THE ROLE OF THE AIRWAY EPITHELIUM IN THE MODULATION OF BRONCHIAL SMOOTH MUSCLE RESPONSIVENESS.

BY

KAREN STUART-SMITH, B.Sc. (Hons.), M.B., Ch.B.

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Based on research conducted at :-

Department of Physiology and Biophysics,
Mayo Clinic and Mayo Foundation,
Rochester,
Minnesota 55905,
U.S.A.

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All experimental protocols were designed by me.
I performed all of the experiments.
I carried out all of the data analysis.
The interpretation of the results, and the theories contained in the discussion are entirely my own.

Therefore the data presented in this thesis and the conclusions drawn from it, are the result of my own work.

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SUMMARY

Asthma is characterised clinically by bronchial hyperreactivity and bronchial spasm, and histologically by thickened oedematous airways, which show extensive epithelial destruction. In asthmatic patients, the degree of airway hyperreactivity correlates with damage to the epithelium. In vitro studies using animal models have shown that removal of the airway epithelium renders the bronchial smooth muscle more responsive to contractile agents, and diminishes the relaxing effect of beta-adrenergic agonists. It has been proposed that there is an epithelium-derived relaxing factor which diffuses from the epithelial cell and alters the responsiveness of the underlying bronchial smooth muscle. The aims of the present study were (i) to determine whether the airway epithelium can modulate the effect of contractile and relaxing agonists, (ii) to investigate whether such an effect shows heterogeneity between different airways, different species and different pharmacological agents, (iii) to investigate the potential role of the metabolites of arachidonic acid, prostaglandins and leukotrienes, and (iv) to use these data to draw conclusions about the distribution, mechanism of action, and physiological role of the proposed epithelium-derived relaxing factor or factors.

In the first part of the study, the effect of epithelium-removal on the pharmacological responses of canine airways was investigated. Second, third and

fourth-order bronchi of the dog were dissected from the lower lobe of the lung, and bronchial segments, with or without epithelium, were place in organ chambers. All tissues showed concentration-dependent contractile responses to acetylcholine, 5-hydroxytryptamine and histamine. For second and third-order bronchi, removal of the airway epithelium caused a significant leftward shift of the concentration-effect curves, indicating that tissues without epithelium were more sensitive to these contractile agents. The maximal contractions achieved, and the contractile response to potassium chloride, were unaffected by epithelium-removal, showing that the bronchial smooth muscle was undamaged. In fourth-order airways, there was no effect of epithelium-removal on the response to any of these agents. All bronchi without epithelium exhibited a diminished relaxation to the beta-adrenergic agonist, isoproterenol. This effect was small in second and third order airways but prominent in fourth order bronchi, where relaxation induced by isoproterenol appeared to be virtually dependent on the presence of the epithelium.

From these data, it is concluded that the airway epithelium of the dog shows two forms of release of the epithelium-derived relaxing factor(s). In the larger airways, basal release of an epithelium-derived relaxing factor reduces the response to contractile agents. In the small airways of the dog, isoproterenol may stimulate beta-adrenoceptors on the epithelium to produce a

factor which enhances the relaxation of the bronchial smooth muscle. This stimulated release is of minor importance in second and third order bronchi.

The second part of the study extended these observations to the airways of the pig. Third, fourth and fifth-order bronchi were examined. Removal of the epithelium caused a significant leftward shift of the concentration-effect curves for acetylcholine and histamine. Unlike the dog, the shift was uniform and distributed evenly throughout the airways. The response to isoproterenol was diminished in airways lacking the epithelium, but in contrast to the dog, the effect of epithelium-removal was similar in the three orders. It is concluded that both basal and stimulated release of the epithelium-derived relaxing factor occurs in porcine airways, but that its effects are more evenly distributed than in the dog. In the pig, the results may be complicated by the presence of an epithelium-derived contracting factor, which enhances the maximal response to potassium chloride, acetylcholine and electrical stimulation in some of the airways. The potential role of such a substance is not clear.

It is known that, in the dog, the degree of relaxation to isoproterenol depends on the contractile agonist used, and the degree of contractile tone achieved. Experiments were performed to determine whether these factors are relevant to the stimulated release of

epithelium derived relaxing factor from fourth-order canine airways. Tissues with and without epithelium were contracted with either low concentrations (ED_{40}) or high concentrations (ED_{80}) of either acetylcholine or 5-hydroxytryptamine, and concentration-effect curves to isoproterenol were obtained. In the presence of 5-hydroxytryptamine, and low concentrations of acetylcholine, there was no effect of epithelium-removal on the relaxation to isoproterenol. At the high concentration of acetylcholine, tissues without epithelium showed a markedly attenuated response to isoproterenol. However, in bronchi with epithelium, the relaxation was preserved such that there was no diminution in the response to the beta-adrenergic agonist. Thus, at levels of acetylcholine greater than the ED_{50} value, the relaxation is dependent on the presence of the epithelium. This effect is not seen at low levels of cholinergic tone or in the presence of 5-hydroxytryptamine, showing heterogeneity in the stimulated release. Relaxation to sodium nitroprusside demonstrated a similar pattern. By contrast, relaxations to forskolin were unaffected by the presence of the epithelium, arguing against a role for cyclic AMP in the response. The bronchi did not relax to nitric oxide. These results demonstrate release of a relaxing factor by a non-cyclic AMP, non-cyclic GMP dependent mechanism.

The response to arachidonic acid was also assessed in canine airways. Second, third and fourth-order bronchi

exhibited epithelium-dependent relaxations to arachidonic acid. The response was abolished by the inhibitors of cyclooxygenase, indomethacin and meclofenamate, and partly attenuated by the inhibitors of lipoxygenase, nordihydroguairetic acid, and BAYG6575. Radioimmunoassay studies revealed that tissues with epithelium release large quantities of prostaglandin E_2 in the presence of arachidonic acid. Bronchi without epithelium show only a small release of prostaglandin E_2, unaffected by the addition of arachidonic acid. It is likely that prostaglandin E_2 mediates the epithelium-dependent relaxations to arachidonic acid. The results obtained with lipoxygenase inhibitors indicate that leukotrienes may also be produced by the airway epithelium and affect prostaglandin E_2 release by an unknown mechanism.

Although prostaglandin E_2 is derived from the airway epithelium, and initiates relaxation of bronchial smooth muscle, it is not the epithelium-derived relaxing factor. Evidence presented in this study, and the evidence of other workers, show that the effects of the epithelium on responses to contractile and relaxing agents are not altered by inhibitors of either cyclooxygenase or lipoxygenase. Therefore, these phenomena cannot be mediated via prostaglandin release. The nature of the epithelium-derived relaxing factor or factors, remains to be elucidated.

INTRODUCTION

CLINICAL BACKGROUND

Asthma is a complex disease with many precipitating causes and clinical presentations (Scadding, 1985). One feature which is common to all forms of asthma is hyperreactivity of the airways (Nadel and Sheppard, 1985, Woolcock and Permutt, 1986). The hyperreactivity consists of an enhanced responsiveness to various bronchoconstrictor agents, as compared to normal individuals (Fig.1). This bronchial hyperreactivity is non-specific, as it is seen in response to such diverse stimuli as inhalation of allergens, cold air, exercise, industrial chemicals, and a number of pharmacological agents (Boushey et al, 1980; Barnes, 1983; Nadel, 1983). Early in the study of the disease it was noted that asthmatic patients exhibit abnormal bronchoconstriction in response to the cholinergic agonist pilocarpine (Alexander and Paddock, 1921) and histamine (Curry, 1946). This phenomenon is seen whether the drugs are administered intravenously, intramuscularly, or by aerosol inhalation (Curry, 1946; Orehek et al, 1977). Asthmatics also demonstrate hyperresponsiveness to other drugs such as acetylcholine, 5-hydroxytryptamine (serotonin) and bradykinin (Boushey et al, 1980).

Although the bronchial hyperreactivity of asthma is well documented, its cause remains unknown (Nadel and Sheppard, 1985; Stephens, 1987). Damage to the airway epithelium has been noted as a pathogonomic feature of asthma for many years (Dunnill, 1960). Extensive

areas of damage and desquamation of epithelial cells, as well as areas of regeneration and squamous metaplasia, have been noted in patients dying in status asthmaticus (Dunnill, 1960). It has been suggested that damage to the airway epithelium may contribute to bronchial hyperresponsiveness (Hogg and Eggleston, 1984; Nadel 1985).

Bronchoscopic examination demonstrates that asthmatic patients show epithelial destruction at all levels of the airways, even when they are in clinical remission; this is correlated with bronchial hyperreactivity to inhaled histamine (Laitinen et al, 1985). A similar study has shown that, in mild asthma, there is a strong positive relationship between the degree of hyperreactivity to methacholine challenge and the number of epithelial cells obtained by bronchoalveolar lavage (Wardlaw et al, 1988). Stimuli such as viral infections and ozone, which cause airway inflammation and consequent epithelial damage, may induce transient hyperresponsiveness even in normal individuals (Empey et al, 1976; Seltzer et al, 1986). Studies performed in a canine model demonstrate that the bronchoconstriction induced by dry air is associated with desquamation of airway epithelial cells and the release of bronchoconstrictor prostaglandins (Freed et al, 1987a). Thus there is considerable circumstantial evidence that damage to the airway epithelium may contribute to increased sensitivity of the bronchial smooth muscle.

EXPERIMENTAL EVIDENCE

1 The existence of an epithelium - derived relaxing factor

In order to investigate the relationship between epithelial damage and bronchial hyperreactivity more closely, in vitro studies have been performed. Animal models are used because of the difficulty of obtaining healthy human tissue. In the first such study, the intrapulmonary bronchi of the dog were examined (Flavahan et al, 1985). Epithelial damage was mimicked by gentle mechanical rubbing of the luminal surface of the bronchi. This procedure removes the epithelial layer without damaging the underlying structures or compromising the ability of the smooth muscle to contract (Flavahan et al, 1985). Paired rings of tissue, with and without epithelium, were placed in organ chambers, and their responses to pharmacological stimuli compared. Tissues without epithelium were significantly more sensitive to the contractile agents acetylcholine, histamine and 5-hydroxytryptamine (Fig.2). Thus, removal of the airway epithelium enhanced the responsiveness of the bronchial smooth muscle to several bronchoconstrictor agents. In addition, there was a significant reduction in the maximal relaxation to the beta-adrenergic agonist isoproterenol. Epithelium removal also affected the response to stimulation of the nerves. Isolated canine bronchi have a complex response to transmural electrical field stimulation. There is an initial rapid contractile response, followed by a gradual fade to a plateau level. The fade occurs in the presence of continuing electrical stimulation (Russell, 1978; Vermeire and Vanhoutte, 1979; Flavahan et al, 1985). The

source of this fade response is unknown. It is not due to stimulation of beta-adrenergic receptors, as it is not affected by propranolol (Flavahan et al, 1985). However, bronchi with epithelium show a greater degree of fade, reaching a lower plateau level than tissues without epithelium, thus demonstrating an epithelial component to the response (Flavahan et al, 1985).

From this evidence, it has been proposed that the airway epithelium releases a 'relaxing factor', which reduces the response to contractile agents and enhances the effect of relaxing agents (Flavahan and Vanhoutte, 1985; Cuss and Barnes, 1987; Vanhoutte, 1987). Epithelium-removal also enhances the effect of various contractile agents on the tracheal smooth muscle of the cow (Barnes et al, 1985), the guinea-pig (Goldie et al, 1986; Holroyde, 1986; Tschirhart and Landry, 1986), and the large intrapulmonary bronchi of the rabbit (Raeburn et al, 1986; Butler et al, 1987). The influence of the epithelium on responses to relaxing agents is more controversial. In bovine airways, the relaxing effect of isoproterenol is reduced by epithelium-removal (Barnes et al, 1985). In the guinea-pig, removal of the airway epithelium may either reduce (Goldie et al, 1986) or enhance (Holroyde, 1986; Farmer et al, 1986) the relaxing effect of the beta-adrenergic agonist.

2. The role of epithelium-derived arachidonic acid metabolites.

Prostaglandins and leukotrienes may play a role in the pathogenesis of asthma (Morris, 1985; Befus, 1987; Burka, 1987). These substances are derived from the

metabolism of arachidonic acid. Arachidonic acid is a cell membrane phospholipid which can be enzymatically cleaved to yield prostaglandins (via the cyclooxygenase pathway) or leukotrienes (via the lipoxygenase pathway). In vivo, stimuli which disrupt the cell membrane promote the breakdown of arachidonic acid. These stimuli include mechanical injury, nerve stimulation, the attachment of immunoglobulins to the cell surface, and various inflammatory mediators (Morris, 1985). In the airways, metabolism of arachidonic acid via the cyclooxygenase pathway yields the bronchoconstricting agents prostaglandin D_2, prostaglandin $F_{2\alpha}$ and thromboxane A_2 (Morris, 1985; Bahkle and Ferreira, 1985; Tamaoki, et al 1987). The relaxing agents prostaglandin E_1 and E_2 are also produced in significant quantities (Orehek et al, 1973; Yamaguchi et al, 1976; Shore et al, 1985). These latter substances are such potent bronchodilators that they have been tested for potential theraputic use in asthma (Gardiner, 1986).

