinea-pigs are referred to as 'control tissues'.

Analysis of results

The data are expressed as % maximum contraction and developed tension (in g). Geometric mean EC_{50} values were calculated from linear regression analyses of probit-transformed data. Results are given as mean ± s.e.mean. Statistical analysis was conducted by use of Student's *t* test for paired or unpaired samples where appropriate; a probability value less than 0.05 was regarded as significant.

Drugs

The following drugs were used: LTC_4, LTD_4, LTE_4, which were synthesized at Smith Kline & French Laboratories (Philadelphia, PA) by Dr J. Gleason and colleagues in the Department of Medicinal Chemistry: the molar concentrations of leukotriene stock solutions were determined from their uv spectra; 5-hydroxytryptamine creatinine sulphate, indomethacin, L-serine, boric acid, L-cysteine (Sigma Chemical Co., St. Louis, MO); U-44069 (9-11-dideoxy-9α, 11α-epoxymethano-prostaglandin $F_{2\alpha}$ (Upjohn Diagnostics, Kalamazoo, MI). Stock solutions of indomethacin and U-44069 were dissolved in ethanol, with subsequent dilutions made using distilled H_2O. The final concentration of ethanol was 0.03% and this was without effect on tissue responsiveness. All other drugs were dissolved in distilled H_2O or 0.9% saline.

Results

The effects of epithelium removal on responses produced by the leukotrienes are summarized in Figure 1 and Table 1. In control tissues removal of the epithelium produced a significant increase in sensitivity to LTC_4 and LTD_4 as reflected by a 1.9 and 1.7 fold decrease in the EC_{50}, respectively (Figure 1a and c; Table 1). In contrast, removal of the epithelium had no effect on LTE_4-induced contractions (Figure 1e; Table 1). Similar results were obtained in sensitized tissues in that there was a significant increase in sensitivity to LTC_4 (1.8 fold) and LTD_4 (3.1 fold) (Figure 1b and d; Table 1), but no effect on contractions produced by LTE_4 (Figure 1f; Table 1). Removal of the epithelium had no effect on the maximum contractions produced by LTC_4, LTD_4 or LTE_4 in either control or sensitized tissues (Table 1).

Indomethacin (3 μM) had contrasting effects on the sensitivity of tissues to the leukotrienes (Table 2). In intact trachea, indomethacin caused an increase in sensitivity, reflected as a decreased EC_{50}, to LTC_4 similar to that produced by epithelium removal (Table 2). In contrast, indomethacin did not alter sensitivity to LTD_4 and had no effect on the increase in sensitivity to LTD_4 produced by epithelium removal. However, in the presence of indomethacin there was a significant decrease in sensitivity to LTE_4, regardless of epithelial integrity (Table 2).

Differential effects of indomethacin on the maximum contractile responses produced by the individual leukotrienes were also evident. If no allowance was made for the indomethacin-induced relaxation, indomethacin produced an apparent increase in the maximum developed responses to LTC_4, LTD_4 and LTE_4 in intact and denuded preparations. However, if the corrected value for the maximum contraction was determined following subtraction of the relaxation produced by indomethacin in individual preparations (see Methods section), the maximum response to LTD_4 (797 ± 98 mg) was unaltered by indomethacin (942 ± 100 mg). Similarly, the maximum responses to LTE_4 in the presence (704 ± 88 mg) and absence of indomethacin (756 ± 104 mg) were not significantly different. Conversely, the maximum response to LTC_4 in the presence of indomethacin was 1315 ± 103 mg, and this value was significantly greater than the control maximum response (980 ± 124 mg). That is, even when the relaxation caused by indomethacin is accounted for, the maximum response to LTC_4 was still increased by approximately 34% by the cyclooxygenase inhibitor.

In contrast to LTC_4 and LTD_4, epithelium removal had no effect on the sensitivity to or the maximum contraction produced by 5-HT in control and sensitized preparations or U-44069 in control tissues (Table 1; Figure 2).

