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Responsiveness of human isolated bronchial segments and its relationship to epithelial loss

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1 The responsiveness of human and bovine bronchi was examined by comparing the magnitude of responses to agonists applied to the adventitial (outside) and to the luminal (inside) surfaces. The development of smooth muscle tone was measured as both an increase in pressure in isovolumic bronchial segments and narrowing in perfused segments.

2 In both closed and perfused human segments, ACh added to the adventitial surface produced highly homogeneous responses while responses to ACh added to the luminal side were extremely variable.

3 Histological examination of the human segments showed that they possessed variable degrees of pre-existing epithelial loss from the mucosal circumference, ranging from 0 to 82%.

4 Denuded human isovolumic segments (exhibiting >30% epithelial loss) were 40 fold more sensitive to ACh inside than intact segments (<30%). Denuded segments were also equally responsive to KCl inside or outside while KCl inside produced a contraction that was 50% of that to KCl outside in intact segments.

5 A strong and highly significant relationship was determined between the proportion of epithelial loss and both the responsiveness and rate of contraction of human segments to ACh and KCl introduced onto the lumen. No relationship was observed between epithelial denudation and responsiveness or contractility to agonists added to the adventitial surface.

6 Mechanical denudation of the epithelium from bovine segments had no effect on responsiveness to ACh or KCl added to the outside while significantly augmenting the sensitivity to ACh (9 fold) and reactivity to KCl introduced into the lumen to the extent that it became the same as outside.

7 The evidence presented here indicates that the response characteristics of *in vitro* airway segments to agonists are highly dependent on the integrity of the epithelial layer which acts as a barrier inhibiting movement of agonists from the lumen to the smooth muscle.

Keywords human bronchial segment bovine bronchial segment bronchial epithelium epithelial denudation epithelial stripping

Introduction

Epithelial damage may lead to heightened responsiveness of airways by exposure of nerve endings (Barnes, 1987), depletion of a putative epithelial derived relaxant factor (EpDRF) (Goldie *et al.*, 1988, 1990; Stuart-Smith & Vanhoutte, 1988, Vanhoutte, 1988), uptake or degradation of agonists (Farmer *et al.*, 1986, Inoue *et al.*, 1992) and/or the destruction of a pre-existing diffusion barrier altering permeability of the epithelial layer (Holroyde, 1986; Mitchell *et al.*, 1990; Munakata *et al.*, 1989; Small *et al.*, 1990; Sparrow & Mitchell, 1991).

Animal models have shown that factors that alter epithelial permeability, such as cigarette smoke, also alter reactivity to inhaled agonists (Hogg, 1990; Nishikawa *et al.*, 1990). Studies undertaken in humans

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however have not supported these findings. O'Byrne *et al.* (1984) showed that while asthmatics were 20-fold more sensitive to inhaled histamine, the permeability of their airways to ^{99m}Tc-DTPA (technetium-labelled diethylenetriamine pentacetic acid) was no different than that of control subjects. Equally as puzzling was the finding that smokers exhibited a greater permeability without demonstrating any changes in responsiveness. This has also been supported by Kennedy *et al.* (1984) who suggest that the reason for this is that smoke induced permeability changes occur in the peripheral airways while responsiveness in asthma occurs in the main central bronchi. It is clear from these numerous contradictory observations that the link between permeability changes in epithelia and hyper-responsiveness of human airways is poorly understood.

Jeffery *et al.* (1989) quantified the proportion of epithelial loss observed in bronchial biopsies from asthmatics and observed a correlation between epithelial loss and reactivity of patients to inhaled methacholine. In this present study we have examined the responsiveness of intact bronchial segments, taken from lungs removed from patients with lung carcinoma. Using this *in vitro* approach, the pressures generated by isovolumic segments and the extent of airway narrowing by perfused segments, were measured in response to agonists applied via the lumen and compared with those obtained by applying the agonists to the outside of the bronchial tube. The integrity of the epithelial layer was quantified to determine the relationship between ciliated epithelial loss and responsiveness. Because of the variability of the responsiveness of human tissue, freshly dissected bovine bronchial segments were also examined to serve as a control and provide a comparison to the responses observed in human segments.

Methods

Isovolumic and perfused segments

The 'isovolumic bronchial segment' consisted of a completely intact segment of airway lying horizontally in a modified organ bath (Figure 1) containing Krebs solution (37° C, pH 7.4, gassed with 5% CO_2 in O_2). The segment was cannulated at both ends and Krebs solution from a reservoir (pressure head) was perfused through the lumen or alternatively closed, via proximal and distal taps. A pressure head of 10 and 6cmH_2O was used for human and bovine segments respectively, these were determined from passive *vs* active pressure curves to be optimum for the segments. When the segment was isolated in this manner the intraluminal volume remained constant (hence 'isovolumic'), therefore any changes in the tone of the bronchial smooth muscle produced alterations in the pressure within the system. Pressure changes were measured via a pressure transducer (type MPX10DP, Motorola semiconductors, Phoenix, USA) located proximal to the segment. The transducer response was linear over a range of pressures (0–100 cmH_2O). Substances dissolved in 0.5–1.0 ml Krebs solution were introduced into the lumen, via the 3 way tap at the proximal end of the segment, when the taps at both ends of the preparation were open, or to the serosa, by direct addition to the bathing medium. After a substance was administered intraluminally the taps were quickly closed, before pressure had begun to develop in the lumen. This took less than 1 second. The segments were also electrically field stimulated (EFS) via encircling platinum ring electrodes placed about 1 cm apart.

