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The effects of epithelium removal on the sensitivity of guinea-pig isolated trachealis to bronchodilator drugs

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1 Mechanical removal of the epithelium increased the sensitivity of tracheal strips to isoprenaline, sodium nitroprusside, and to adenosine (only in the presence of inhibitors of its uptake and metabolism). Epithelium removal was without effect on sensitivity to salbutamol or papaverine.

2 Preincubation of tracheal strips with an inhibitor of extraneuronal uptake, corticosterone (50 μM), had no effect on tissue sensitivity to either salbutamol or papaverine. However, the steroid both increased sensitivity to isoprenaline, and abolished the effect of epithelium removal on sensitivity to this catecholamine.

3 These results suggest that in the guinea-pig, the tracheal epithelium is a major source of extraneuronal uptake for catecholamines. Furthermore, the increase in trachealis sensitivity to isoprenaline following epithelium removal is probably due to loss of these sites of extraneuronal uptake.

4 The fact that sensitivity to salbutamol, papaverine and adenosine (in the absence of metabolic inhibitors) was not increased by denuding the epithelium indicates that loss of a diffusion barrier to drugs is not the mechanism of increased sensitivity.

5 Adenosine (and possibly nitroprusside) may cause the epithelium to release a smooth muscle excitatory factor. Thus, removal of the epithelium attenuates this excitatory influence and enhances smooth muscle responsiveness to adenosine.

6 These results provide further evidence that the epithelium has an important role in modulating the sensitivity of guinea-pig trachealis to drugs.

Introduction

Mechanical removal of the epithelial layer increases the responsiveness of isolated trachealis from several species, including humans, to various bronchoconstrictor drugs and it has been suggested that epithelial cells secrete an inhibitory factor which modulates airway smooth muscle tone (Flavahan *et al.*, 1985; Hay *et al.*, 1985; Barnes *et al.*, 1985; Raeburn *et al.*, 1986a). However, the effect of epithelium removal on responses to the β-adrenoceptor bronchodilator isoprenaline shows marked species variation. Dog bronchus and bovine trachealis are less sensitive to the spasmolytic action of isoprenaline following epithelium removal (Flavahan *et al.*, 1985; Barnes *et al.*, 1985), whereas a recent study by Goldie and co-workers (1986) found no change in sensitivity of guinea-pig trachea to isoprenaline after the epithelium was removed, though there was a reduction in the maximum response. Conversely, Holroyde (1986) and studies in our laboratory with guinea-pigs have demonstrated increased tracheal responsiveness to isoprenaline following epithelium removal (Farmer *et al.*, 1986; Hay *et al.*, 1986a, b). This is possibly due to the extraneuronal uptake of catecholamines being lost with the epithelium. It has been shown that guinea-pig trachea contains an extraneuronal uptake system for catecholamines, which are subsequently *O*-methylated (Foster, 1969; O'Donnell & Saar, 1973; Pun *et al.*, 1973; Anning *et al.*, 1979). The first objective of the present study therefore, was to determine if the increased sensitivity of the tissues to isoprenaline after epithelium removal involves a loss of extraneuronal uptake. These studies involved an examination of the

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effects of epithelium removal on the ability of corticosterone, an inhibitor of extraneuronal uptake (Gillespie, 1976; Bryan & O'Donnell, 1981), to alter sensitivity to isoprenaline and to the β_2-selective agonist salbutamol, which is not a substrate for extraneuronal uptake (McFadden, 1981).

Secondly, we wished to examine the possibility that epithelium removal might alter trachealis sensitivity to other bronchodilator drugs, and we therefore examined adenosine, papaverine and sodium nitroprusside.

Some of the results have been presented previously in preliminary form (Farmer *et al.*, 1986; Hay *et al.*, 1986a).

Methods

Tissue preparation

Male, English short-haired guinea-pigs (350–500 g; Camm Research Institute, Wayne, New Jersey) were killed by a blow to the head and exsanguinated. The trachea was removed and placed in modified Krebs-Henseleit solution (composition mM: NaCl 113, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, glucose 5.5). Following removal of adhering fat and connective tissue the trachea was slit open along its longitudinal axis, directly opposite the smooth muscle, and strips consisting of two adjacent cartilage rings were prepared. The strips were suspended in 10 ml organ chambers containing modified Krebs-Henseleit solution at 37°C and gassed with 95% O_2 and 5% CO_2. Tissues were connected to force-displacement transducers for the measurement of isometric tension responses. Before commencement of each experiment the tissues were equilibrated for 60 min under an optimal resting load of 1 g, and washed with fresh solution every 15 min.