The major lipoxygenase products are leukotrienes B_4, C_4 and D_4 (Bahkle and Ferreira, 1985; Morris, 1985). Leukotriene B_4 appears to have no direct effect on bronchial smooth muscle, but stimulates recruitment of mast cells and neutrophils (Burka, 1987). These then release inflammatory mediators such as histamine and 5-hydroxytryptamine, which promote bronchoconstriction (O'Byrne et al, 1985; Befus, 1987). Leukotrienes C_4 and D_4 are the components of slow-reacting substance of anaphylaxis (SRS-A) (Murphy et al, 1979). SRS-A is a

powerful bronchocontricting agent in guinea-pig and human airways (Dahlen et al, 1980; Ghelani et al, 1980; Burka and Saad, 1984; Samhoun and Piper, 1986). However, its effects in other species are more variable (Krell et al, 1981). In normal mongrel dogs leukotrienes C_4 and D_4 have minimal effect (Hirshman et al, 1983) although dogs with hyperactive airways do show an enhanced response to leukotriene D_4 (Hirshmann et al, 1983).

Preliminary studies have shown that arachidonic acid evokes relaxations in canine bronchi which are dependent on the presence of the epithelium (Flavahan et al, 1986). Similar observations have been made in the rabbit (Butler et al, 1987) and the guinea-pig (Tschirhart et al, 1987). In the rabbit, prostaglandin E_2 has been implicated as the arachidonic acid product mediating this response (Butler et al, 1987). These results provide evidence that the airway epithlium is able to release prostaglandins which can influence the contractile behaviour of the underlying smooth muscle. However, the exact role of epithelium-derived metabolites of arachidonic acid remains to be established.

The aims of this study are as follows:-

(a) To examine whether the effect of epithelium-removal shows heterogeneity along the respiratory tree. To investigate this question, three orders of intrapulmonary bronchi of the dog will be examined in vitro: second order (lobar bronchus), third order (segmental bronchus) and fourth order (subsegmental bronchus). Concentration-effect curves will be obtained to the following contractile

agents: 5-hydroxytryptamine, histamine, acetylcholine and potassium chloride. The relaxing effect of isoproterenol will also be examined. The effect of epithelium-removal on the responses to these agents will be compared between orders of bronchi.

(b) To examine whether the effect of the epithelium shows heterogeneity between species, the intrapulmonary bronchi of the pig will be studied. Three orders of bronchi will be chosen which have the same outside diameter as those studied in the dog. These are: third order (segmental) bronchus and fourth and fifth order (subsegmental) bronchi. Paired rings of tissue, with and without epithelium, will be placed in organ chambers. Concentration-effect curves will be obtained to acetylcholine, histamine, potassium chloride and isoproterenol. Pig bronchi do not respond to 5-hydroxytryptamine (Goldie et al, 1982). The influence of the epithelium on the responsiveness of the bronchial smooth muscle will be compared both between orders of bronchi and between species.

(c) The effect of epithelium-removal on the response to relaxing agents will be studied in more detail, with special reference to the beta-adrénergic agonist, isoproterenol. Removal of the epithelium is said to either reduce (Flavahan et al, 1985; Goldie et al, 1986) or enhance (Holroyde, 1986; Farmer et al, 1986) the relaxation of airway smooth muscle to isoproterenol. In canine airways, the degree of relaxation obtained to isoproterenol depends on (i) the contractile agent employed to produce active force

(Russell, 1984) and (ii) the level of contraction induced by that agent (Torphy et al, 1983; Torphy et al, 1985). An attempt will be made to determine whether either of these factors might explain the conflicting results obtained by different workers. In addition, the influence of the epithelium on the responses to other relaxing agents will be examined.

In these experiments, fourth order bronchi, with and without epithelium, will be placed in organ chambers. Concentration-effect curves for the contractile agents, acetylcholine and 5-hydroxytryptamine, will be obtained. From these curves, the concentration of agonist giving 40% (EC_{40}) and 80% (EC_{80}) of the maximal response to each agonist will be determined. Tissues will then be contracted to either the EC_{40} or the EC_{80} value for either acetylcholine or 5-hydroxytryptamine. Concentration-response curves will be obtained to the following agents: isoproterenol, prostaglandin E_2, forskolin and sodium nitroprusside. In the case of isoproterenol and sodium nitroprusside, these protocols will be repeated in the presence of indomethacin, to assess the contribution of endogenous prostaglandins, and in the presence of LY83583, an inhibitor of cyclic GMP accumulation, to determine whether the effect of the epithelium is mediated via guanylate cyclase.

(d) The mechanisms whereby arachidonic acid metabolites released from the airway epithelium may influence the responsiveness of the bronchial smooth muscle will be investigated. Second, third and fourth

order canine bronchi will be examined. Concentration-response curves to arachidonic acid will be obtained in the presence and in the absence of the epithelium. These experiments will then be repeated in the presence of inhibitors of cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism. An attempt will be made to assay products of arachidonic acid metabolism, in order to determine which of these may mediate epithelium-dependant relaxations in canine bronchi.

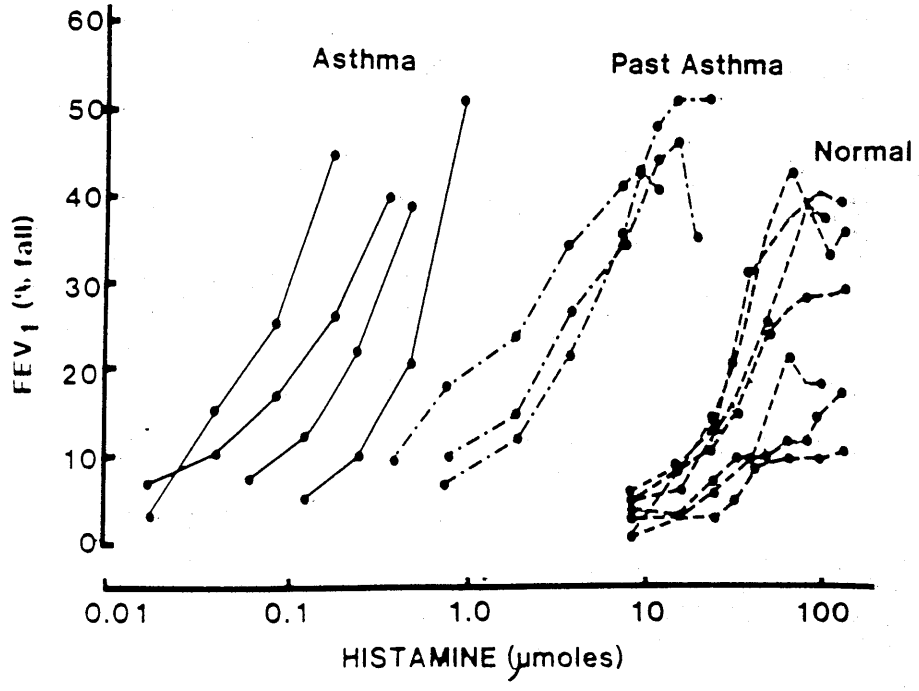

Figure 1: Bronchospasm is induced by the constricting agent, histamine. Dose-effect curves to histamine are shown for: normal individuals (right), asthmatics currently in remission (middle) and asthmatics in the active phase of their disease (left). There is a progressive leftward shift of the dose-effect curve to histamine with increasing severity of the disease. Bronchospasm in measured as the percent fall in the forced expiratory volume in one second (FEV_1) caused by the presence of increasing doses of histamine. (Reproduced, with permission, from Woolcock, A.J. (1986). Bronchial Hyperresponsiveness, in Handbook of Physiology. The Respiratory System. Mechanics of Breathing Part 2. American Physiological Society, Bethesda, MD).

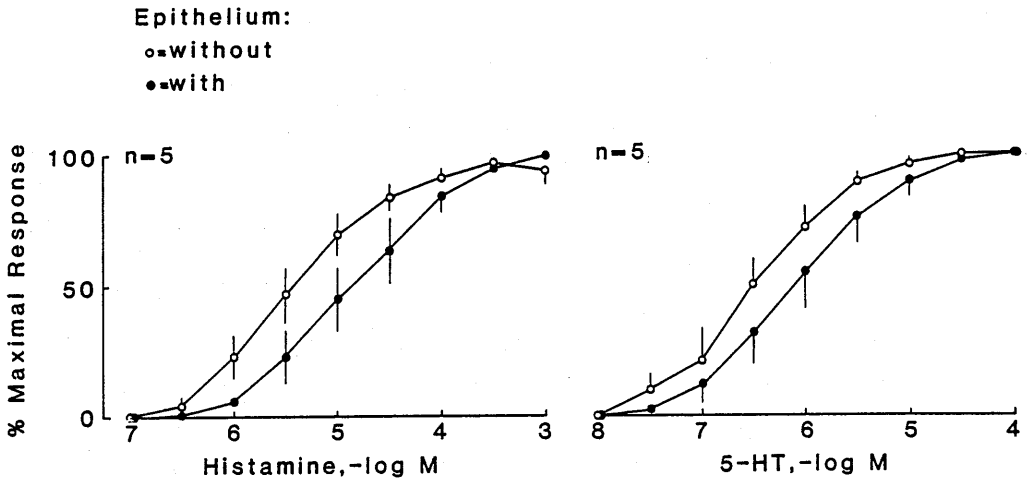

Figure 2: The effect of epithelium-removal on the concentration-effect curves to histamine (left) and 5-hydroxytryptamine (5-HT, right) in third order canine bronchi. •, Rings with epithelium; o, rings without epithelium. (Reproduced, with permission, from Flavahan et al, (1985) Journal of Applied Physiology, 58, 834 - 838).

CHAPTER 1

Heterogeneity in the Effects of Epithelium Removal in the Canine Bronchial Tree

INTRODUCTION

In vitro studies have shown that, in the dog, removal of the airway epithelium augments the responsiveness of the bronchial smooth muscle to the contractile agonists 5-hydroxytryptamine, histamine and acetylcholine. In addition, the relaxing effect of the beta-adrenergic agonist isoproterenol is decreased (Flavahan *et al*, 1985). Subsequent studies have demonstrated a similar effect of epithelium-removal on the tracheal smooth muscle of the guinea-pig (Goldie *et al*, 1986; Holroyde, 1986), the cow (Barnes *et al*, 1985) and the rabbit (Raeburn *et al*, 1986; Butler *et al*, 1987). So far, no attempt has been made to map the distribution of this phenomenon within the respiratory tree. Furthermore, the influence of the epithelium in smaller bronchi has not been investigated. This study explores the effect of epithelium removal in three orders of canine bronchi.

METHODS

Experiments were performed on bronchi taken from mongrel dogs of either sex (15-20 kg), anaesthetized with pentobarbital sodium (30 mg/kg i.v.) and killed by exsanguination. Lungs which showed evidence of infection with heart worms or bronchopneumonia were rejected from the study. Tissues were taken from either the right or

left lower lung lobe (lobus diaphragmaticus). This lobe was chosen for ease of dissection, and to eliminate any variation arising from the use of different lobes. In each dog, three orders of bronchus were examined; second order (lobar bronchus), third order (segmental bronchus) and fourth order (subsegmental bronchus) (Miller et al, 1964). The outside diameters of these airways are given in Table 1. All bronchi used in this study were shown histologically to contain cartilagenous elements. Bronchial segments (8-10 mm long) were dissected free of the surrounding parenchyma, and paired rings (4-5mm long) were prepared from each segment. In one ring of each pair, care was taken not to damage the epithelium; in the other ring, the tips of a pair of watchmaker's forceps were inserted into the lumen and the epithelial layer removed by gently rolling the tissue back and forth on saline-loaded filter paper (Flavahan et al, 1985). Epithelium-removal was confirmed histologically at the end of each experiment (Fig 3).

The rings were suspended in organ chambers filled with 25ml of Krebs-Ringer bicarbonate solution (millimolar composition: NaCl, 118.3; KCL, 4.7; $CaCl_2$, 2.5;$MgSO_4$, 1.2; KH_2PO_4,1.2; $NaHCO_3$, 25.0; calcium disodium edetate, 0.026; glucose, 11.1) maintained at 37^{o}C and aerated with 95%0_2/5%CO_2 (pH=7.4) (Fig.4). Each ring was suspended by two stainless steel clips passed through the lumen. One clip was anchored to the bottom of the organ chamber, the other was connected to a strain gauge (Statham Gould UC2) for the measurement of isometric force. This procedure

caused damage only to the small area of epithelium where the clips were in contact with the tissue, as confirmed by histology. For transmural nerve stimulation, two platinum-plate electrodes were placed parallel to the preparation (distance between electrodes: 7mm) (Fig.4). Electrical impulses (2 ms, 16Hz) were provided by a stimulator (Grass S09) and a D.C. current amplifier (Mayo Clinic, Section of Engineering).