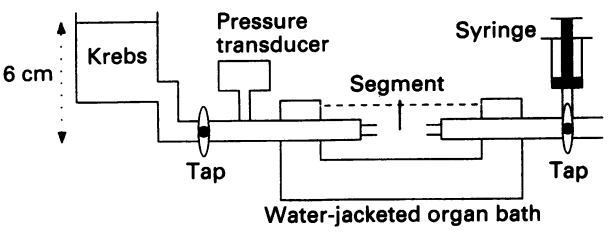

Figure 1 Diagram of the isovolumic preparation.

The narrowing of 'perfused segments' was measured via the perfusion apparatus described by Mitchell *et al.* (1989). Krebs was perfused through the lumen of segments from a 5 cm H_2O pressure head and the flow rate was measured with a differential pressure transducer. Drugs were added to the inside via the perfusate or to the outside bathing medium.

Collection and preparation of airway segments

Segments of human airway were obtained from post-operative lung tissue from patients that had undergone lung resection due to lung carcinoma. The mean age of the donors was 66.5 ± 4.7 years (n = 9) and all were previous smokers. From each lung specimen segments were obtained from regions of the lungs that were devoid of any bronchogenic growths and were stored in cold phosphate buffered saline pH 7.4 (PBS) for use the following day. The time between removal and storage in PBS was less than 1 h. Bovine lungs were obtained from the local abattoir, the time between death and collection being not more than 15 min where upon the whole lungs were packed in ice.

Parenchyma attached to the bronchi was bluntly dissected away and the side branches tied off and cut. As the availability of human lung tissue was considerably limited it was impossible to standardize totally the size of the segments used. While the length was kept constant at 15 mm the external diameter varied between 3 and 6 mm. Bovine bronchial segments of 4 mm external diameter and 20 mm length were obtained from the upper lobes of the lungs. The bovine segments were denuded by a inserting a moistened cotton bud into the lumen and gently rubbing of the epithelium.

Protocols

As the human tissues were stored they required 2 h to equilibrate while bovine segments required 1 h. After equilibration the segments were primed by adding a submaximal concentration (10 μM) of ACh to the Krebs solution bathing the adventitia, after washout and a further 10–20 min recovery time the segments were electrically field stimulated (20 Hz,60 V, bovine 0.5 ms, human 1.0 ms), which elicited a contraction that was

approximately 30–50% of E_{max}. This was given at regular intervals during an experiment to monitor the responsiveness of the tissue. The Krebs solution bathing the segments was exchanged for fresh solution at regular 10 min intervals throughout the experiment. In the case of the isovolumic technique the Krebs solution in the lumen was also replaced at the same time. In the perfused setup there was a continuous flow of Krebs through the lumen.

Concentration-response curves to ACh added on the adventitial surface (outside) were recorded cumulatively. From this the maximal response to ACh (E_{max}) was determined and used to standardise the responses to both ACh and KCl observed in the segments. Due to the nature of the isovolumic apparatus it was not possible to increase the concentration of drug added to the lumen without initially releasing the intraluminal pressure whilst injecting the drug. For this reason the responses to agonists introduced into the lumen were obtained non-cumulatively by introducing single concentrations of drug inside the tube. The drugs were washed out by perfusing with Krebs solution from the reservoir and after recovery the preparations were then challenged again with a higher concentration. In perfused human segments cumulative concentration-response curves were generated to both luminal and adventitial addition of ACh.

The rate at which the segments generated an increase in transmural pressure ($\Delta P\ s^{-1}$) was estimated at the inflexion point (steepest point) of the observed response. This rate of contraction was then expressed as a percentage of the maximum amount of pressure that was produced by the segment ($\%\Delta P\ s^{-1}$).

Histology

To investigate the structure of the various segments the tissues were fixed in 4% formaldehyde for 72 h then transfered to 40% sucrose for a further 24 h and embedded in CRYO-M-BED (Bright). Each segment was cut into distal, central and proximal regions and 15 μm sections were made in triplicate from each region and stained with haemotoxylin and chromotrope 2R. Morphometric measurements from each section were made by tracing the desired features using a *Chromatic*—colour image analysis system (Leading Edge Pty Ltd, Bellevue Heights, South Australia).

In each segment areas devoid of columnar epithelium were grouped together and classified as epithelial loss. An estimate of the amount of epithelial loss exhibited by each of the segments was obtained by dividing them into proximal, central and distal regions. One representative section from each region was then examined and the amount of epithelial loss quantified. The average of the three regions was then used as an estimate of the overall epithelial integrity of the segment. Other morphometric parameters including area (adventitial/smooth muscle/mucosal), perimeter (internal/external) and diameter were estimated in the same fashion.

Solutions

Krebs solution had the following composition (mM): NaCl 121, KCl 5.4, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.5 and $CaCl_2$ 2.5. The PBS contained (mM): NaCl 150, NaH_2PO_4 5, Na_2HPO_4 15. Depolarising Krebs solutions contained 121 mM KCl in place of NaCl. Acetylcholine chloride was obtained from Sigma Chemicals and prepared in Krebs solution on the day of each experiment. All drug solutions were kept on ice.