The epithelium was removed by gently rubbing the luminal surface (over both the smooth muscle and cartilage areas) of alternate strips with a cotton-tipped applicator. We have previously confirmed the effectiveness of this procedure (Hay *et al*, 1986b; Raeburn *et al.*, 1986b). Unrubbed, adjacent strips served as paired controls.

Experimental protocol

In some experiments, following the equilibration period, tissues were precontracted with equieffective concentrations of methacholine (tissues with epithelium, 2 μM; tissues without epithelium, 1 μM). These concentrations represent approximately the respective EC_{60} values for methacholine. Cumulatively increasing concentrations of bronchodilators were then added to the bath. Only one concentration-response curve was obtained from each tissue. In all experiments with adenosine, and some with isoprenaline, papaverine or nitroprusside, tissues were not precontracted, and relaxations of basal tone were obtained.

The effect of corticosterone (50 μM) on responses to isoprenaline, salbutamol or papaverine was determined in tissues which had been incubated with the steroid for 30 min. Similarly, concentration-response curves for adenosine were obtained in the presence or absence of both the adenosine uptake blocker dipyridamole (0.5 μM) and the adenosine deaminase inhibitor erythro-9-2-hydroxy-3-nonyl adenine (EHNA; 1 μM), which were added to the bath 30 min previously.

Results are expressed as a percentage of the maximum relaxation to each agonist. Geometric mean EC_{50} values (the concentration (M) producing 50% of the maximum response) were determined using linear regression analysis of probit-transformed data. The data were evaluated for differences by means of Student's t tests for paired or unpaired samples, as appropriate. The 0.05 level of probability was regarded as significant.

Drugs

Unless otherwise mentioned, drugs were freshly dissolved on the day of the experiment in 0.9% w/v NaCl solution (saline). Acetyl-β-methylcholine chloride (methacholine), (−)-isoproterenol (+)-bitartrate (isoprenaline), salbutamol, papaverine hydrochloride, adenosine hemisulphate and corticosterone 22-acetate were purchased from the Sigma Chemical Co. EHNA was obtained from Burroughs Wellcome Co., dipyridamole from Boehringer Ingelheim Ltd, and sodium nitroprusside from Fisher Scientific Co.

Isoprenaline stock solutions were made up in saline containing 14 mM ascorbic acid. Final concentrations of ascorbic acid never exceeded 13.8 μM in the bath. Corticosterone and dipyridamole were each dissolved in ethanol; the final concentration of 0.01% in the bath was without effect on the tissues.

Results

Regardless of whether epithelium removal had any effect on trachealis sensitivity to the various drugs used in this study, it was a consistent observation that relaxations developed more quickly and reached a steady level sooner in tissues free of the epithelium than in intact tissues.

Papaverine and nitroprusside

Epithelium removal had no effect on the sensitivity or maximum response to papaverine of either precon-

tracted tissues (Table 1 and Figure 1), or in tissues with basal tone (+ epithelium: $pD_2 = 6.13 \pm 0.11$, $n = 6$; − epithelium: $pD_2 = 6.06 \pm 0.04$, $n = 6$). Similarly, there was no effect of epithelium removal on the sensitivity of tissues with basal tone to nitroprusside (+ epithelium: $pD_2 = 6.78 \pm 0.08$, $n = 8$; − epithelium: $pD_2 = 6.84 \pm 0.13$, $n = 8$). In tissues precontracted with methacholine, epithelium removal produced a small but significant leftward shift (1.55 ± 0.15 fold) in the concentration-response curve for nitroprusside, though the maximum response was unaffected (Table 1 and Figure 1).

Adenosine

In the absence of dipyridamole and EHNA, the pD_2 value for adenosine in intact trachealis was not different from that in epithelium-denuded tissues (Figure 2 and Table 1). However, with the inhibitors of adenosine uptake and degradation present, epithelium removal caused a significant, 4.21 ± 1.26 fold, leftward shift of the adenosine concentration-response curve (Figure 2 and Table 1). Epithelium removal caused a significant increase in the maximum relaxation to adenosine in the absence of dipyridamole and EHNA (Table 1). Conversely, the maximum relaxation induced by adenosine in the presence of the inhibitors was unaffected by epithelium removal (Table 1).