The tissues were allowed to equilibrate for one hour. At the end of this period, the bronchial rings were stretched progressively until the response to a standard electrical stimulus (9V, 2ms, 16Hz 10s, applied every 15 minutes) was maximal. These stimulation parameters produce maximal contractions in airways of the sizes used in this study (Russell, 1978). Tissues which did not respond to transmural electrical stimulation were rejected from the protocol. When optimal resting tensions were achieved (Table 1), the bronchial rings were allowed to equilibrate for a further hour prior to the administration of drugs. To ensure that the integrity of the smooth muscle had not been compromised by removal of the epithelium, concentration-effect curves for potassium chloride were obtained for each order of bronchus. Within each order, the concentration of potassium chloride producing 50% of the maximal response (EC_{50}) was not significantly different for tissue with and without epithelium (Table 1).

Experimental Protocols

The following drugs were used: 5-hydroxytryptamine, histamine, acetylcholine and isoproterenol. For each drug, concentration-effect curves were obtained by increasing the concentrations in the organ chambers cumulatively in half-log increments (Van Rossum, 1963). In one protocol, the effects of 5-hydroxytryptamine and histamine were studied. A concentration-effect curve to 5-hydroxytryptamine was first obtained. This drug was then washed out and the tissues allowed to return to resting tension. Histamine is known to have a weak effect at cholinergic receptors in this tissue, which is blocked by the addition of 10^{-8}M atropine (Shore et al, 1983). Therefore, prior to the administration of this agonist, the bronchial rings were incubated in atropine (10^{-8}M) for 30 minutes. A concentration-effect curve for histamine was then obtained. Atropine remained in contact with the tissues throughout this period. In a separate protocol, concentration-effect curves to acetylcholine were obtained, and the drug was washed out of the preparations. The bronchial rings were incubated for 30 minutes with cocaine (5×10^{-6}M), hydrocortisone (3×10^{-5}M) and phentolamine (10^{-6}M) to block neuronal uptake, extraneuronal uptake and alpha-adrenoceptors, respectively (Furchgott, 1972). These drugs remained in contact with the tissues throughout the experiment. Each ring was then contracted to its individual EC_{50} value for acetylcholine, and a cumulative concentration-effect curve to isoproterenol was obtained. Relaxations to the beta-adrenergic agonist are

expressed as percent of the contractile response to acetylcholine.

Histology

At the end of each experiment, all tissues were fixed for 24 hours in a 10% buffered formaldehyde solution (pH= 7.0). They were then embedded in paraffin wax (Tissue Prep 2, Fair Lawn, NJ) and sectioned at 5 μm. Sections were stained in haematoxylin and eosin, and examined under light microscopy to assess the presence or absence of airway epithelium.

Drugs Used

Acetylcholine chloride (Sigma Co., St. Louis, MO.); atropine sulphate (Sigma); cocaine hydrochloride (Yar Lang, LaCrosse, WI); histamine hydrochloride (Sigma); hydrocortisone 21-sodium hemisuccinate (Sigma); 5-hydroxytryptamine creatinine sulphate (Sigma); dl-isoproterenol hydrochloride (Sigma); papaverine (Sigma) phentolamine mesylate (Ciba-Geigy Corp., Summit, NJ); and potassium chloride (Fisher, Fair Lawn, NJ).*

Data Analysis

For each drug, concentration-effect curves were obtained in the range 10^{-9}M to 10^{-4}M. Relaxations to isoproterenol are expressed as percent relaxation of the contractile response to acetylcholine. For the contractile agonists 5-hydroxytryptamine, histamine and acetylcholine, results are expressed as percent of the response to a 10^{-4}M concentration of the agonist. EC_{50} values (i.e. that concentration of agonist giving 50% of the

*Unless otherwise stated, all drugs were dissolved in distilled water, and kept on ice.

contractile response to 10^{-4}M) were obtained by regression analysis of the concentration-effect curve, and are presented as -log EC_{50}. To determine the degree of shift in the concentration-effect curves for contractile agonists caused by epithelium removal, concentration-ratios were calculated as follows; antilog (-log EC_{50} with epithelium)/(-log EC_{50} without epithelium).

The results are expressed as means $\pm$ SE. Statistical analysis of EC_{50} values and concentration-ratios was by Student's t-Test for paired observations. P smaller than 0.05 was considered to be statistically significant. The contractile response achieved to a 10^{-4}M concentration of agonist showed a wide variation between tissues. Therefore, for these values, the Wilcoxon matched-pairs signed-ranks test was used to determine whether there was a significant difference between tissues with and without epithelium; α smaller than 0.05 was considered to be statistically significant. In all experiments, n equals the number of dogs.

RESULTS

Contractile Agents. 5-hydroxytryptamine, histamine and acetylcholine evoked concentration-dependent contractions in the three orders of bronchi. For the second and third order, removal of the epithelium caused a significant leftward shift of the concentration-effect curve for each agonist (Figs. 5,6,7; Table 2). The degree of shift obtained was comparable for each agonist, as determined by concentration-ratios (Table 2). In fourth order bronchi, epithelium-removal did not significantly

affect the concentration-effect curves to any of the contractile agents tested (Figs. 5,6 and 7; Table 2). Within each order the contractile reponse to the maximal concentration of each agonist used showed no significant difference between tissues with and without epithelium, as determined by the Wilcoxon matched-pairs signed-ranks test (Table 2).

Isoproterenol. Each tissue was contracted with its individual EC_{50} value for acetylcholine. The degree of active force development was comparable for each order of bronchus with and without epithelium. Isoproterenol caused concentration-dependent relaxations in the three orders of bronchi. In second order bronchi, the relaxant effect was greater in tissues with epithelium, although this difference was significant only at high concentrations of isoproterenol (10^{-5}M - 10^{-4}M) (Fig. 8). Third order bronchi showed a similar response, with intact preparations showing a significantly greater degree of relaxation at high concentrations of the beta-adrenergic agonist (3×10^{-6}M - 10^{-4}M) (Fig. 8). Fourth order bronchi with epithelium exhibited a marked relaxation to isoproterenol (Fig. 8). By contrast, fourth order bronchi without epithelium showed no significant relaxation to the beta-adrenergic agonist, but relaxed completely in response to 3×10^{-4} papaverine (Fig. 9). The difference between tissues with and without epithelium was significantly greater than that for second or third order bronchi.

DISCUSSION

This study confirms that, in canine airways, removal of the airway epithelium augments the responsiveness of bronchial smooth muscle to 5-hydroxytryptamine, histamine and acetylcholine, and reduces the effect of isoproterenol (Flavahan et al, 1985). In all tissues studied, potassium chloride caused concentration-dependent contractions which were similar in bronchi with or without epithelium, demonstrating that epithelium-removal did not impair the ability of the smooth muscle to contract. Tissues without epithelium relaxed completely to papaverine, indicating that the smooth muscle was capable of relaxation.

The major findings can be summarized as follows: (a) with decreasing diameter, the potentiating effect of epithelium-removal on contractile agonists is reduced; and (b) in contrast, with decreasing diameter, the modulating influence of the epithelium on the beta-adrenergically mediated relaxation becomes more prominent. The implication of these results is that if airway epithelium releases a factor which promotes relaxation of bronchial smooth muscle (Flavahan and Vanhoutte, 1985; Vanhoutte, 1987), there is considerable heterogeneity in the release or the effect of the factor along the respiratory tree.

In larger airways (second and third order) it appears that the major effect of epithelium-removal is to increase the efficacy of contractile agonists. This is a non-selective phenomenon, as the concentration-effect curves obtained for 5-hydroxytryptamine, histamine and

acetylcholine are shifted in a comparable manner. By contrast, in small airways (fourth order) the major effect is a pronounced enhancement of the relaxation to isoproterenol. A logical explanation for these observations may be that in larger airways, there is a continuous, basal release of an epithelium-derived relaxing factor, which exerts a tonic restraint of the underlying smooth muscle, reducing sensitivity to constricting agents. The absence of potentiation of the response to contractile agonists in the smaller bronchi then would mean that at the level of these airways, the basal release of epithelium-derived relaxing factor is minimal.

That the epithelium of these smaller airways can release inhibiting substances is demonstrated by the effect of epithelium-removal on the response to isoproterenol. Significant numbers of beta-adrenoceptors have been detected in the airway epithelium of several species [the dog (Davis et al, 1979); the ferret (Barnes et al, 1982); the rat, (Xue et al, 1983), the guinea-pig (Goldie et al, 1986), and the pig (Goldie et al, 1986)) These beta-adrenoceptors can modulate secretion across the airway epithelium (Davis et al, 1979; Smith et al, 1982; Welsh, 1986). The dependency of the relaxation to the beta-adrenergic agonist on the presence of epithelium would then be explained if isoproterenol were to stimulate beta-adrenoceptors on the epithelial cells to cause active release of an inhibitory substance which relaxes bronchial smooth muscle (Fig. 10). If this interpretation is correct,

the present results strongly suggest that the epithelial beta-adrenoceptors are more numerous, or more effective, in smaller than in larger airways.

Thus, the airway epithelium modulates the responsiveness of the bronchial smooth muscle at several levels of the respiratory tree. This modulation may be achieved by the release of epithelium-derived relaxing factors. These factors show heterogeneity in their release and/or their effect within bronchi of varying diameter.

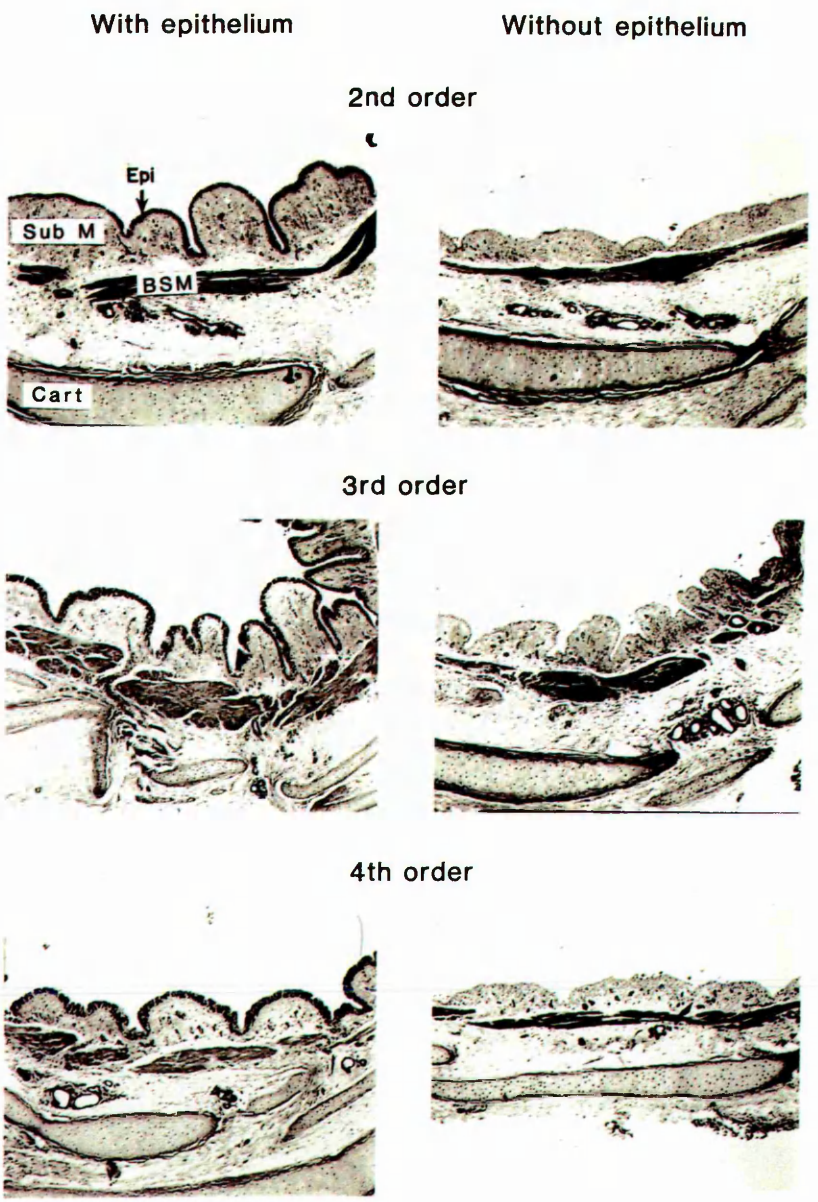

Figure 3: Histological sections of second-order (top), third-order (middle) and fourth-order (bottom) canine bronchi, demonstrating that the epithelium was successfully removed by gentle mechanical rubbing of the luminal surface (right). In control tissues, the epithelium remained intact (left). Epi, epithelium; subM, submucosa; BSM, bronchial smooth muscle; cart, cartilage. Haemotoxylon and eosin stain; Magnification x 100.