Statistical analysis

Data shown are mean ± s.e. mean with n = number of preparations. The significance of the difference between means was compared using the Student's t-test (paired). Correlations were determined using a regression analysis. $P < 0.05$ was considered significant.

Results

EFS (1–5 ms, 10–20 Hz, 60 V) and addition of acetylcholine (ACh) and K^+ depolarizing solution to either the inside or outside of human bronchial segments, caused a prompt increase in pressure (in isovolumic segments) or reduction in flow rate in perfused segments. From each concentration-response curve the sensitivity (EC_{50}) and maximum effect was recorded. EC_{50}s were then transformed into pD_2 (see below).

Supra-maximal stimulation of human segments to ACh added to the adventitial side gave a mean E_{max} of 8.9 ± 1.5 cmH_2O (n = 9) in isovolumic segments and a 80.4 ± 4.2% reduction in flow rate in perfused segments (n = 5). The sensitivity of bronchial segments to ACh placed on the outside was highly consistent; for human isovolumic segments the mean pD_2 ± s.e. mean was 4.00 ± 0.29 (n = 9). However, when placed on the inside, responses to ACh and to K^+ depolarizing solution showed marked variability in different segments. For example, in isovolumic segments, pD_2 values ranged from 2.82 to 6.50 representing almost a 10,000 fold range in sensitivities. Figure 2 shows representative chart

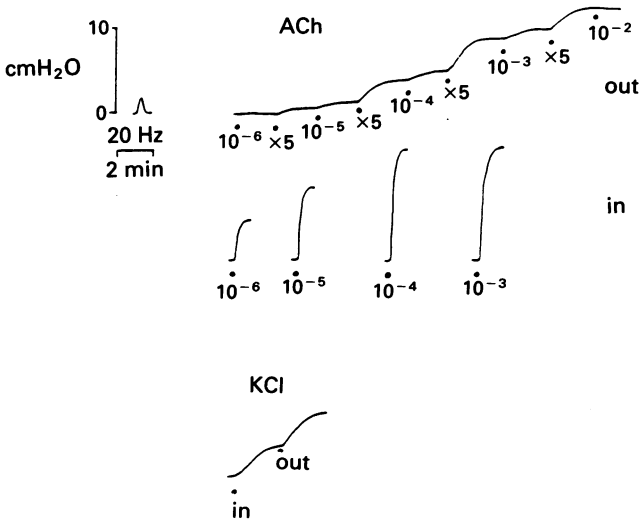

Figure 2 Increases in intraluminal pressure, above resting, of human bronchial segments contracting isovolumically to electrical field stimulation (20Hz, 1.0 ms, 60V) and to ACh and K^+depolarising solution (KCl). A resting luminal pressure of 10 cmH_2O was maintained throughout.

Table 1 Morphometric data from sections of human isovolumic segments and the corresponding coefficients of correlation (r) from a regression analysis between pD_2 values for ACh IN and OUT and morphometric measurements from individual segments. Morphometric data are expressed as mean ± s.e. mean ($n = 9$). * indicates a significant correlation ($*P < 0.005$)

	Mean	*Coefficient of correlation* (r) OUT	IN
Epithelial damage %	31.1 ± 7.93	0.20	0.88*
External diameter (mm)	4.2 ± 0.5	0.42	0.16
Internal perimeter (mm)	7.5 ± 1.1	0.32	0.05
External perimeter (mm)	11.7 ± 1.0	0.18	0.21
Mucosal area (mm^2)	0.9 ± 0.2	0.005	0.14
Smooth muscle area (mm^2)	0.4 ± 0.1	0.09	0.004
Adventitial area (mm^2)	5.5 ± 1.0	0.05	0.34
Total area (mm^2)	6.8 ± 1.2	0.26	0.15

recordings of responses to ACh and KCl in human isovolumic bronchial segments.

Human bronchial segments used above were subsequently examined morphometrically (see Methods) and the data are presented in Table 1. The range of diameters of segments studied was 2–5 mm i.d. and all airways contained cartilage. The most notable difference between different segments was the extent of damage or loss of the columnar epithelium. Within each bronchial segment there were usually some regions of intact ciliated columnar epithelium, and some regions exhibiting either squamous cell metaplasia (SCM) or complete denudation of epithelial cell types (Figure 3). For each section examined, all areas of mucosa that were devoid of columnar epithelial cells were classified as epithelial loss. The proportion of damaged to normal epithelium was usually consistent in the multiple sections from an individual airway segments (7 of the 9 isovolumic segments and all perfused segments exhibited <35% variation between sections) but it was highly variable between segments from different patients.

Areas of SCM were very different in appearance from normal epithelium (see Figure 3b) and consisted of two or more layers of non ciliated flattened cells (Figure 3c). Regions that were completely denuded of epithelium were usually associated with ulcers of chronic inflammation of the mucosa exhibiting cellular infiltration, oedema as well as collagen deposition (Figure 3a).

Since the median % loss of epithelium was 30% we grouped the isovolumic bronchial segments into those with extensive (>30% [range 32–82%], $n = 4$) epithelial loss i.e. 'denuded', and those with slight epithelial loss (<30% [range 0–30%], $n = 5$) i.e. 'intact'. Responses to ACh and to K^+ depolarizing solution in the two groups were then compared. The sensitivity to ACh added to the serosal surface (Figure 4a) was not different between the two groups (pD_2 values were 4.20 ± 0.36 [intact] *vs* 3.74 ± 0.50 [denuded]). In contrast, the denuded human segments were on average 40 fold more sensitive to ACh introduced via the lumen (Figure 4b) than the intact segments (pD_2 values were 5.00 ± 0.63 *vs* 3.41 ± 0.32 respectively [$P < 0.05$]). With K^+ depolarizing solution, responses were independent of the route of exposure in denuded preparations, but in intact segments responses were significantly attenuated when the KCl was introduced via the luminal surface (Figure 5).