Discussion

The effects of epithelium removal on the reactivity of mammalian airway smooth muscle has been attributed to the loss of a modulatory factor(s) which is normally released from the epithelial cell layer (see Introduction). On the other hand it has been suggested (Holroyde, 1986) that, at least in guinea-pig trachea, the effects of epithelium removal result solely from the loss of a diffusional barrier, which subsequently facilitates the access of bronchoactive agents to the smooth muscle layer. However, as has been reported recently for a number of relaxant agents (Farmer *et al.*, 1986b), the present study demonstrates that there is selectivity in the modulatory influence of the epithelium on the reactivity of the guinea-pig trachealis to contractile agents. Thus, epithelium removal was without effect on responses produced by LTE_4, 5-HT and U-44069, while it increased the sensitivity to LTC_4 and LTD_4. This unique agonist-selectivity seems to be at odds with the notion that the effects of epithelium removal on airway smooth muscle responsiveness are due solely to loss of a diffusion barrier.

It has been reported that there is an increased sensitivity to 5-HT in dog bronchus (Flavahan *et al.*, 1985) and bovine trachea (Barnes *et al.*, 1985) denuded of epithelium. An increase in the maximal contractile response in denuded bovine trachea was also apparent (Barnes *et al.*, 1985). We can offer no explanation for our observation that epithelium removal had no effect on guinea-pig tracheal responsiveness to 5-HT, whereas a previous report (Holroyde, 1986) found a marked increase in sensitivity of the same preparation to this drug. Possibly strain differences in the guinea-pigs account for this discrepancy.