Isoprenaline and salbutamol

In tissues with basal tone the pD_2 for isoprenaline was 8.62 ± 0.16 ($n = 4$), whereas in tissues stripped of their epithelium, the pD_2 for isoprenaline was 9.22 ± 0.16 ($n = 4$). These values were significantly different and

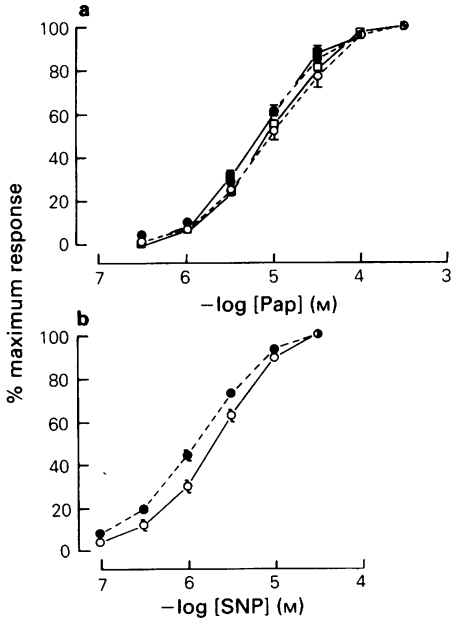

Figure 1 Concentration-response curves for (a) papaverine (Pap) and (b) sodium nitroprusside (SNP) in precontracted trachealis. Effect of epithelium removal and corticosterone (50 μM). (○) Intact tissues; (●) epithelium-free tissues; (□) intact tissues in presence of corticosterone; (■) epithelium-free tissues in presence of corticosterone. Each point is the mean of at least 6 observations and vertical lines show s.e.mean.

Table 1 Effect of epithelium removal on the sensitivity of guinea-pig trachealis to relaxant drugs

	+ *Epithelium*	− *Epithelium*
Papaverine (6)		
pD_2†	5.06 ± 0.07	5.23 ± 0.07
Maximum§	1307 ± 194	1402 ± 191
Nitroprusside (8)		
pD_2	5.85 ± 0.08	6.02 ± 0.09**
Maximum	910 ± 72	1080 ± 72
Isoprenaline (9)		
pD_2	7.70 ± 0.09	8.10 ± 0.10***
Maximum	1360 ± 60	1459 ± 91
Salbutamol (7)		
pD_2	7.51 ± 0.07	7.54 ± 0.12
Maximum	1220 ± 80	1360 ± 150
Adenosine (9)		
pD_2	3.60 ± 0.10	3.72 ± 0.06
Maximum	530 ± 62	644 ± 72*
Adenosine (0.5 μM dipyridamole + 1.0 μM EHNA) (8)		
pD_2	4.04 ± 0.11	4.55 ± 0.09**
Maximum	624 ± 40	686 ± 62

Data are expressed as means ± s.e.mean. Numbers in parentheses are the number of separate observations.
†pD_2 values were calculated as $-\log_{10}EC_{50}$ and are given in molar terms.
§Maximum relaxation response in mg. For each agent, pD_2 and maximum response values for paired intact and epithelium-denuded strips were compared using paired analysis. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared to + epithelium. All tissues, except those exposed to adenosine, were precontracted with methacholine (see Methods). EHNA = erythro-9-2-hydroxy-3-nonyl adenine.

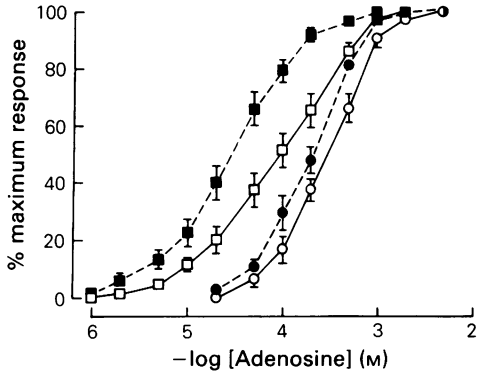

Figure 2 Concentration-response curves for adenosine in guinea-pig isolated trachealis. Effect of epithelium removal, and dipyridamole (0.5 μM) and erythro-9-2-hydroxy-3-nonyl adenine (EHNA, 1 μM). (○) Intact tissues; (●) epithelium-free tissues; (□) intact tissues in presence of dipyridamole and EHNA; (■) epithelium-free tissues in presence of dipyridamole and EHNA. Each point is the mean of at least 8 observations and vertical lines show s.e.mean.