To study possible mechanisms of this variability in

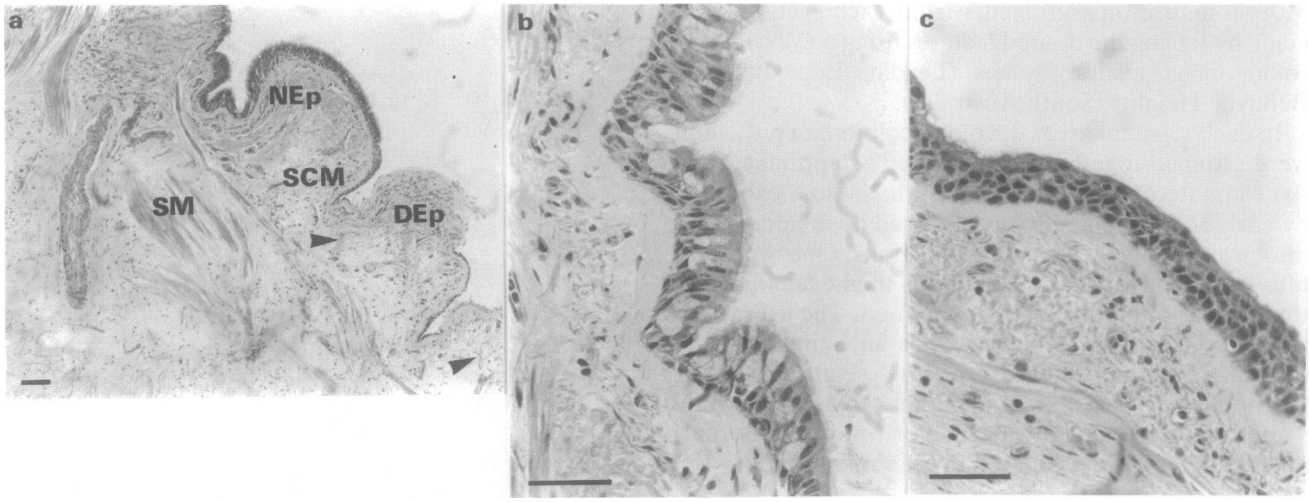

Figure 3 Photomicrographs of human bronchial mucosa depicting; a) a section of airway wall including smooth muscle (SM) showing normal epithelium (NEp), some squamous cell metaplasia (SCM) and a region denuded of epithelium (DEp) associated with a blister of inflammation in which collagen fibres are present (arrowheads), b) normal intact ciliated epithelium under high magnification, and c) a region of squamous cell metaplasia. Scale bars represent 25 μ.

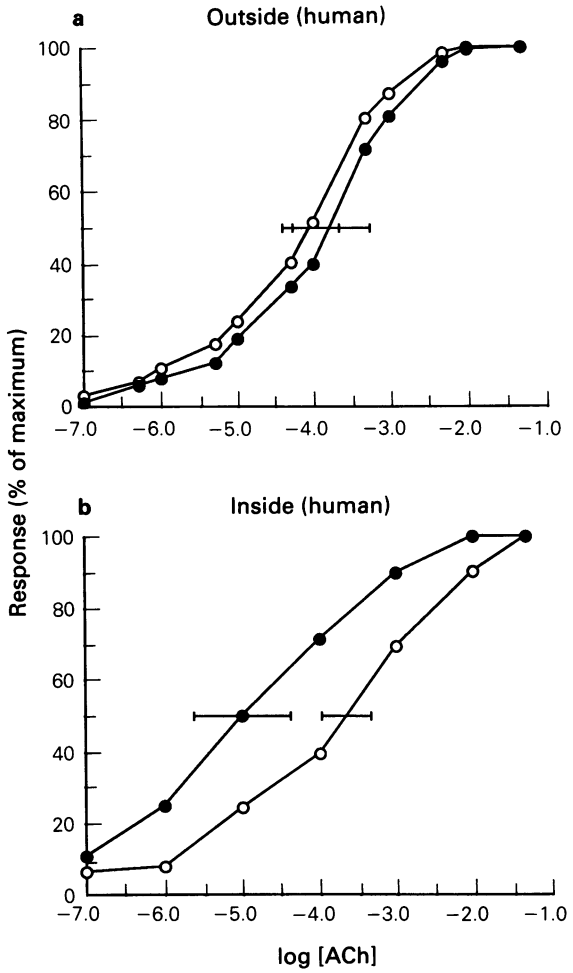

Figure 4 The concentration-response relationships to ACh from 'intact' (ranging from 0–30% epithelial loss $n = 5$) or 'denuded' (ranging from 32–82% epithelial loss, $n = 4$) human segments under isovolumic conditions. a) ACh added outside and b) ACh inside. ○-represent 'intact', ●-represent 'denuded'.