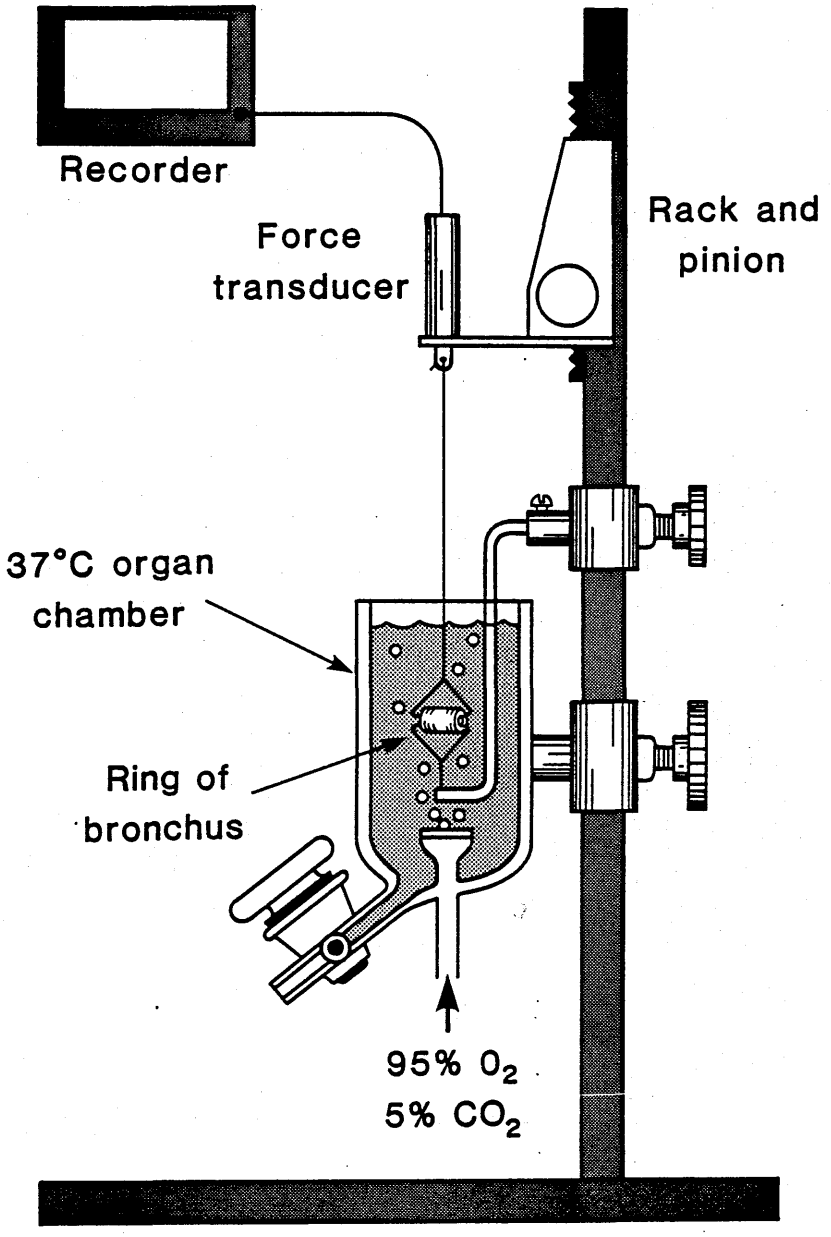

Figure 4: Experimental set-up.

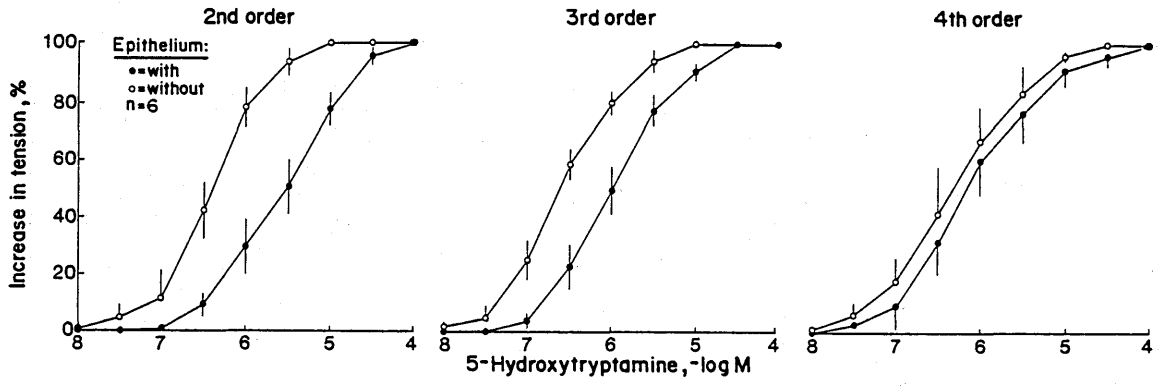

Figure 5: The effect of epithelium-removal on the concentration-effect curve to 5-hydroxytryptamine in second order (left), third-order (middle), and fourth-order (right) canine bronchi. Data (means ± SE) are expressed as percent of the response to 10^{-4}M 5-hydroxytryptamine; n=6. ●, Rings with epithelium; 0, rings without epithelium.

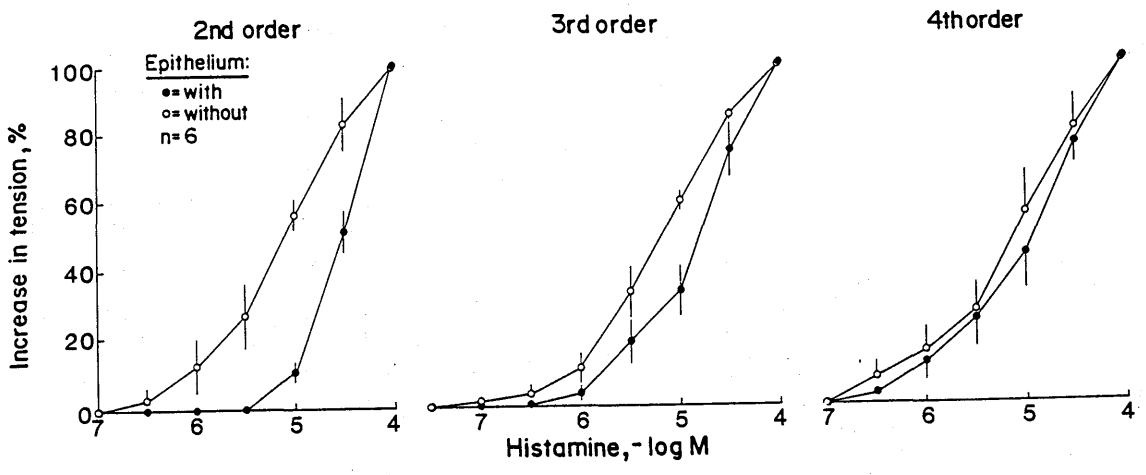

Figure 6: The effect of epithelium-removal on the concentration-effect curve to histamine in second-order (left), third-order (middle) and fourth-order (right) canine bronchi. Data (means ± SE) are expressed as percent of the response to 10^{-4}M histamine; n=6. ●, rings with epithelium; 0, rings without epithelium.

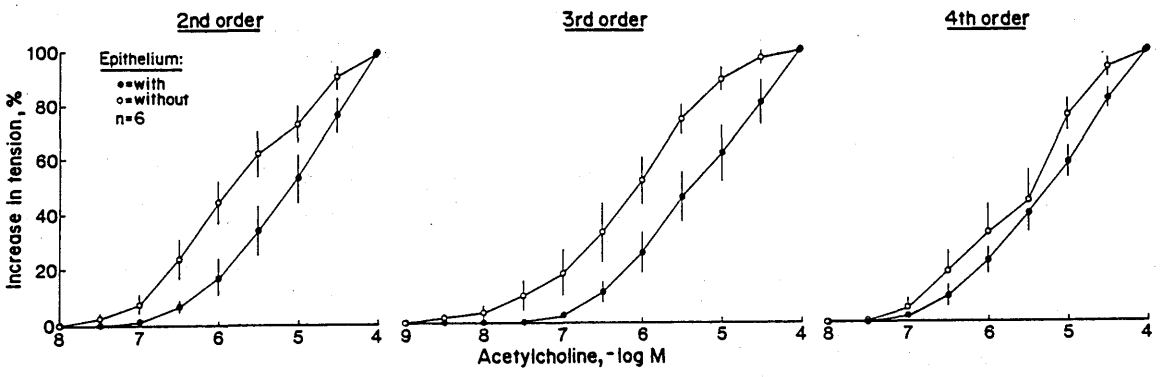

Figure 7: The effect of epithelium-removal on the concentration-effect curve to acetylcholine in second order (left), third-order (middle), and fourth-order (right) canine bronchi. Data (means ± SE) are expressed as percent of the response to 10^{-4}M acetylcholine; n=6.

●, Rings with epithelium; 0 rings without epithelium.

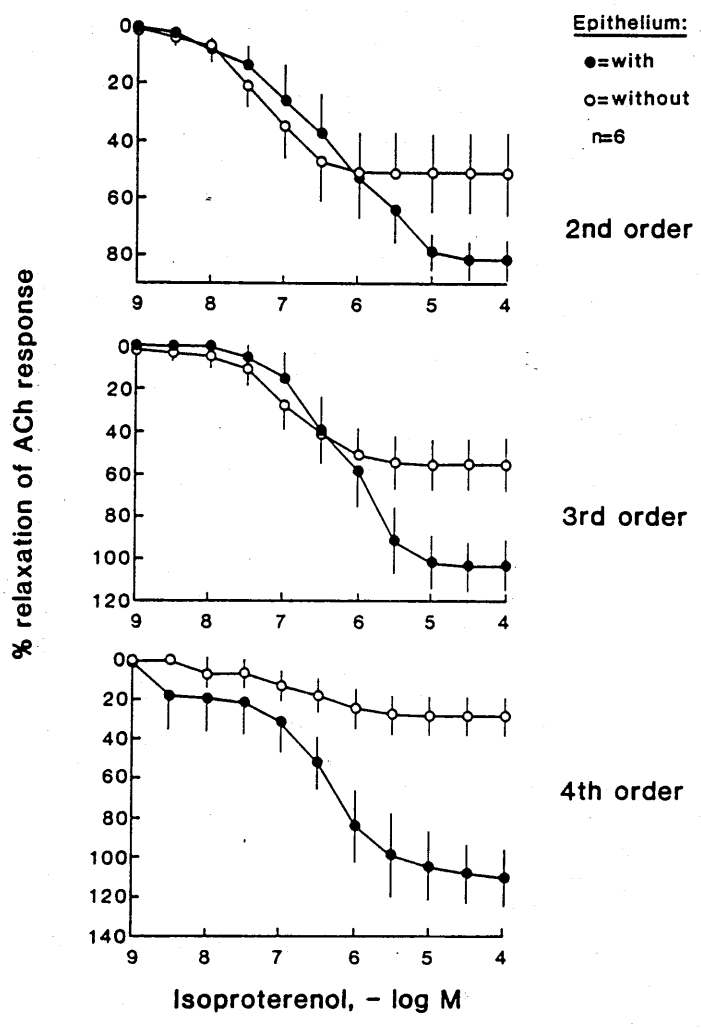

Figure 8: The effect of epithelium-removal on the concentration-effect curve to isoproterenol in second-order (top), third-order (middle) and fourth-order (bottom) canine bronchi. Rings were contracted to equal tensions using the concentration of acetylcholine producing 50% of the maximal response to this agonist. Data (means ± SE) are expressed as percent relaxation of the response to acetylcholine; n = 6. ●, Rings with epithelium; 0, rings without epithelium.

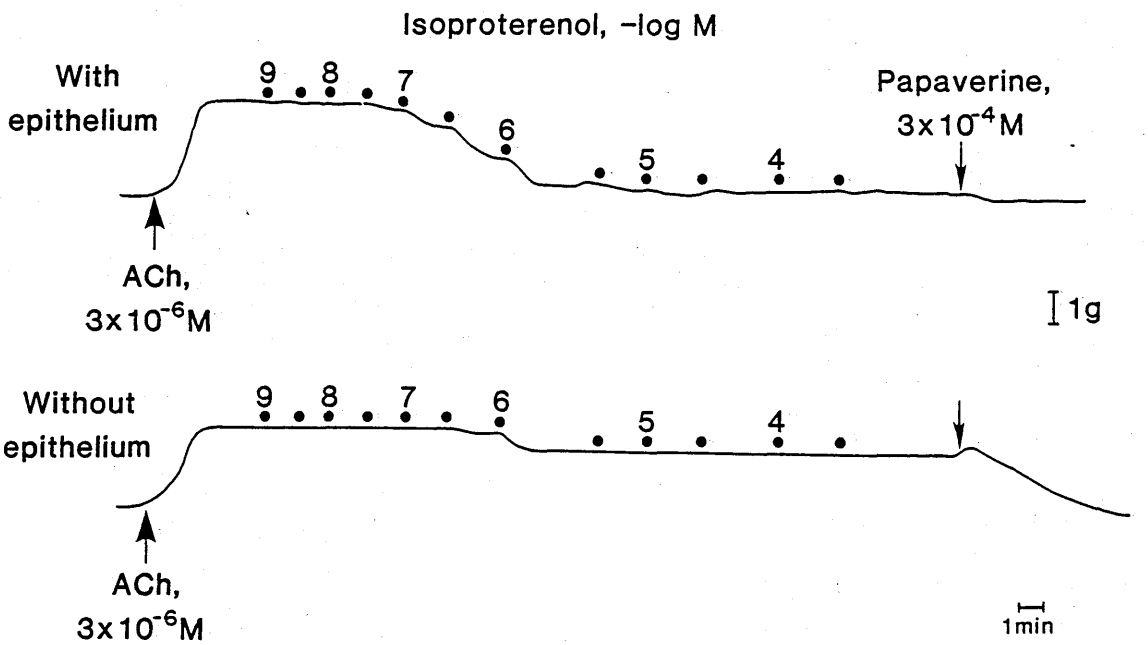

Figure 9; Representative tracing of the effect of isoproterenol on paired rings of fourth-order bronchi with (top) and without (bottom) epithelium. Each ring was contracted with the EC_{50} dose for acetylcholine.