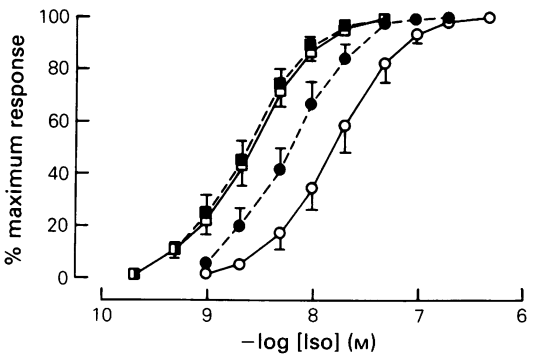

Figure 3 Concentration-response curves for isoprenaline (Iso) in precontracted trachealis. Effect of epithelium removal and corticosterone (50 μM). (○) Intact tissues; (●) epithelium-free tissues; (□) intact tissues in presence of corticosterone; (■) epithelium-free tissues in presence of corticosterone. Each point is the mean of at least 8 observations and vertical lines show s.e.mean.

represent a 4.06 ± 0.36 fold shift to the left of the isoprenaline concentration-response curve following epithelium removal. In tissues precontracted with methacholine, epithelium removal caused a significant, 2.66 ± 0.31 fold, leftward shift in the isoprenaline concentration-response curve (Figure 3 and Table 1).

In contrast to the results with isoprenaline, removal of the epithelial layer did not alter the sensitivity of the precontracted trachealis to salbutamol (Figure 4 and Table 1). The maximum relaxation induced by either of the β-adrenoceptor agonists was unaffected by the epithelium (Table 1).

Corticosterone

In 14 out of 16 intact tracheal strips, the addition of 50 μM corticosterone to the organ bath caused a transient relaxation of 233 ± 51 mg. Likewise, in 14 out of 16 epithelium-denuded strips, the steroid caused a similar relaxation of 244 ± 37 mg. In the remaining 4 tissues, corticosterone was without effect on basal tone.

In intact tissues with basal tone the pD_2 for isoprenaline, in the presence of corticosterone was 9.82 ± 0.12 ($n = 5$), whereas in epithelium-denuded tissues the pD_2 for isoprenaline was 9.72 ± 0.08 ($n = 5$). These values were not significantly different. Thus, although corticosterone caused 16.7 fold and 3.6 fold leftward shifts in the isoprenaline concentration-response curves for intact and denuded tissues respectively (Table 2), the steroid abolished the increased sensitivity to the β-agonist caused by epithelium removal.

Similarly, pretreatment of precontracted tracheal strips with corticosterone induced a leftward shift in the concentration-response curves for isoprenaline (Table 2 and Figure 3). In precontracted, intact trachealis corticosterone caused a 7.2 fold leftward shift in the isoprenaline concentration-response curve, and in denuded tissues the steroid caused a 3.2 fold shift. Again, however, in the presence of corticosterone there was no significant difference in the pD_2 values for isoprenaline in precontracted, intact and denuded tracheal strips (Table 2 and Figure 3).

In contrast to isoprenaline, incubation of tissues (intact and epithelium-free) with corticosterone was without effect on the responsiveness of the tissues to either salbutamol (Table 2 and Figure 4) or papaverine (Table 2 and Figure 1).

Discussion

The marked increase in sensitivity to isoprenaline following epithelium removal from guinea-pig tracheal strips is probably due to loss of epithelial sites of extraneuronal uptake. This is evidenced by the fact that removing the epithelium did not affect tissue sensitivity to salbutamol which, unlike isoprenaline, is not a substrate for extraneuronal uptake (McFadden, 1981; Reed, 1985). Moreover, while corticosterone caused a large leftward shift in the isoprenaline concentration-response curve, it was without effect on

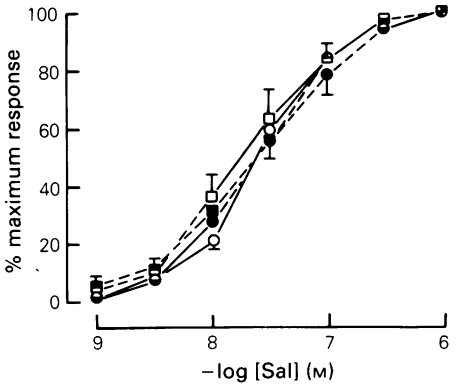

Figure 4 Concentration-response curves for salbutamol (Sal) in precontracted trachealis. Effect of epithelium removal and corticosterone (50 μM). (○) Intact tissues; (●) epithelium-free tissues; (□) intact tissues in presence of corticosterone; (■) epithelium-free tissues in presence of corticosterone. Each point is the mean of at least 7 observations and vertical lines show s.e.mean.