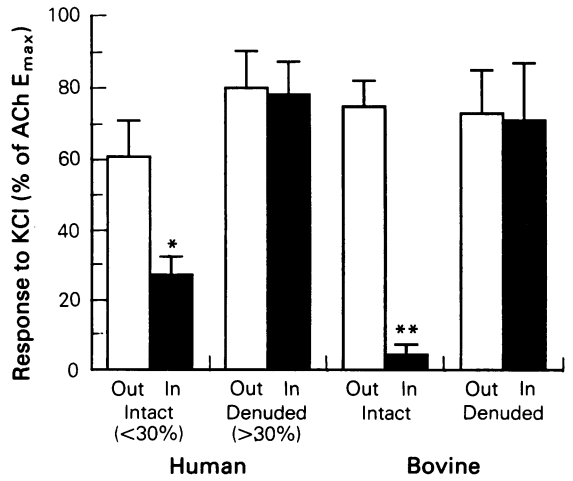

Figure 5 The reactivity to KCl depolarising solution (In *vs* Out) from human 'intact' (0–30% epithelial loss) and 'denuded' (32–82% epithelial loss) ($n = 4$) and bovine intact (0% epithelial loss) and denuded (> 90% epithelial loss) ($n = 6$) isovolumic segments. Data are expressed as a % of the maximum response generated by ACh. * In is significantly different from Out ($^*P < 0.05$, $^{**}P < 0.0005$).

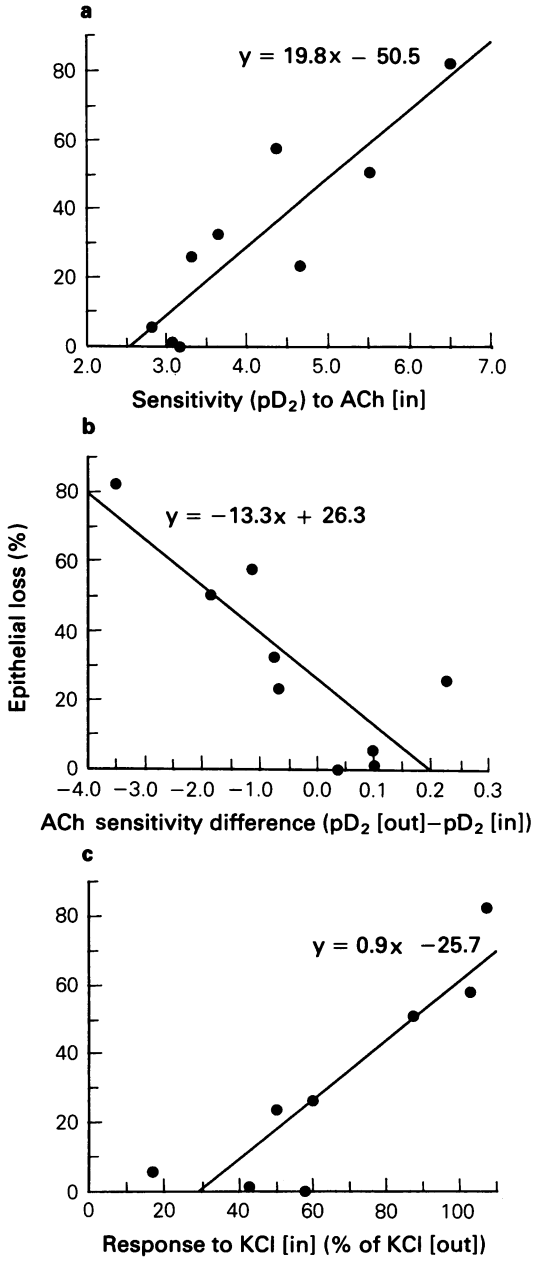

Figure 6 The correlation between the proportion of epithelial loss observed in human isovolumic bronchial segments and the; a) sensitivity to ACh introduced into the lumen [in] ($r = 0.885$, $P < 0.005$, $n = 9$), b) difference between pD_2 [in] and pD_2 [out] ($r = 0.821$, $P < 0.005$, $n = 9$) and c) the reactivity to KCl [in] ($r = 0.894$, $P < 0.005$, $n = 8$).

intraluminal responses of isovolumic segments we sought correlations between responsiveness (e.g. pD_2) and morphological properties of the airway wall described above. Responses of isovolumic segments to ACh and K^+ depolarizing solution inside were strongly correlated with the extent of loss of normal epithelium (Figure 6a, c). Responses to ACh and K^+ on the outside were not correlated with epithelial loss, neither was the sensitivity significantly correlated to other dimensions of the airway (Table 1). In addition, there was no correlation between the maximal response to either ACh or KCl and any of the morphometric parameters measured. The rate of pressure development after exposure to ACh or KCl to the inside was also correlated in the same fashion as sensitivity (Figure 7). Subtraction of the ACh pD_2