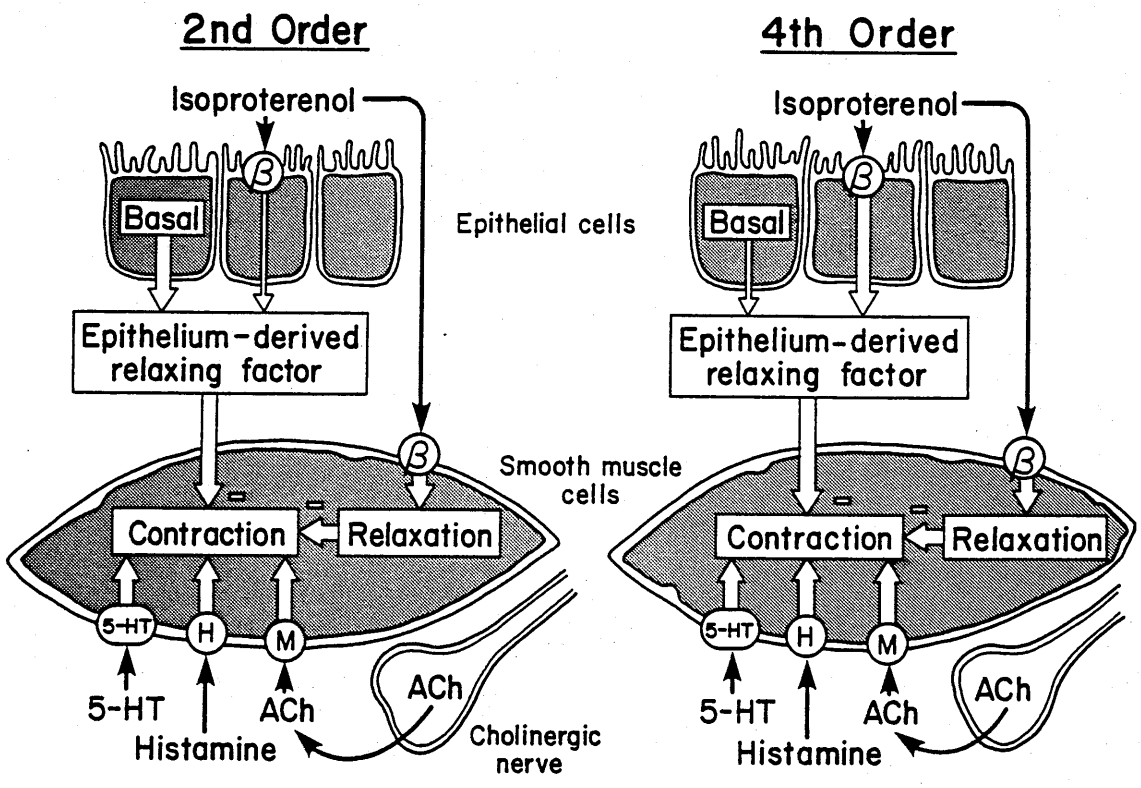

Figure 10: Proposed model for the heterogeneity in the release of the epithelium-derived relaxing factor in second order (left) and fourth order (right) canine bronchi. In the second order airways, the most prominent effect of the epithelium is the basal release of an epithelium-derived relaxing factor, which reduces the response to contractile agents. There is also a stimulated release of this factor, which is mediated via beta-adrenoceptors on the epithelial cells. This route is of minor importance in large airways. In fourth order bronchi, basal release is minimal or absent, and stimulated release assumes a far greater importance. 5-HT = 5-hydroxytryptamine. ACh = acetylcholine. β = beta-adrenoceptor. H = histamine receptor. M = muscarinic receptor.

TABLE 1. Characteristics of the three orders of canine bronchus used.

	Outside Diam. mm	Optimal Resting Tension, g	EC_{50} for KCl, mM		Maximal Response to KCl, g	
			With epithelium	Without epithelium	With epithelium	Without epithelium
Second Order (lobar bronchus)	8-10	0.5-2	18.86±1.2	16.18±0.26	7.4±2.16	5.62±1.38
Third Order (segmental bronchus)	4-5	0.5-2	14.88±0.4	13.66±0.64	6.31±2.7	5.83±1.74
Fourth Order (subsegmental bronchus)	1-3	0.5-1	14.18±1.62	15.06±1.13	3.5±1.33	2.7±0.8

Value are means ± SE. Outside diameters and optimal resting tensions are approximate ranges based on 12 experiments; for response to KCl, n = 6. EC_{50} concn producing 50% of maximal response.

Table 2. Effect of epithelium removal on response to 5-hydroxytryptamine, histamine, and acetylcholine in the three orders of canine bronchi.

		-Log EC_{50}			Response to 10^{-4}M agonist, g	
	Bronchi	With epithelium	Without epithelium	Concentration Ration	With epithelium	Without epithelium
5-Hydroxytryplamine	2nd order	5.58±0.15	6.44±0.16a	8.32±2.0	5.9±1.78	4.3±0.7
	3rd order	6.02±0.13	6.63±0.10a	4.55±0.86	5.47±3.3	5.28±1.99
	4th order	6.15±0.26	6.36±0.24	1.88±0.54	3.93±0.82	2.47±0.94
Histamine	2nd order	4.53±0.05	5.14±0.21a	3.12±0.62	4.46±2.6	5.38±1.78
	3rd order	4.82±0.13	5.24±0.08a	3.18±1.0	4.98±2.24	6.7±1.5
	4th order	4.99±0.16	5.07±0.15	1.39±0.30	3.98±2.0	2.84±1.4
Acetycholine	2nd order	5.16±0.20	5.83±0.22a	4.20±0.66	7.3±3.22	4.9±2.23
	3rd order	5.39±0.22	6.18±0.21a	5.69±1.67	4.8±2.69	4.15±2.3
	4th order	5.25±0.14	5.63±0.14	1.99±0.78	2.88±0.75	1.43±0.67

Values are means ± SE n = 6 for each agonist. EC_{50} concn producing 50% of maximal response. [a]Significantly different from tissues with epithelium.

CHAPTER 2

The Airway Epithelium Modulates the Responsiveness of Porcine Bronchial Smooth Muscle

INTRODUCTION

The previous study demonstrated that, in the dog, the airway epithelium can modulate the responsiveness of the underlying smooth muscle. This effect of the epithelium shows heterogenity along the canine respiratory tree: in large airways the major effect of epithelium-removal is to enhance the effect of contractile agonists, while in small airways the major effect is the reduction of relaxing responses to isoproterenol. It is postulated that the epithelium secretes a relaxing factor, which has a continuous, basal release, and whose release may also be stimulated by beta-adrenergic agents. Other studies have indicated that the epithelium may enhance the relaxation to electrical field stimulation in isolated canine bronchi (Flavahan et al, 1985). The aims of the following study were (a) to determine whether the heterogeneity in the modulatory influence of the airway epithelium could be demonstrated in the intrapulmonary bronchi of another species, and (b) to investigate the effect of transmural electrical field stimulation, in order to compare the responses of porcine bronchi with those previously obtained in the dog. (Flavahan et al, 1985).

METHODS

Experiments were performed on bronchi, taken from male Yorkshire pigs (30-50kg) anaesthetized with ketamine hydrochloride (30.0mg intramuscularly), followed by pentobarbital sodium (12.5 mg/kg intravenously) and exsanguinated. Tissues were taken from either the right or left lower lung lobe (caudal lobe) (Nickel et al, 1973). This lobe was chosen to remove any variation arising from the use of different lobes, and to allow comparison with the lower lobe of the dog used in the previous study. None of the tissues studied showed evidence of infection on either gross or histological examination. In each pig, three orders of bronchus were examined; third order (segmental bronchus), fourth order and fifth order (subsegmental bronchi). These bronchi correspond to the diameters of those studied in the dog. The morphology of the airway epithelium is directly related to the absolute size of the airway (Weibel 1985). Thus, by choosing bronchi of similar diameter to those of the dog, rather than bronchi of similar anatomical origin, an attempt was made to standardize the epithelial cell types present in the two species. All bronchi used in this study were shown histologically to contain cartilagenous elements. The outside diameters of the airways used are given in Table 3.

Bronchial segments (8-10 mm long) were dissected free of the surrounding parenchyma, and paired rings (4-5 mm long) were prepared from each segment. In one

ring of each pair, care was taken not to damage the epithelium; in the other ring, the tips of a pair of watchmaker's forceps were inserted into the lumen and the epithelial layer removed as previously described for the dog. Epithelium-removal was confirmed histologically at the end of each experiment.

The rings were suspended in organ chambers identical to those used for the canine bronchi and allowed to equilibrate for one hour. At the end of this period, the bronchial rings were streched in 0.5 g increments until the response to a standard electrical stimulus (16Hz, for 30s, applied in every 10 minutes) was maximal. Preliminary experiments indicated that these stimulus parameters produce 80-90% of the maximal contraction to electrical stimulation obtainable in this tissue. When optimal resting tensions were achieved (Table 3), the bronchial rings were allowed to equilibrate for a further hour prior to the administration of drugs. The optimal resting tensions were similar in tissues with and without epithelium.

Experimental protocols

The following drugs were used: acetylcholine, isoproterenol, histamine and potassium chloride. Concentration-effect curves were obtained by increasing the concentrations in the organ chambers cumulatively in half-log increments (Van Rossum, 1963). In one series of experiments, concentration-effect curves to acetylcholine were obtained, and the drug was washed out of the preparations. The bronchial rings were then incubated for

thirty minutes with cocaine (5×10^{-6}M), hydrocortisone (3×10^{-5}M) and phentolamine (10^{-6}M) to block neuronal uptake, extraneuronal uptake and alpha-adrenoceptors, respectively (Furchgott, 1972). These drugs remained in contact with the tissues throughout the experiment. Each ring was then contracted to its individual EC_{50} value for acetylcholine, and a cumulative concentration-effect curve to isoproterenol was obtained. At the end of each experiment, a maximal relaxation to sodium nitroprusside (10^{-5}M) was obtained in all tissues. In a separate experimental series, concentration-effect curves to histamine were performed in the three orders of bronchi; the drug was washed out, the preparations allowed to relax to baseline, and concentration-effect curves for potassium chloride obtained.

Transmural nerve simulation. Experiments were conducted to confirm the presence of excitatory cholinergic neurotransmission in pig bronchi. Transmural nerve stimulation was applied in the presence of either atropine (10^{-6}M), tetrodotoxin (10^{-6}M) or hexamethonium (5×10^{-6}M) (Vermeire and Vanhoutte, 1979). Each of these drugs was in contact with the tissue for 30 minutes prior to the experiment, and remained in the solution during nerve stimulation. Atropine and tetrodotoxin abolished the response to transmural nerve stimulation in the three orders of pig bronchi. However, the ganglion-blocking agent hexamethonium had no significant effect ($n = 2$). These results imply that the contractile response to transmural nerve stimulation in the pig is mediated via

release of endogenous acetylcholine from postganglionic cholinergic nerves. Frequency-effect curves were constructed in a non-cumulative manner. The following stimulus parameters were employed: 9V, 2ms, 0.5 to 64Hz. Preliminary experiments indicated that this voltage and pulse duration were maximal. The bronchial rings were stimulated at each frequency for three minutes at ten minute intervals (Flavahan et al, 1985). For those experiments in which the effect of propranolol was examined, a 5 x 10^{-6}M concentration of the beta-adrenergic antagonist was added 30 minutes prior to the start of the protocol. A frequency-effect curve to transmural nerve stimulation was then conducted. The antagonist remained in contact with the tissue throughout the experiment.