the salbutamol curve. This confirms previous findings (Pun *et al.*, 1973; Geddes *et al.*, 1974; O'Donnell & Wanstall, 1976) that hydrocortisone, another glucocorticoid inhibitor of extraneuronal uptake, increased the sensitivity of guinea-pig trachealis to isoprenaline but had no effect on responses to salbutamol. The effect of corticosterone is unlikely to be due to an increase in the number of β-adrenoceptors (Rinard *et al.*, 1983; Salonen, 1985), since the sensitivity to salbutamol was unaffected. Furthermore, Foster (1969) and Anning *et al.* (1979) showed that drugs which inhibit extraneuronal catecholamine uptake inhibit both the accumulation of, and potentiate responses to, isoprenaline in guinea-pig trachea. That corticosterone had no effect on trachealis sensitivity to papaverine is also indicative that the action of this steroid, in our study, was not a non-specific one. The fact that corticosterone abolished the difference in sensitivity to isoprenaline between tissues containing or lacking an intact epithelium suggests that the epithelium is a major source of extraneuronal uptake of isoprenaline.

To our knowledge, no one has previously described an extraneuronal uptake system for catecholamines in airways epithelial cells. Further, O'Donnell & Saar (1973), using a histochemical technique and noradrenaline as substrate, only rarely observed fluorescence in the epithelium and concluded that, in the intact guinea-pig trachea, extraneuronal uptake occurred mainly in the smooth muscle and cartilage. However, noradrenaline has a high affinity for neuronal uptake but it is not transported as efficiently as isoprenaline by the extraneuronal uptake carrier (Trendelenburg, 1979). In the present study, the observation that corticosterone increased the sensitivity of epithelium-free trachea to isoprenaline (albeit to a much smaller extent than in intact tissues) is evidence that in guinea-pig airways the smooth muscle (and probably also the cartilage) is a site of appreciable extraneuronal uptake (O'Donnell & Saar, 1973; Anning *et al.*, 1979). Catechol *O*-methyltransferase and presumably a related catecholamine extraneuronal uptake mechanism, has been localized in the epithelial cells of several organs including those of the brain, liver, kidney and vas deferens (Inoue *et al.*, 1977). The present results

Table 2 Effect of corticosterone on the sensitivity of precontracted guinea-pig trachealis to bronchodilators

	+ *Epithelium*		− *Epithelium*
		pD_2	
Isoprenaline			
Control (9)	7.70 ± 0.09		8.10 ± 0.10***
Corticosterone (8)	8.56 ± 0.07†††		8.61 ± 0.07†††
Salbutamol			
Control (7)	7.51 ± 0.07		7.54 ± 0.12
Corticosterone (7)	7.65 ± 0.14		7.70 ± 0.12
Papaverine			
Control (6)	5.06 ± 0.07		5.23 ± 0.07
Corticosterone (6)	5.08 ± 0.09		5.18 ± 0.08

Data are expressed as means ± s.e.mean. Numbers in parentheses are the number of separate observations.
pD_2 values for tissues containing or lacking epithelium were compared using paired analysis. ***$P < 0.001$, significantly different from + epithelium.
pD_2 values for tissues in the presence and absence of corticosterone (50 μM) were compared using non-paired analysis. †††$P < 0.001$, significantly different from control.

indicate that the extraneuronal uptake system seems to be located to a large extent in the epithelial cells of the guinea-pig trachea.

It has been proposed recently (Holroyde, 1986) that the increase in responsiveness of airway smooth muscle to contractile and relaxant drugs following epithelium removal is due to the loss of a diffusion barrier, and not, as proposed by others (Flavahan *et al.*, 1985; Hay *et al.*, 1985; 1986b; Barnes *et al.*, 1985), to loss of a modulatory factor(s) secreted by the epithelium. Certainly, loss of a diffusion barrier may contribute to the effect of epithelium removal, but it does not explain the present findings that stripping the epithelium had no effect on the responsiveness to salbutamol, papaverine, adenosine (in the absence of dipyridamole and EHNA), nitroprusside (in basal-tone tissues), or to isoprenaline in the presence of corticosterone. In canine trachealis removal of the epithelium not only potentiated responses to contractile agents, but it also attenuated responses to isoprenaline (Flavahan *et al.*, 1985), an effect which cannot be attributed to loss of a diffusion barrier. Furthermore, responses were quantitated at the 'plateau' level, i.e., at a time when the interaction between each drug and the tissue had reached dynamic equilibrium. The frequent observation in the present study that responses to each agent developed more quickly in rubbed preparations may manifest the loss of an epithelial barrier to drug diffusion. However, though an epithelial diffusion barrier may increase the time taken for each response to develop, it should not alter, under equilibrium conditions, the agonist concentrations producing equivalent responses (i.e., tissue sensitivity). It seems more likely, therefore, that epithelial cells produce one or more factors which modulate smooth muscle responsiveness.