Perhaps the most interesting finding in the present

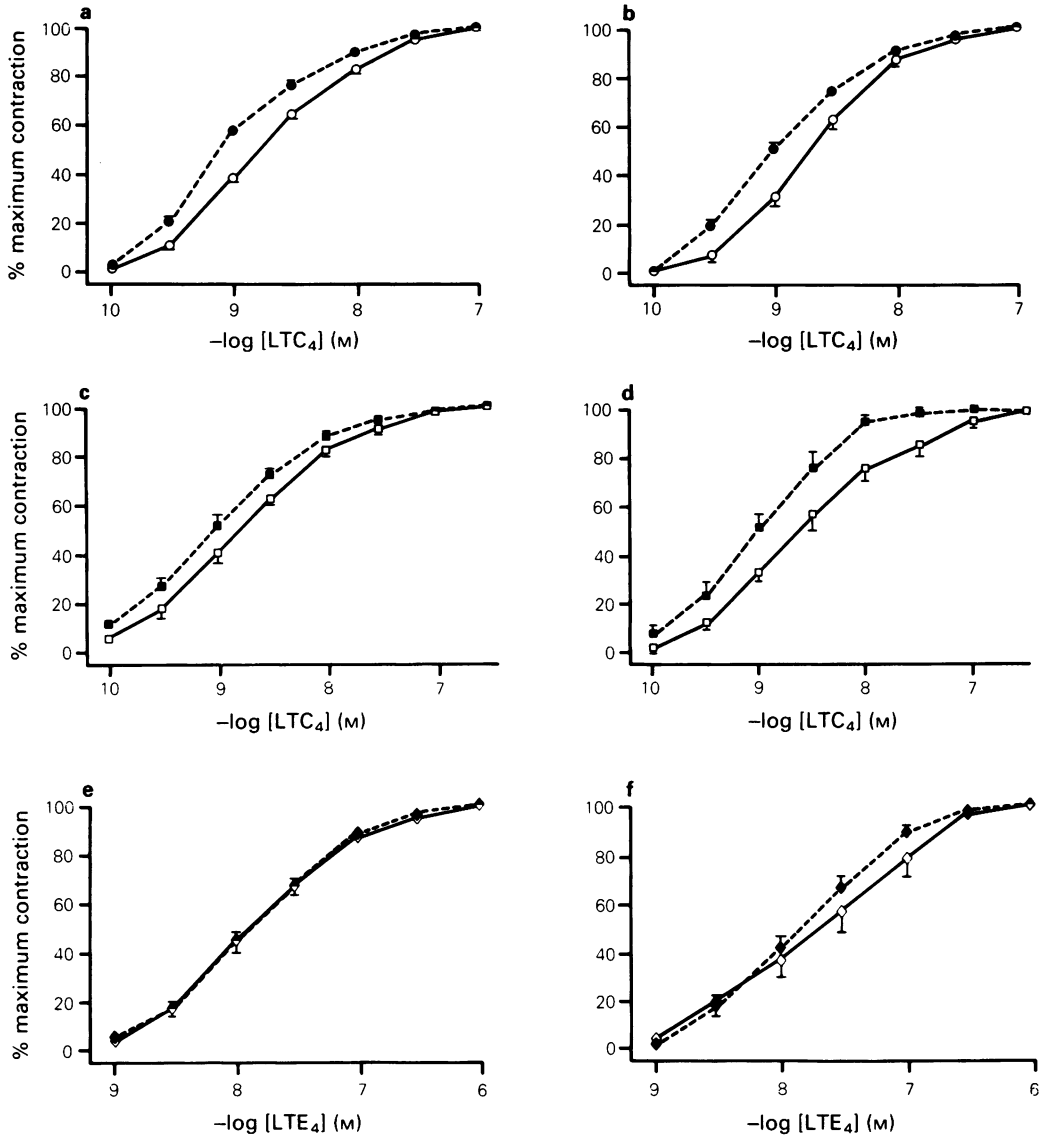

Figure 1 Effects of epithelium removal on concentration-response curves to leukotriene C_4 (LTC_4; ●, ○; a,b), LTD_4 (■, □; c,d) and LTE_4 (◆, ◇; e,f) in strips of trachealis from control (a,c,e) and ovalbumin-sensitized (b,d,f) guinea-pigs. Results are expressed as % of the maximum contraction and each point is the mean with s.e.mean indicated by vertical lines; *n*: (a) = 20; (b) = 11; (c) = 19; (d) = 10; (e) = 23; (f) = 9. Open symbols, solid line = + epithelium; filled symbols, broken line = − epithelium.

study was the lack of influence of the epithelium on responses produced by LTE_4, in contrast to the increases in sensitivity to LTC_4 and LTD_4. This is surprising given that evidence from biochemical and functional studies strongly suggests that, in the guinea-pig trachea, LTC_4, on the one hand, and LTD_4 and LTE_4, on the other, interact with two separate receptor populations (Hogaboom *et al.*, 1983; Fleisch *et al.*, 1984; Cheng *et al.*, 1985). Therefore, one might have anticipated that any selectivity exhibited by the epith-

Table 1 Effects of epithelium removal on contractions produced by leukotrienes, 5-hydroxytryptamine and U-44069 in tracheal strips from control and ovalbumin-sensitized guinea pigs

	Control			*Ovalbumin-sensitized*		
Agonist	n	*+ Epithelium*	*− Epithelium*	n	*+ Epithelium*	*− Epithelium*
			EC_{50} (− log M)			
LTC_4	20	8.65 ± 0.05	8.94 ± 0.04*	11	8.69 ± 0.06	8.93 ± 0.06*
LTD_4	19	8.74 ± 0.09	8.98 ± 0.09*	8	8.54 ± 0.12	9.03 ± 0.09*
LTE_4	23	7.81 ± 0.07	7.80 ± 0.08	9	7.67 ± 0.16	7.82 ± 0.11
5-HT	9	6.69 ± 0.14	6.84 ± 0.12	9	6.63 ± 0.07	6.77 ± 0.01
U-44069	5	7.49 ± 0.31	7.23 ± 0.09	—	N.D.	N.D.
			Maximum contraction (g)			
LTC_4	20	1.24 ± 0.07	1.29 ± 0.09	11	1.25 ± 0.13	1.31 ± 0.11
LTD_4	19	0.70 ± 0.07	0.64 ± 0.07	8	0.66 ± 0.14	0.60 ± 0.08
LTE_4	23	0.61 ± 0.05	0.68 ± 0.08	9	0.79 ± 0.10	0.66 ± 0.08
5-HT	9	0.43 ± 0.07	0.42 ± 0.11	9	0.36 ± 0.05	0.39 ± 0.08
U-44069	5	0.62 ± 0.10	0.62 ± 0.11	—	N.D.	N.D.