Drugs Used

Acetylcholine chloride (Sigma Co., St. Louis, MO); atropine sulphate (Sigma); cocaine hydrochloride (Yar Lang, LaCrosse, WI); hexamethonium bromide (K + K Labs., Inc., Plainview, NY); histamine hydrochloride (Sigma); hydrocortisone 21-sodium hemisuccinate (Sigma); dl - isoproterenol hydrochloride (Sigma); phentolamine mesylate (Ciba-Geigy Corp., Summit, NJ); dl -propranolol hydrochloride (Sigma); sodium nitroprusside (Sigma) and tetrodotoxin (Sigma). *

Data Analysis

For acetylcholine, concentration-effect curves were obtained in the range 10^{-9}M to 10^{-4}M. For histamine, curves were constructed in the range 10^{-7}M to 10^{-4}M. For

* Unless otherwise stated, all drugs were dissolved in distilled water, and kept on ice.

these agonists, the results are expressed as percent of the response to a 10^{-4}M concentration of the agonist in each individual preparation. For potassium chloride, contractile responses were obtained in the range 10 - 40 mM. Relaxations to isoproterenol (in the range 10^{-9} - 10^{-4}M) are expressed as percent relaxation of the contractile response to acetylcholine. EC_{50} values (i.e. the concentration of agonist giving 50% of the contractile response obtained with 10^{-4}M) were obtained by regression analysis of the concentration-effect curve, and are presented as -log EC_{50}.

The response to transmural nerve stimulation was measured at two points: the initial peak response (30 secs after the start of stimulation) and the fade response (3 mins after the start of stimulation). Both points are expressed as a percent of the maximal peak response to electrical stimulation (i.e.at 32 Hz) in each bronchial ring.

The results are expressed as means $\pm$ S.E.M. Statistical analysis was by Student's t-test for paired observations. P smaller the 0.05 was considered to be statistically significant. In all experiments, n equals the number of pigs.

RESULTS

Potassium chloride: Concentration-effect curves for potassium chloride were obtained for each order of bronchus. For third and fourth order bronchi, the maximal response to potassium chloride was reduced significantly by the removal of the epithelium (Fig. 11). However, the EC_{50} values for potassium chloride were not significantly

different between tissues with and without epithelium (Table 3). In fifth order bronchi, neither the maximal response nor the EC_{50} values for potassium chloride were significantly affected by epithelium-removal (Fig.11, Table 3).

Acetylcholine and histamine: The two agonists evoked concentration-dependent contractions in the three orders of bronchi. For third and fourth order bronchi, removal of the epithelium caused a significant leftward shift of the concentration-effect curve for acetylcholine as determined from EC_{50} values (Fig. 12, Table 4). For these two orders, there was no significant difference in the response to 10^{-4}M acetylcholine (Fig.11). For fifth order bronchi, there was no significant difference in E_{50} values between tissues with and without epithelium (Table 4). However, there was a significant leftward shift in the concentration-effect curve for fifth order tissues without epithelium at lower concentrations of acetylcholine (3×10^{-7}M, 10^{-6}M) (Fig.12). In addition, the maximal response achieved to acetylcholine was significantly reduced by epithelium-removal (Fig.11).

Removal of the epithelium caused a significant leftward shift of the concentration-effect curve for histamine in third and fifth order bronchi, as determined from EC_{50} values (Fig.13, Table 4). For fourth order bronchi, although the EC_{50} values were similar for tissues with and without epithelium, there was a significant leftward shift of the curve for tissues without epithelium at lower concentrations of histamine (3×10^{-6}M) (Fig.13).

There was no significant difference in maximal response achieved to 10^{-4}M histamine in tissues with and without epithelium for any of the three orders (Fig.11).

Isoproterenol. The degree of active force developed in response to the EC_{50} value of acetylcholine was similar for each order of bronchus with and without epithelium (data not shown). Isoproterenol caused concentration-dependent relaxations in the three orders of bronchi (Fig.14). The relaxation obtained to the beta-adrenergic agonist was significantly reduced in tissues without epithelium in the following dose-ranges: third order: 3 x 10^{-7}M-10^{-4}M; fourth order: 3 x 10^{-7}M-10^{-4}M; and fifth order: 10^{-7}M-10^{-4}M (Fig.14). For the three orders, the maximal relaxation achieved was similar in tissues with epithelium. There was no significant difference between the three orders in the maximal relaxation achieved in tissues without epithelium. All tissues relaxed to basal tension in the presence of sodium nitroprusside (10^{-5}M).

Transmural nerve stimulation. In all rings, transmural nerve stimulation evoked a biphasic response, consisting of an initial peak response, followed by a gradual fade (Fig.15). The peak responses was frequency-dependent, and reached a maximum at 32Hz (Table 5). Further increases in frequency caused a decline in the response (Table 5). For third and fourth order bronchi, tissues without epithelium showed a rightward shift of the frequency-effect curve which was significant at the following frequencies: 3rd order: 1Hz, 2Hz, 8Hz, 16Hz; 4th order: 0.5Hz, 1Hz, 2Hz (Fig. 16, Table 5). Fifth order

bronchi without epithelium also showed a rightward shift of the frequency-effect curve, but this was significant only at 1Hz (Table 5). The maximal response to electrical stimulation was similar in tissues with and without epithelium in the three orders of bronchi (Fig.11). The degree of fade of the peak response during the 3-minute stimulation period was also frequency-dependent, i.e. the response showed a greater degree of reduction at higher stimulus frequencies (Fig 15). This fade response was not affected by the beta-adrenergic antagonist propranolol (5×10^{-6}M) (n = 2, data not shown). For third and fourth order bronchi, the fade response was similar in tissues with and without epithelium. In fifth order bronchi without epithelium, the degree of fade of the response was significantly less than that for rings with epithelium at 8Hz and 16Hz (Fig. 17, Table 5).

DISCUSSION

These results imply that, as in the dog, the airway epithelium of the pig is able to modulate the responsiveness of the underlying smooth muscle to both exogenously applied agonists and nerve stimulation, possibly via the production of substances from the epithelium. The distribution of this effect shows heterogeneity along the porcine respiratory tree, but the pattern of this heterogeneity differs from that of the dog. In canine airways, the potentiating effect of epithelium-removal on the contractile agonists acetylcholine and histamine is reduced as airway diameter

decreases. In the pig, removal of the epithelium causes a significant leftward shift in the concentration-response curve for acetylcholine. The shift is most prominent in third and fourth order bronchi, and is least in fifth order tissues. The response to histamine is also enhanced significantly by epithelium-removal. The degree of shift of the concentration-effect curve to histamine is similar in third and fifth order bronchi, and is reduced in fourth order bronchi. Overall, the data suggest that the shifts in the concentration-effect curves for acetylcholine and histamine are comparable for the three orders. Thus, it is likely that in the pig, unlike the dog, the epithelium of bronchi of different diameters exhibits a comparable basal release of relaxing factors. As in the dog, the factor is non-specific, as similar shifts occur for the concentration-response curves to acetylcholine and histamine with epithelium-removal.

The relaxing effect of isoproterenol is considerably enhanced in the presence of the epithelium. The degree of shift of the concentration-effect curve caused by epithelium-removal was similar in the three orders of bronchi. This is in contrast to the dog, where the influence of the epithelium on isoproterenol-induced relaxations is most prominent in the smallest airways. It has been proposed that isoproterenol acts on beta-adrenoceptors of the airway epithelium to stimulate active release of a relaxing factor (Chapter 1). Significant numbers of beta-adrenoceptors have been demonstrated in the epithelium of porcine airways (Goldie

et al, 1986). It is probable that isoproterenol stimulates these receptors to produce an epithelium-derived relaxing factor. If so, the data suggest that the distribution of these epithelial beta-adrenoceptors may be more uniform in the pig than in the dog.

The response to electrical stimulation in pig bronchi is abolished by atropine and tetrodotoxin, but not by hexamethonium. This means that transmural nerve stimulation causes a contractile response via the release of endogenous acetylcholine from postganglionic cholinergic nerves, as previously described in the dog (Vermeire and Vanhoutte, 1979). In the dog, the peak response to electrical stimulation shows a significant leftward shift in the absence of the epithelium (Flavahan et al, 1985). In the pig, epithelium-removal causes a significant shift to the right of the peak response to nerve stimulation. This is in contrast to the effect of exogenous acetylcholine in this animal. The implication of these results is that there is an epithelium-derived contracting factor, which is released during transmural nerve stimulation, and augments the contractile response to endogenous acetylcholine. An epithelium-dependent contractile response to electrical stimulation has been described in human airways (de Jongste et al, 1987). For third and fourth order bronchi, there was no difference in the fade response between tissues with and without epithelium. In fifth order bronchi, epithelium removal diminished the degree of decline of the peak response. This latter phenomenon has been observed also in third

and fourth order canine bronchi, where it has been attributed to loss of an epithelium-derived relaxing factor (Flavahan et al, 1985). Thus, it appears that the response of the pig bronchus to transmural nerve stimulation may be influenced by the production of both epithelium-derived contracting and relaxing factors.

Removal of the airway epithelium of the pig reduced the maximal response to potassium chloride in third and fourth order bronchi, and that to acetylcholine in fifth order bronchi. This may indicate that the process of epithelium-removal has damaged the underlying smooth muscle. However, the histological examination of the tissues showed no disruption of the smooth muscle in bronchi without epithelium. For third and fourth order bronchi, the maximal responses to acetylcholine and histamine were similar in tissues with and without epithelium. Fifth order bronchi, which might be expected to undergo greater mechanical damage during epithelium-removal than larger airways, showed no reduction in the maximal response to either potassium chloride or histamine. For the three orders, removal of the epithelium did not affect the maximal response to electrical stimulation. All tissues without epithelium relaxed completely in response to sodium nitroprusside, indicating that the smooth muscle was capable of relaxation. Therefore, it is reasonable to assume that removal of the airway epithelium did not damage the integrity of the bronchial smooth muscle. The reason for the diminution of the maximal contractile response

to certain agonists in certain orders of bronchi is not clear. One possible explanation might be that there is release of an epithelium-derived contracting factor(s), similar to that which may be produced in response to electrical stimulation. This may potentiate the maximal response to certain agonists in certain bronchi. For example, potassium chloride may stimulate release of contracting factors in third and fourth order bronchi, thus potentiating the maximal response to this substance. Similarly, in fifth order bronchi,high concentrations of acetylcholine may cause the release of a contracting factor from the epithelium, which potentiates the maximal response to the contractile agonist, and also attenuates any effect of a relaxing factor. Histamine does not appear to evoke release of a contracting factor, as the maximal response to the agonist is similar in the three orders with and without epithelium.

In conclusion, these results demonstrate that the airway epithelium of the pig may release both relaxing and contracting factors. The distribution of these factors shows heterogeneity along the porcine respiratory tree. Their release and action show qualitative and quantitative differences compared to previous observations made in the dog.

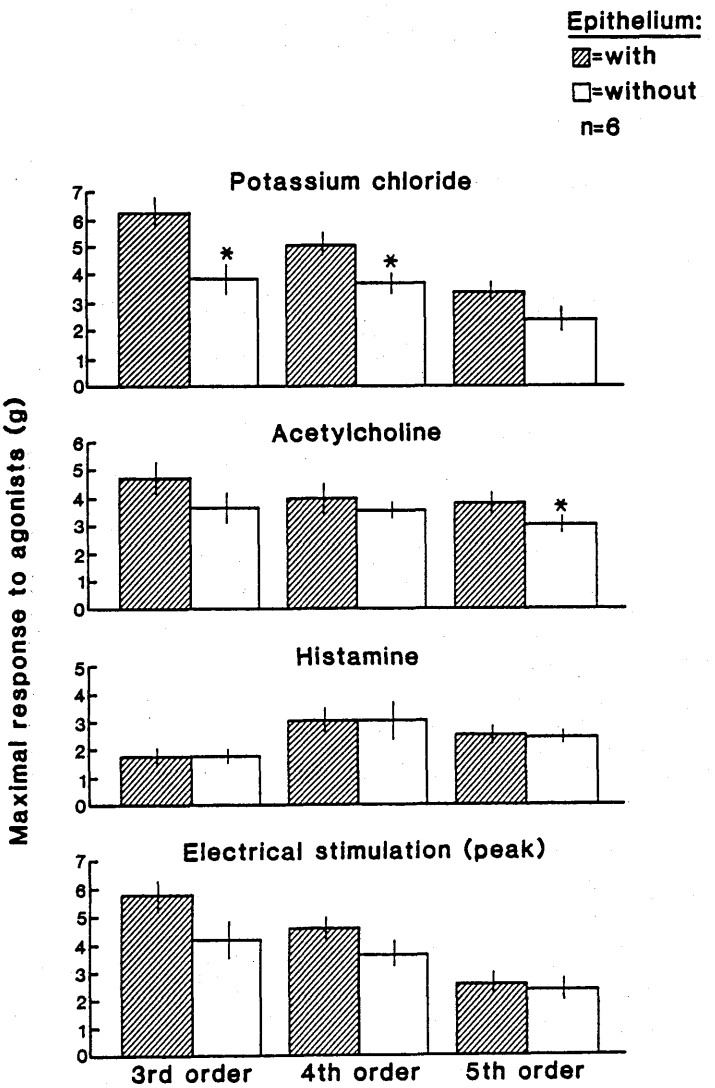

Figure 11: The effect of epithelium-removal on maximal responses to potassium chloride, acetylcholine, histamine, and electrical stimulation (peak response) in third order (left), fourth order (middle) and fifth order (right) porcine bronchi. Data (means ± SE) are expressed as maximal tension developed (in grams). *; Significant difference between tissues with and without epithelium.