Previous studies with canine (Flavahan *et al.*, 1985), guinea-pig (Hay *et al.*, 1985; Goldie *et al.*, 1986), bovine (Barnes *et al.*, 1985), rabbit (Raeburn *et al.*, 1986b) and human (Raeburn *et al.*, 1986a) airways have demonstrated increased responsiveness to contractile agents such as cholinoceptor agonists, histamine, 5-hydroxytryptamine and electrical field stimulation following removal of the epithelium. It has been proposed by several groups of investigators that airways smooth muscle responsiveness to contractile drugs may be reduced by one or more inhibitory factors released from the epithelial cells. Thus, removal of the epithelium eliminates this inhibitory influence and consequently increases responsiveness to bronchoconstrictors (Barnes *et al.*, 1985; Flavahan *et al.*, 1985; Hay *et al.*, 1986b; Goldie *et al.*, 1986; Raeburn *et al.*, 1986a, b). This situation may be analogous to the potentiation of vascular smooth muscle responsiveness to noradrenaline and 5-hydroxytryptamine after loss of the endothelium (Cocks & Angus, 1983), due to removal of the inhibitory effect of released endothelium-derived relaxant factor (ERDF).

It is important to note that the most prevalent features of asthma are severe bronchoconstriction and bronchial hyperreactivity (Garland, 1984). The hyperreactivity may be connected with the fact that the airways epithelium in these patients is damaged or lost (Laitinen *et al.*, 1985). Also, it is known that during respiratory viral infections there is both bronchial hyperreactivity (Empey *et al.*, 1976) and loss of bronchial epithelium (Hers, 1966). We have recently demonstrated that human isolated airways smooth muscle responsiveness to methacholine *in vitro* is increased by removing the epithelium (Raeburn *et al.*, 1986a).

In contrast to the β-adrenoceptor agonists, the effects of epithelium removal on responsiveness to adenosine and nitroprusside are more difficult to interpret. Recently, Holroyde (1986) showed increased sensitivity of guinea-pig trachea to the relaxant action of adenosine following epithelium removal. This study also demonstrated that in rubbed tissues, concentrations of adenosine of less than 100 μM produced a contraction. Our results differ in two respects. Firstly, we did not observe the contractile action of the purine at any concentration. This may reflect differences in the resting tone of the preparations, since it has previously been shown that adenosine may cause either contraction or relaxation of guinea-pig trachea, depending on the tone of the tissue (Fredholm *et al.*, 1979; Advenier *et al.*, 1982). Secondly, we found that epithelium removal increased the sensitivity to adenosine only in the presence of dipyridamole and EHNA. If the potentiating effects of epithelium loss on responsiveness were due simply to removal of a diffusion barrier, one might expect an increased sensitivity to adenosine whether or not the inhibitors are present. Rather, it is possible adenosine causes the secretion by epithelial cells of some factor which stimulates airway smooth muscle. Thus, removal of the epithelium may result in a loss of this excitatory influence and in a subsequent potentiation of the direct action of adenosine. The potentiation, albeit a small one, of nitroprusside-induced tracheal relaxation in the present study might be similarly explained. Indeed, in rat aortic smooth muscle, removal of the endothelium enhances the vasodilatation produced by nitroprusside (Shirasaki & Su, 1985). We can offer no explanation for the observation that epithelium removal potentiated the adenosine-induced relaxant responses only in the presence of dipyridamole and EHNA, or that sensitivity to nitroprusside was increased only in tissues which were precontracted. Further investigation in this area is required.

In conclusion, while the effect of epithelium removal on sensitivity to isoprenaline can be explained by a loss

of extraneuronal catecholamine uptake, the increase in sensitivity to other agents cannot be explained in the same way. We think it unlikely that the effect of epithelium removal is due to the loss of a diffusion barrier since the responsiveness to several agents is not altered.

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