Results are expressed as EC_{50} values (− log M) and maximum contractile response (g) and are the means ± s.e.mean.
*Significantly different from + epithelium.
N.D. Not determined.

elium removal on sensitivity to the leukotrienes would be exerted against either LTC_4, or both LTD_4 and LTE_4. The reason(s) for the discrepancy in the present study is unclear but may be connected to recent evidence that LTE_4, unlike LTC_4 or LTD_4, may be a partial agonist in the guinea-pig trachea (Muccitelli *et al.*, 1986; Mong *et al.*, 1986a,b) and might, therefore, have a reduced ability to release the postulated epithelium-derived inhibitory factor. To clarify this it will be necessary to determine whether the factor is released under basal conditions, and also whether a correlation exists between the ability of an agonist to stimulate its release and the ability of epithelium removal to modulate the contractile response. A differentiation between maximum responses to LTC_4, LTD_4 and LTE_4 was evident in that indomethacin induced an increase in the maximum response to LTC_4, but not to the latter leukotrienes. This suggests that LTC_4 may stimulate the release of an inhibitory prostanoid which could antagonize physiologically the direct contractile action on the smooth muscle. Conversely, the indomethacin-induced decrease in sensitivity to LTE_4 suggests that responses to this leukotriene are mediated in part by an excitatory prostanoid.

Table 2 Effects of indomethacin (3 μM) on contractions produced by the leukotrienes, LTC_4, LTD_4 and LTE_4 in guinea-pig tracheal strips in the absence and presence of epithelium

	EC_{50} (− log M)			
Agonist	*Control*		*+ Indomethacin*	
	+ Epithelium			
LTC_4	8.84 ± 0.09	(5)	9.16 ± 0.11	(4)*
LTD_4	9.57 ± 0.08	(7)	9.42 ± 0.10	(7)
LTE_4	8.25 ± 0.12	(6)	7.96 ± 0.05	(5)*
	− Epithelium			
LTC_4	9.37 ± 0.04	(5)	9.40 ± 0.09	(4)
LTD_4	9.72 ± 0.09	(7)	9.67 ± 0.10	(7)
LTE_4	8.32 ± 0.10	(6)	7.97 ± 0.12	(5)*

Results are expressed as EC_{50} (− log M) and are the means ± s.e.mean; *n* values are given in parentheses.
*Significantly different from control.

The observation that indomethacin mimicked the effects of epithelium removal on sensitivity to LTC_4 is similar to previous findings using methacholine as the agonist (Hay *et al.*, 1986a), and suggests that the postulated inhibitory factor is a product of the cyclo-oxygenase pathway or, alternatively, that the release of the factor is modulated by a prostanoid. However, we have previously found that indomethacin is without effect on the sensitivity to histamine (Hay *et al.*, 1986a) and the present study indicates similar results with LTD_4. The reason(s) for the selectivity in the effects of indomethacin on agonist-induced responses that are affected by removal of the epithelium is unknown. One possibility is that more than one inhibitory factor is released by the guinea-pig tracheal epithelium. In view of the evidence that LTD_4 and LTE_4 act via the same receptor population in guinea-pig airways (Fleisch *et al.*, 1984), the finding that indomethacin decreased the sensitivity to LTE_4 but not LTD_4 was unexpected, and no obvious explanation is apparent.