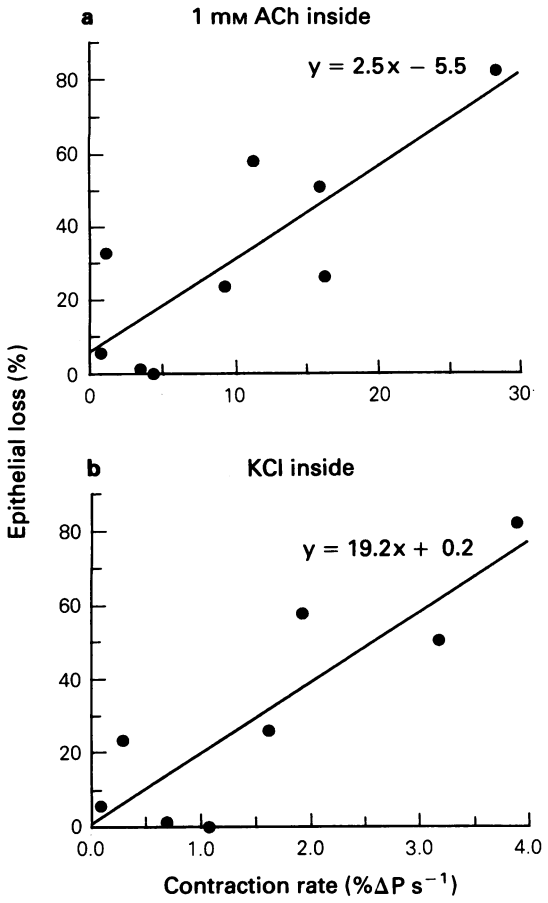

Figure 7 The correlation between the proportion of epithelial loss observed in human isovolumic segments and the contraction rate of the segments to: a) ACh [in] ($r = 0.816$, $P < 0.01$, $n = 9$) and b) KCl [in] ($r = 0.870$, $P < 0.005$, $n = 8$).

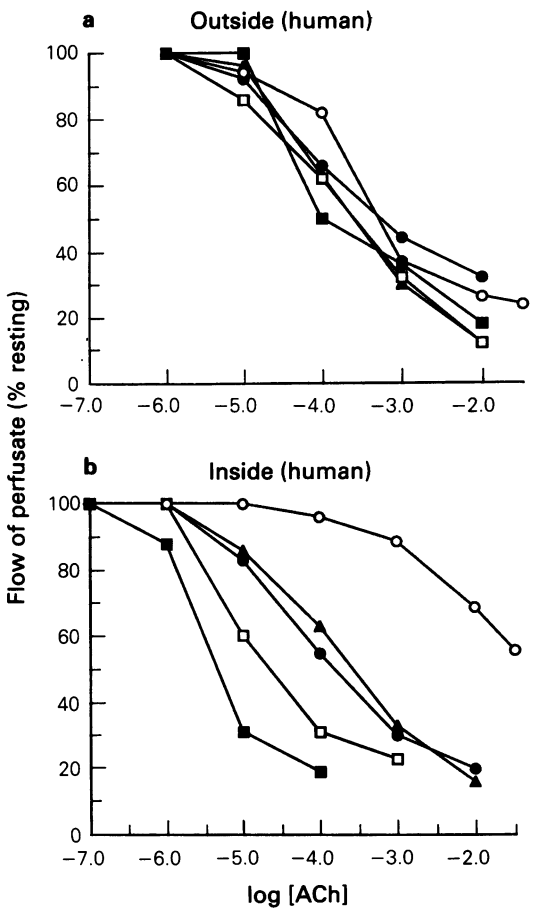

Figure 8 Airway narrowing in response to ACh from five separate human perfused bronchial segments (A–E), a) ACh added outside and b) ACh inside.

[IN] from the $_pD_2$ [OUT], to correct for inherent sensitivity differences between segments, again showed that augmented sensitivity was directly related to epithelial loss (Figure 6b).

In each of five perfused bronchial segments studied the sensitivity to ACh on the outside was consistent. However, on the inside, there was approximately a 1000 fold range in sensitivities in different segments (Figure 8). This was positively correlated to the epithelial loss ($r = 0.842$, $P < 0.05$). Responses to K^+ depolarizing solution on the outside averaged 52.4 ± 3.5% reduction in flow but on the inside responses were also highly variable in different preparations (from 0% to 57% flow reduction). Responses to K^+ depolarizing solution on the inside were positively correlated ($r = 0.883$, $P < 0.05$) with the sensitivity (pD_2) for intraluminal ACh. As observed with the isovolumic preparation the sensitivity of perfused segments to ACh did not correlate with any other morphometric parameters of the airway wall.

In view of the striking effect that the presence of columnar epithelium had on the responsiveness of human segments the effect of mechanical denudation of bovine segments was next examined. Histological examination of the bovine segments showed that the controls had an intact epithelium while all six mechanically denuded segments exhibited greater than 90% removal of the columnar epithelial layer. No differences in the magnitude of responses to EFS (20Hz) were observed between epithelial intact and epithelial denuded segments (responses to EFS [% of Emax] were 38.30 ± 8.3% [intact] and 37.26 ± 7.07% [denuded], $n = 6$). The concentration responses to ACh (both inside and outside) of bovine intact and denuded segments are shown in Figure 9. Removal of the epithelial layer did not significantly alter the sensitivity to ACh added to the adventitial surface of the bovine segments (pD_2 values were 3.18 ± 0.17 [intact] *vs* 3.11 ± 0.09 [denuded], $n = 6$). In contrast denuded bovine segments were on average 9 fold more sensitive to ACh introduced into the lumen than intact segments (pD_2 values were 1.81 ± 0.06 [intact] *vs* 2.76 ± 0.16 [denuded], $P < 0.001$, $n = 6$). Denuded bovine segments were also greatly more responsive to KCl introduced via the luminal side (see Figure 5).