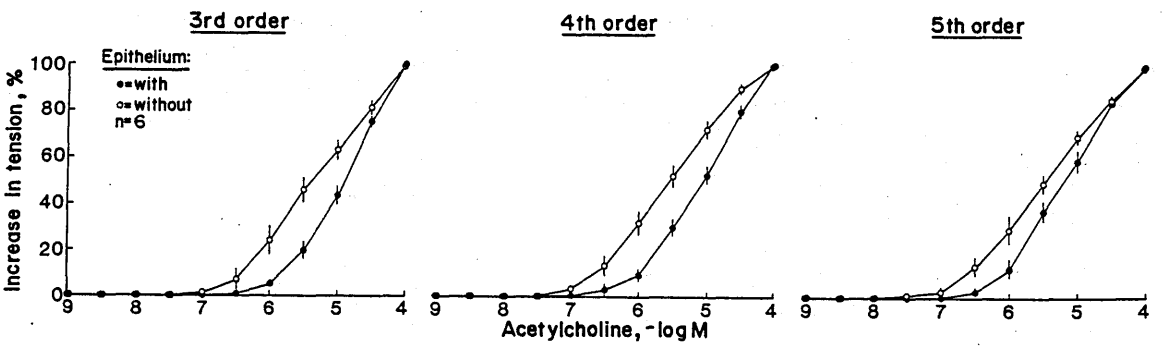

Figure 12: The effect of epithelium-removal on the concentration-effect curve to acetylcholine in third-order (left), fourth-order (middle) and fifth-order (right) porcine bronchi. Data (means ± SE) are expressed as percent of the response to 10^{-4}M acetylcholine. ●, Rings with epithelium; O, rings without epithelium.

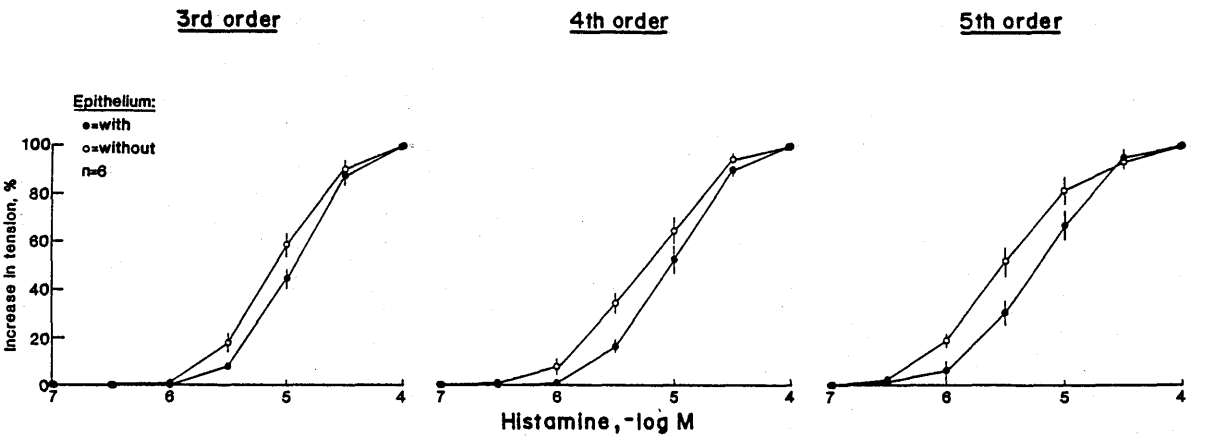

Figure 13: The effect of the epithelium-removal on the concentration-effect curve to histamine in third-order (left), fourth order (middle) and fifth order (right) porcine bronchi: ●, Rings with epithelium; O, Rings without epithelium.

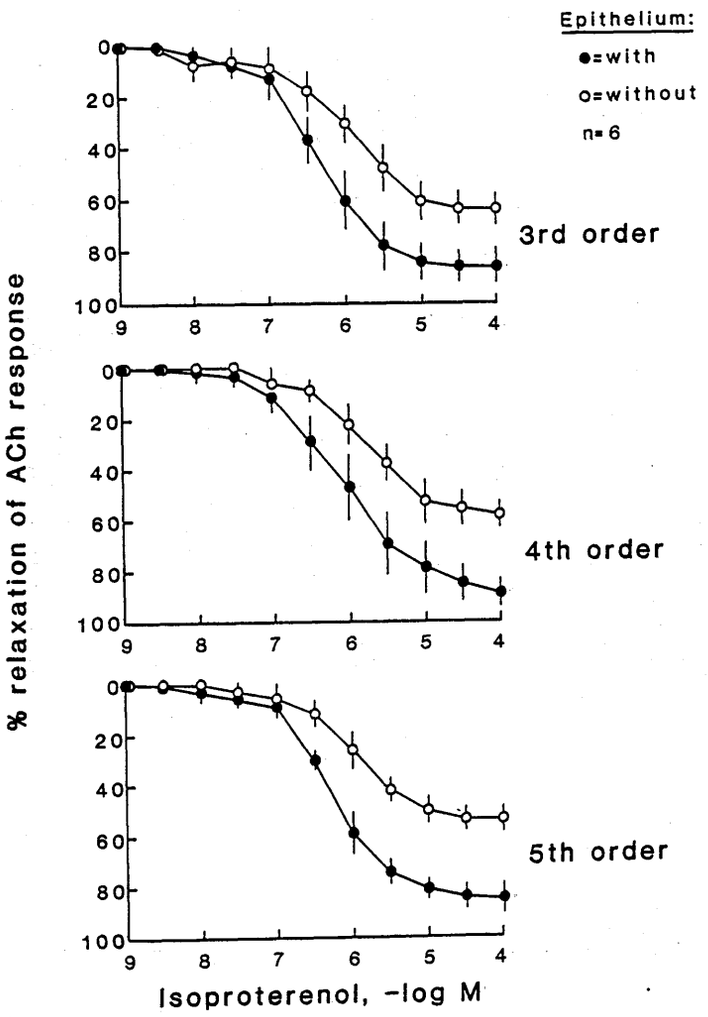

Figure 14: The effect of epithelium-removal on the concentration-effect curve to isoproterenol in third-order (top), fourth-order (middle) and fifth-order (bottom) porcine bronchi. Rings were contracted to equal tensions using the concentration of acetylcholine producing 50% of the maximal response to this agonist. Data (means ± SE) are expressed as percent relaxation of the response to acetylcholine; n=6. ●, Rings with epithelium;. O, Rings without epithelium.

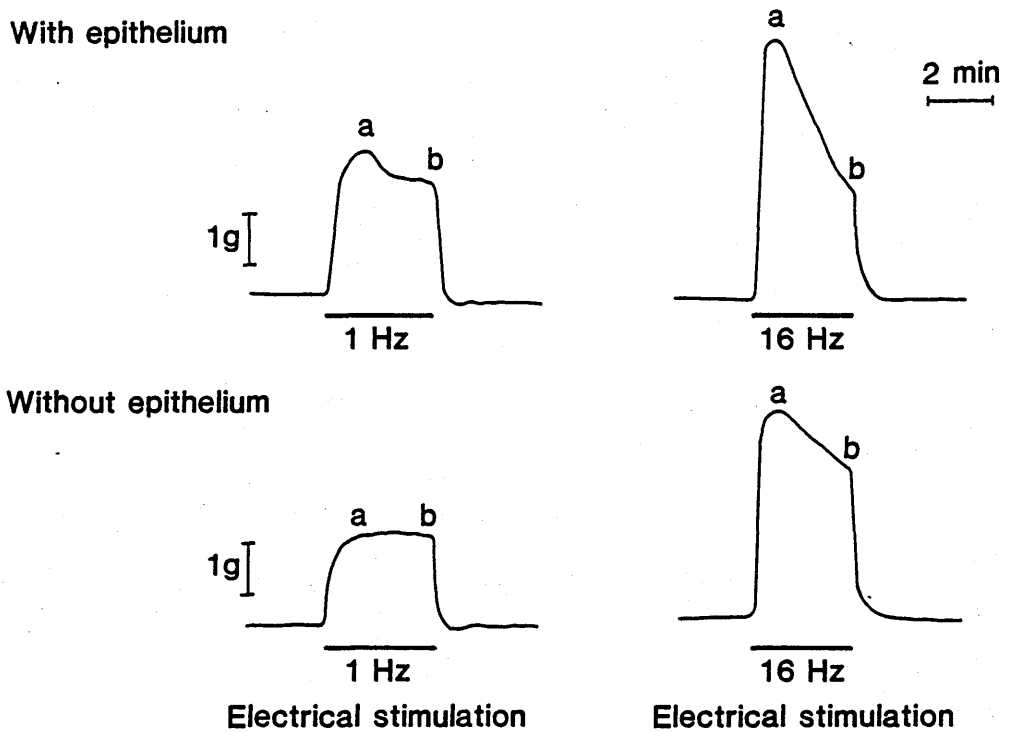

Figure 15: Representative tracings of effect of transmural nerve stimulation on paired rings of fourth-order bronchi with and without epithelium. A low stimulus frequency (1 Hz) and a high stimulus frequency (16 Hz) are shown. Peak response (a) was measured 30 seconds after the start of stimulation. Fade response (b) was measured 3 minutes after the start of stimulation.

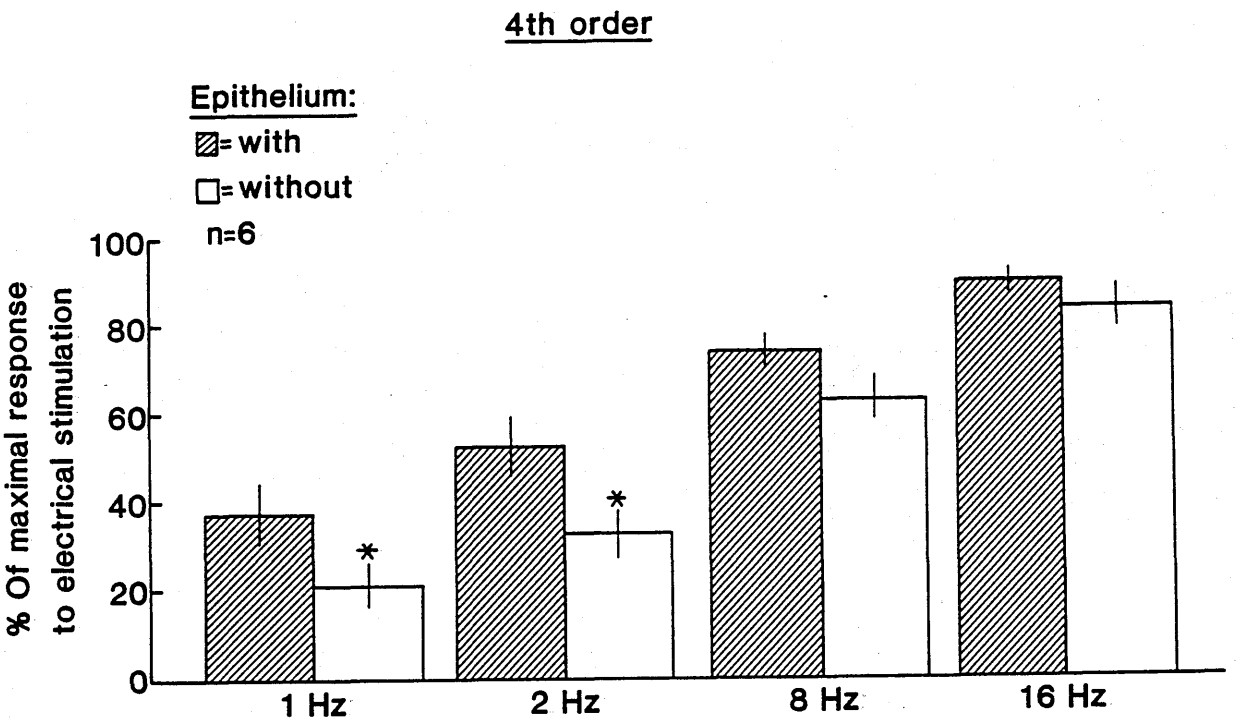

Figure 16: The peak response to electrical stimulation at 1, 2, 8, and 16 Hz in fourth-order porcine bronchi. Data (means $\pm$ SE) are expressed as percent of the maximal peak response. *Significant difference between tissues with and without epithelium.