The response to antigen challenge *in vitro* in tracheal

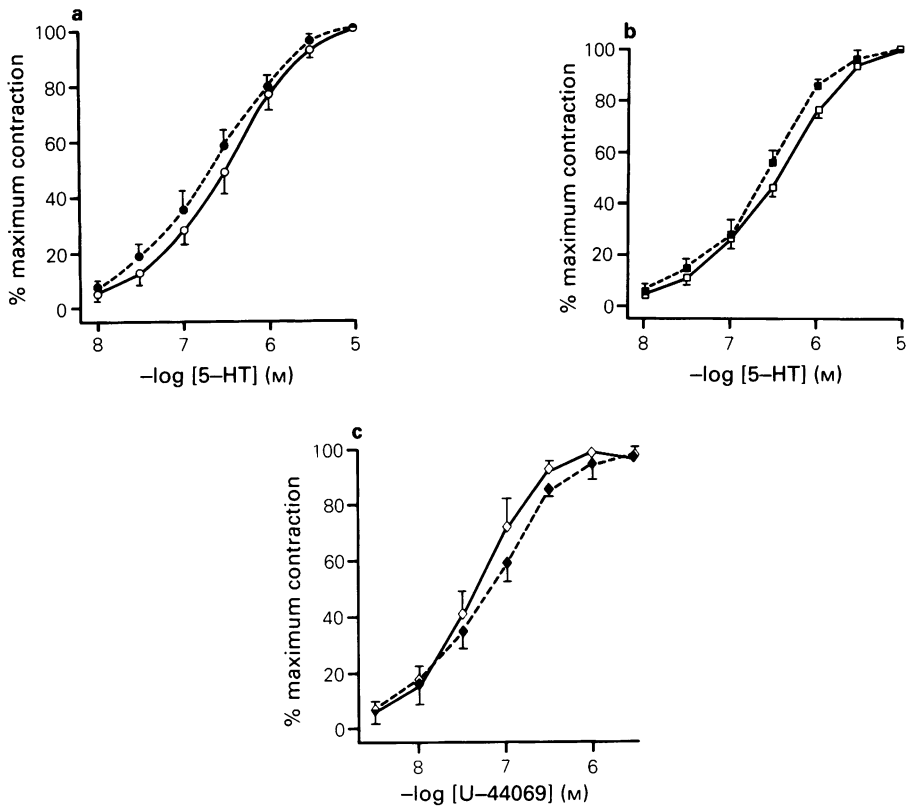

Figure 2 Effects of epithelium removal on concentration-response curves to 5-hydroxytryptamine (5-HT; a,b) and U-44069 (c) in tracheal strips from control (a,c) and ovalbumin-sensitized (b) guinea-pigs. Results are expressed as % of the maximum contraction and each point is the mean with s.e.mean indicated by vertical lines; *n*: (a) = 9; (b) = 9; (c) = 5. Open symbols. solid line = + epithelium; filled symbols, broken line = − epithelium.

preparations from ovalbumin-sensitized guinea-pigs is biphasic and appears to involve exclusively histamine (mediating the initial rapid phase) and leukotrienes (responsible for the secondary sustained portion of the response) which are released from mast cells (Adams & Lichtenstein, 1979). Thus, the contraction is abolished by a combination of an H_1-histamine receptor antagonist and a leukotriene receptor antagonist (Wasserman *et al.*, 1986). We have previously demonstrated that removal of the epithelium produced a greater increase in sensitivity to antigen than to histamine (Hay *et al.*, 1986b). It was anticipated that the sensitivity to leukotrienes would be affected to a similar extent by epithelium removal as the sensitivity to antigen. However, the increase in sensitivity to LTC_4 and LTD_4 (approximately 2 to 4 fold) in denuded preparations was smaller than that observed for antigen and similar to that previously determined for histamine (Hay *et al.*, 1986a,b). The reason for the greater influence of epithelium removal on responses produced by antigen challenge, when compared with those induced by the individual mediators, histamine and leukotrienes, alone is unclear. It is possible that the inhibitory factor, in addition to modulating directly the bronchoconstrictor effects of leukotrienes and histamine, also may decrease the release of these mediators from mast cells.

Although ovalbumin-sensitization has been shown to produce increased responsiveness to histamine in guinea-pigs *in vivo* (Iwayama *et al.*, 1982), it does not produce airway hyperreactivity *in vitro*; in fact, a decreased responsiveness to histamine and methacholine has been reported in trachea from ovalbumin-sensitized guinea-pigs (Cheng & Townley, 1983; Hay *et al.*, 1986b). In the present study, both the sensitivity and the maximum contractile response to LTC_4, LTD_4, LTE_4, 5-HT and U-44069 were the same in preparations (intact and rubbed) from control and ovalbumin-sensitized guinea-pigs. This confirms the ineffectiveness of ovalbumin-sensitization as an *in*

vitro model of airway hyperreactivity.

In summary, the selectivity in the effects of removal of the epithelium on responses produced by leukotrienes, 5-HT and U-44069, together with that previously reported for several bronchorelaxants (Farmer *et al.*, 1986b), provides evidence that the effects of epithelium removal on the responsiveness of airway smooth muscle are due to a differential influence on epithelium-derived modulatory factors rather than solely the loss of a diffusion barrier. In view of the evidence that the leukotrienes are directly involved in the pathophysiology of asthma, the present results suggest that epithelial disruption, a feature of this disorder (Laitinen *et al.*, 1985), may increase the responsiveness to these mediators and contribute to the characteristic airway hyperreactivity.

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