Discussion

In this study we have examined the responsiveness of human bronchial segments to ACh and KCl and compared this with a number of morphometric parameters. When the agonists were introduced to the adventitial surface of the segments the responses were homogeneous and no significant correlations with parameters related to segment size (i.e. diameter, perimeter and cross-sectional area) were observed. The mucosa of the different human segments exhibited highly variable loss of ciliated

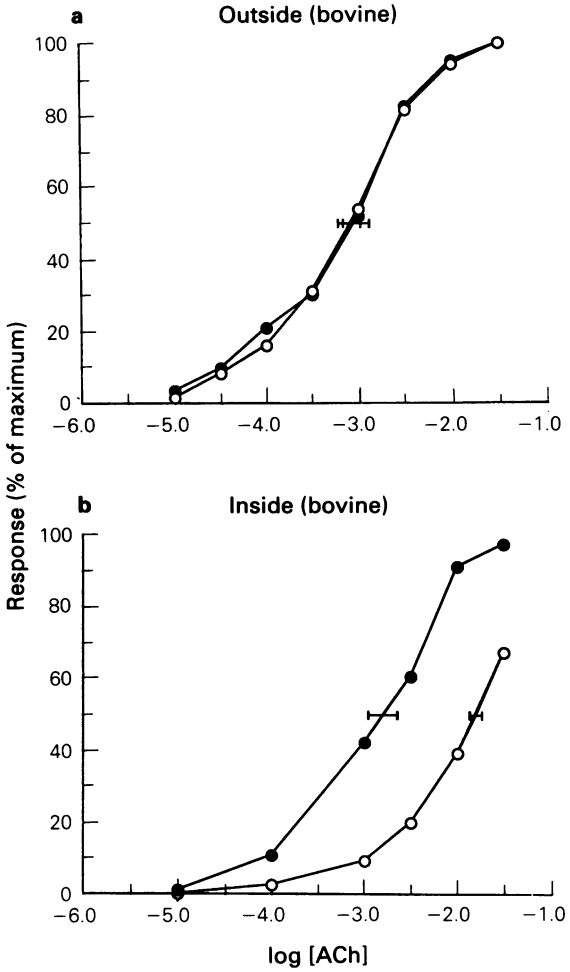

Figure 9 The dose response relationships to ACh from both intact and denuded bovine isovolumic bronchial segments. a) ACh added to the serosal side and b) ACh introduced into the lumen (n = 6). ○-represent intact, ●-represent denuded.

columnar epithelial cells along with squamous cell metaplasia (SCM). These findings are consistent with previous observations on the airways of smokers describing SCM as well as the coexistence of both inflammation and epithelial loss (Auerbach *et al.*, 1961; Leube & Rustad, 1991; Niewoeher *et al.*, 1974). Epithelial loss may be associated with the actions of activated inflammatory cells in the airway (Barnes, 1990; Frigas & Gleich, 1986; Hogg, 1990; Motojima *et al.*, 1989) or alternatively SCM, as the cells exhibit only loose desmosomal cell-cell attachment and are easily sloughed off (Leube & Rustad, 1991). The sensitivity, reactivity and rate of contraction to ACh and KCl introduced via the luminal surface was strongly correlated with the extent of this epithelial loss.

It has been postulated that airway epithelial cells may alter responsiveness by releasing a putative epithelial derived relaxant factor (EpDRF). EpDRF may alter the contractility of ASM directly (Vanhoutte *et al.*, 1988) or indirectly by dilating the microvasculature of the airway wall and increasing the clearance of spasmogens (Fernandes & Goldie, 1990). In a recent study by Xie *et al.* (1992) it was observed that the smooth muscle cells of epithelial denuded canine airways were more depolarised than epithelial intact preparations, and that when the denuded preparations were exposed to epithelial cell suspensions they rapidly hyperpolarised. This study also demonstrated an epithelial dependent inhibition of EFS responses that was due to the release of leukotrienes. Yu *et al.* (1992) showed that supernatants from canine cultured epithelial cells contained the cyclo-oxygenase product PGE_2 which caused relaxation of histamine and 5-HT contractions (canine TSM). This evidence indicates that the airway epithelium may constantly be producing putative substances that alter the electrophysiological and pharmacological properties of airway smooth muscle. In the present study, using bovine and human tubular bronchial segments we were unable to detect the effects of any endogenous epithelial derived factors from human airways because there was no correlation between columnar epithelial loss and either sensitivity or contractility to ACh added to the serosal side. Similarly, the stripping of bovine segments did not alter their responsiveness to adventitially added ACh or KCl indicating that the process of mechanically removing the epithelium did not cause the release of any long lasting bronchoactive substances. In the case of human bronchi the loss of epithelium was not brought about by mechanical perturbation (in the laboratory) so it is unlikely that freshly elaborated bioactive substances were responsible for the observed phenomenon.

The epithelium may also function as a metabolic barrier, reducing the amount of agonist reaching the smooth muscle layer via mechanisms such as the extra-neural uptake of catecholamines (Farmer *et al.*, 1986) or through the effects enzymes such as neutral endopeptidase (Inoue *et al.*, 1992) and acetylcholinesterase (Lev *et al.*, 1990) that are localised in the epithelium. While the correlation between epithelial loss and sensitivity to ACh in the current study may be influenced by endogenous ACh-esterase, the similar relationship between epithelial loss and KCl responses (unaffected by ACh-esterase) indicates that epithelially mediated hydrolysis of ACh may only exert a small effect, if any. In addition, other studies using segment preparations have demonstrated that the responsiveness to carbachol (a carbamic ester resistant to hydrolysis by ACh-esterase) is augmented by epithelial stripping, in a similar fashion as ACh (Small *et al.*, 1990; Sparrow & Mitchell, 1991).

The epithelium may also be a physical barrier preventing agonist molecules from gaining access to the smooth muscle layer (Holroyde, 1986; Mitchell *et al.*, 1990; Munakata *et al.*, 1989; Pavlovic *et al.*, 1989; Small *et al.*, 1990; Sparrow & Mitchell, 1991). Sparrow & Mitchell (1991) observed that epithelial disruption of porcine perfused segments potentiated the responsiveness to ACh (added via the lumen) by some 30 fold and concluded that the epithelial lining of airways was an effective barrier preventing the access of substances to the smooth muscle layer. In addition they were also able to show that the epithelial layer is indeed a substantial barrier to the movement of small inorganic ions like K^+ and vanadate (VO_3-). When added to the lumen such ions have virtually no effect compared with a substantial response when added to the adventitial surface. The response to K^+ ions added via the lumen can be enhanced by exposure to a Ca-free Krebs containing EGTA (chelator of Ca), this treatment denatures the tight junctions between epithelial cells but does not damage the cells themselves, leading to increased permeability

of K^+ (Omari & Sparrow, 1992). Other studies that have examined the effects of epithelial stripping on the responsiveness of airway muscle strips to KCl and have suggested that K^+ is not a releaser of EpDRF (Barnes *et al.*, 1985; Goldie *et al.*, 1986; Holroyde, 1986; Lennart-Lundblad & Persson, 1988). Our findings show that both human and bovine segments denuded of epithelium exhibit a large increase in sensitivity to ACh and reactivity to KCl inside. Our data also suggests (Figure 6a and b) that the pD_2 to intraluminal ACh, in human isovolumic and perfused segments with an intact epithelium, is approximately 2.5. This is more than 10 times less sensitive than the outside and is similar to the sensitivity difference reported here in bovine segments and previously in porcine segments (Sparrow & Mitchell, 1991). This suggests that the epithelium exerts a similar barrier effect in airways from different species (including man).

If epithelial cells act as a protective barrier, any alteration in their structural integrity would augment the ability of agonists to penetrate the mucosa and interact with receptors on the smooth muscle. Ciliated columnar epithelial cells are bonded together by tight junctions that exhibit a multilaminar necklace arrangement around the apex of the cell (Matsumura & Setoguti, 1989) which may serve to prevent the movement of substances between adjacent cells. Indeed substances that alter the integrity of tight junctions (e.g. NO_2) cause bronchial hyper-responsiveness (Gordon *et al.*, 1986). Such inter-epithelial junctional complexes may be damaged by cigarette smoke (Simani *et al.*, 1974). Squamous cell metaplasia is the transformation of normal cilliated columnar epithelial cells into flattened squamous cells. As previously mentioned such cells are attached to each other by desmosomes but do not exhibit tight junctions (Leube & Rustad, 1991). Squamous epithelial cells are normally present in the alveoli and capillaries where rapid diffusion of substances is essential. It is therefore conceivable that the process of SCM may augment the ability of substances to cross the epithelial layer by removing the elaborate cell-cell connections (tight junctions) known to exist between columnar epithelial cells. The epithelial permeability of airways *in vivo* has previously been assessed by measuring the clearance of radioactively labelled aerosols such as ^{99m}Tc-DTPA (technetium-labelled diethylenetriamine pentacetic acid). These studies have observed increases in epithelial permeability in smokers (Kennedy *et al.*, 1984; Kohn *et al.*, 1990; O'Byrne *et al.*, 1984). The use of animal models has also shown that cigarette smoke potentiates both permeability and reactivity to inhaled agonists (Hogg, 1990; Nishikawa *et al.*, 1990).

Bronchial responsiveness of patients undergoing lung resection has previously been examined *in vivo* and compared with the *in vitro* responsiveness of bronchial strips from the same patients (Armour *et al.*, 1984; Roberts *et al.*, 1984; Vincenc *et al.*, 1983). Each of these investigations showed that while the *in vivo* responsiveness was highly variable in different patients, the *in vitro* responsiveness of bronchial strips was very homogeneous and concluded that the variation *in vivo* is not the result of an intrinsic abnormality of the bronchial smooth muscle. In bronchial strip or ring preparations, agonists can easily come into contact with the smooth muscle without interacting with the epithelium. In contrast, segment techniques separate the adventitial and mucosal surfaces, so that agonists introduced into the lumen must first pass through the epithelial lining before making contact with the smooth muscle. By utilizing bronchial segment techniques we have shown that the responses of human segments *in vitro* were not homogeneous when agonists where added to the lumen. The strong link between epithelial loss and responsiveness *in-vitro* indicates that variations previously observed *in vivo* may also be explained in terms of epithelial loss.

We conclude that alterations in the integrity of the epithelial layer of human bronchial segments, will specifically determine the *in vitro* response characteristics of the segments to agonists when introduced via the lumen and not the adventitial side. Epithelial loss and the subsequent removal of a permeability barrier to diffusion may therefore be a major contributing factor in *in vivo* hyper-responsiveness.

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