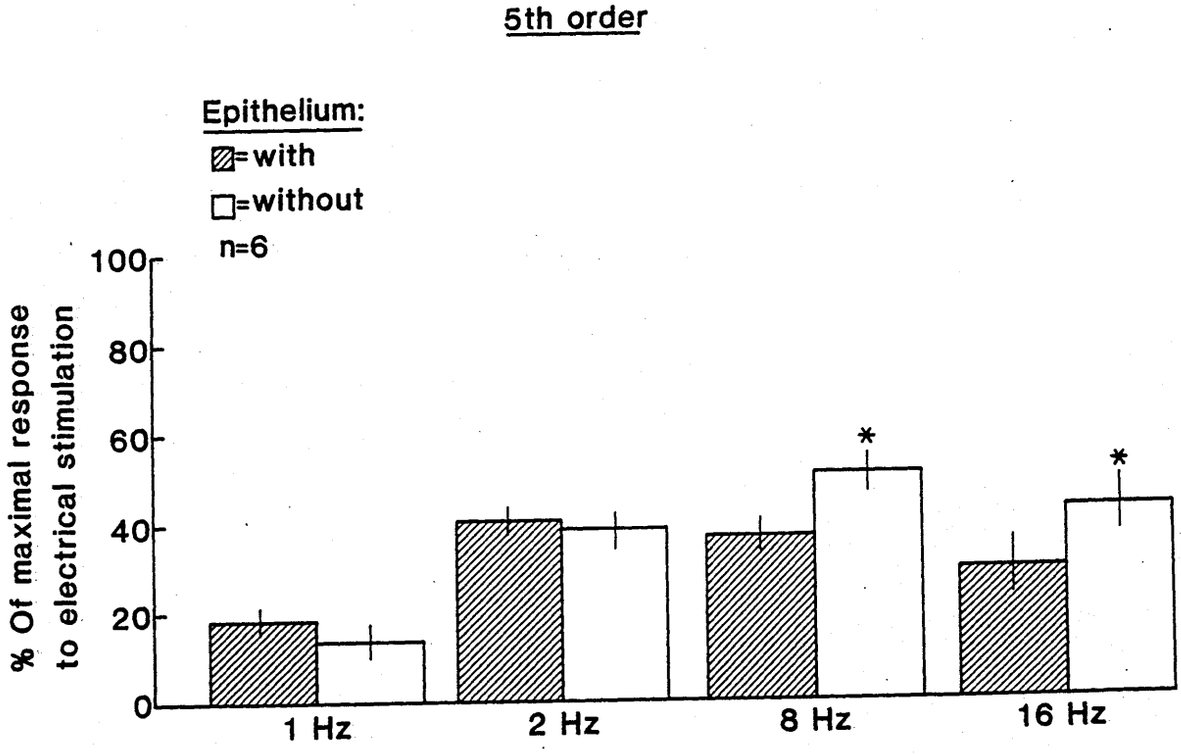

Figure 17: The fade response to electrical stimulation at 1, 2, 8 and 16 Hz in fifth-order bronchi. Data (means ± SE) are expressed as percent of the maximal peak response. *Significant difference between tissues with and without epithelium.

TABLE 3. Characteristics of the 3 orders of porcine bronchi

	Outside Diam, mm	Optimal Resting Tension, g	EC_{50} for KCl, mM	
			With Epithelium	Without Epithelium
3rd Order (Segmental Bronchus)	8-10	1-2	14.9±0.9	14.8±0.7
4th Order (Subsegmental Bronchus)	5-6	0.5-1	13.0±1.5	14.0±1.4
5th Order (Subsegmental Bronchus)	3-4	0.5-1	13.1±1.4	12.3±1.4

Values are means ± SE. Outside diameters and optimal resting tensions are approximate ranges based on 18 experiments; for responses to KCl, n = 6. Outside diameters were measured before application of force.

Table 4. Effect of epithelium removal on sensitivity to acetylcholine and histamine in 3 orders of porcine bronchi.

	-Log EC_{50}	
	With Epithelium	Without Epithelium
Acetylcholine		
3rd order	4.9±0.1	5.4±0.1a
4th order	5.1±0.1	5.6±0.1a
5th order	5.2±0.1	5.5±0.1
Histamine		
3rd order	4.9±0.04	5.1±0.04a
4th order	5.0±0.06	5.2±0.1
5th order	5.2±0.1[b]	5.5±0.1a,b

Values are means ± SE; n = 6. EC_{50} concentration producing 50% of maximal response. a significantly different from tissues with epithelium. [b] Significantly different from 3rd and 4th Order bronchi.

TABLE. 5. Effect of epithelium removal on responses to transmural nerve stimulation in porcine bronchi

	Response, % of maximal response							
	0.5 Hz	1 Hz	2 Hz	4 Hz	8 Hz	16 Hz	32 Hz	64 Hz
Peak								
3rd Order								
With Epithelium	7.0±1.1	21.4±2.3	37.4±3.3	47.5±3.7	64.1±3.0	91.0±2.4	100	82.9±5.7
Without Epithelium	5.0±0.9	11.8±3.1[a]	24.7±3.7[a]	38.8±4.2	55.2±3.3[a]	79.0±4.3[a]	99.4±0.6	92.1±4.9
4th Order								
With Epithelium	23.1±7.0	38±7.8	52.3±6.8	61.5±5.8	74.8±4.3	90.3±3.2	100	71.4±9.4
Without Epithelium	10.2±3.5[a]	21±4.7[a]	33.6±5.6[a]	47±5.3	63.4±5.4	83.8±4.6	100	89.5±3.6
5th Order								
With Epithelium	20.6±2.0	42±2.2	54±3.5	70.2±6.2	75.3±4.1	90.6±2.9	100	71.3±9.0
Without Epithelium	19.2±2.6	27.8±4.0[a]	42.7±3.6	55.1±2.7	67.7±3.0	86.3±3.0	100	83.0±4.8
Fade								
3rd Order								
With Epithelium	6.0±2.4	19.3±2.9	27.4±2.5	31.6±3.5	35.0±4.0	23.4±3.5	23.4±2.8	25.8±3.0
Without Epithelium	2.4±1.4	9.05±3.4	19.6±5.6	28.7±5.8	39.3±5.3	35.8±5.1	34.7±3.8	19.4±2.2
4th Order								
With Epithelium	19.1±6.4	33.0±7.9	43.9±7.9	52.1±7.7	54.4±6.6	34.8±5.4	34.3±3.3	26.7±4.6
Without Epithelium	8.4±3.8	17.2±5.9	29.8±7.2	41.7±7.2	50.1±7.2	45.7±6.7	34.4±4.4	28.2±4.5
5th Order								
With Epithelium	18.9±3.2	33.5±2.4	41.6±2.2	51.8±3.8	38.9±3.7	30.1±6.6	28±4.4	30.2±7.1
Without Epithelium	13.7±3.8	24.7±4.5	38.2±4.6	48.2±3.8	51.7±4.7[a]	44±6.7[a]	33.3±6	25.6±2.6

Values are means ± SE; n = 6. [a] Significantly different from tissues with epithelium.

CHAPTER 3

Epithelium, Contractile Tone and Responses to Relaxing Agonists in Canine Bronchi

INTRODUCTION

In canine airway smooth muscle, the relaxation to isoproterenol is partially dependent on the contractile agonist employed to induce tone, and on the degree of contraction achieved (Torphy *et al*, 1983; Russell, 1984; Torphy *et al*, 1985; White *et al*, 1988). Canine tracheal tissue is significantly more sensitive to isoproterenol during contractions induced by 5-hydroxytryptamine, as compared to acetylcholine (Russell, 1984). The relaxations induced by isoproterenol and forskolin are smaller during near-maximal contractions to methacholine, as compared to lower levels of cholinergic activation (Torphy *et al*, 1983; Torphy *et al*, 1985).

Removal of the airway epithelium reduces the responsiveness of canine bronchial smooth muscle to the beta-adrenergic agonist isoproterenol (Flavahan *et al*, 1985; Chapter 1). A similar effect of epithelium-removal is seen in the cow (Barnes *et al*, 1985) and the pig (Chapter 2). The aim of the present study was to explore whether the effect of the epithelium on relaxation of canine airways is affected by the contractile agonist employed, or by variations in the level of contraction obtained prior to the administration of relaxing agents. As the influence of the epithelium on relaxations to isoproterenol is most marked in the fourth order bronchi of the dog (Chapter 1), this tissue was used.

METHODS

Experiments were performed on bronchi taken from mongrel dogs of either sex (15-20 kg) anaesthetised with pentobarbital sodium (30mg/kg intravenously) and exsanguinated. Lungs which showed evidence of infection with heart-worms or bronchopneumonia were rejected from the study. Tissues were taken from the lower lung lobes. The fourth order (subsegmental bronchus, outer diameter 1-3mm) was examined. Paired rings of tissue, with and without epithelium, were placed in organ chambers, and brought to the optimal point of their resting tension, as described in Chapter 1.

Experimental Protocols

Concentration-effect curves to acetylcholine were obtained in bronchi with and without epithelium, by increasing the concentration of acetylcholine in the organ chambers in half-log increments (Van Rossum, 1963). From these curves, the concentration of acetylcholine giving a contraction which was either 40% (EC_{40}) or 80% (EC_{80}) of the response to a 10^{-4}M concentration of the agonist was calculated. After washing out, the tissues were allowed to equilibrate for 30 minutes. At the end of this period, in one pair of tissues (with and without epithelium), the EC_{40} of acetylcholine was added; in a second pair the EC_{80} was used. When the contraction had stabilized, a concentration-effect curve (10^{-9} to 10^{-4}M) was obtained for sodium nitroprusside. The preparations were then washed by changing the solution at least six times over a

period of one hour. Cocaine (5×10^{-6}M), hydrocortisone (3×10^{-5}M) and phentolamine (10^{-6}M) were added to the organ chambers to block neuronal uptake, extraneuronal uptake, and alpha-adrenoceptors, respectively (Furchgott, 1972); preliminary experiments indicated that the relaxation to isoproterenol was not affected by the presence or absence of inhibitors of catecholamine-uptake. The inhibitors were present in the organ chambers for 30 minutes prior to the further administration of drugs, and remained in contact with the preparation throughout the experiment. The bronchi were contracted to either the EC_{40} or the EC_{80} level for acetylcholine, and a concentration-effect curve to isoproterenol (10^{-9} to 10^{-4}M) was obtained.

In a second series of experiments, the tissues were contracted to either the EC_{40} or the EC_{80} level for acetylcholine and a concentration-effect curve for forskolin [10^{-9} to 10^{-5}M (a stimulator of adenylate cyclase, Seamon and Daly, 1981)] was obtained. In separate, but similar, protocols, the effects of sodium nitroprusside, isoproterenol and forskolin were determined in bronchi (with and without epithelium) contracted with the individually determined EC_{40} and EC_{80} for 5-hydroxytryptamine.

In certain experiments, after obtaining concentration-effect curves to either acetylcholine or 5-hydroxytryptamine, the tissues were incubated with either an inhibitor of cyclooxygenase, indomethacin (10^{-5}M), or the inhibitor of cyclic GMP accumulation LY83583 (10^{-5}M) (Schmidt et al, 1985) for 30 minutes prior to the administration of drugs. The bronchi were then contracted with either acetylcholine or

5-hydroxytryptamine and concentration-effect curves were obtained to either sodium nitroprusside (10^{-9} to 10^{-4}M) or isoproterenol (10^{-9} to 10^{-4}M).

Nitric Oxide

A gas bulb fitted with a silicon injection septum was filled with nitric oxide from a cylinder (Union Carbide, Chicago, IL). An appropriate volume (10-1000μl) was removed with a syringe and injected into 100ml of distilled water, that had been gassed with helium for approximately 3 hours, giving stock solutions of nitric oxide of 4×10^{-5}M and 4×10^{-4}M (Palmer et al, 1987). Aliquots of these stock solutions were drawn into a glass syringe and injected into the organ chambers, to obtain a concentration-effect curve to nitric oxide in the concentration-range 3×10^{-9} to 10^{-4}M. Prepared in this way, nitric oxide induces profound relaxation of vascular smooth muscle (Komori et al,1988).

Drugs

Acetylcholine-chloride (Sigma Co, St.Louis, MO); cocaine hydrochloride (Yar Lang, La Crosse, WI); forskolin (Sigma); hydrocortisone 21-sodium hemisuccinate (Sigma); 5-hydroxytryptamine creatinine sulfate (Sigma); indomethacin (Sigma); dl-isoproterenol hydrochloride (Sigma); LY83583 (6-anilino-5, 8-quinolinediene) (Eli Lilly and Company, Indianapolis, IN); phentolamine mesylate (Ciba-Geigy, Summit NJ); and sodium nitroprusside (Sigma). Unless otherwise specified, drugs were prepared daily in distilled water, and kept on ice. Stock solutions of indomethacin were prepared in equal molar

concentrations of Na_2CO_3. Stock solutions of forskolin were dissolved in 100% dimethylsulfoxide and dilutions were prepared in distilled water. Preliminary experiments indicated that the solvent had no effect on the bronchi at the concentration used.

Data analysis

Responses to forskolin, sodium nitroprusside and isoproterenol are expressed as percent reduction of the response to the contractile agonist. The degree of relaxation obtained varied widely between agonists, and between tissues with and without epithelium. Some tissues without epithelium showed minimal relaxation under certain circumstances. For this reason, that concentration of agonist giving either 25% or 50% relaxation of the initial contraction (IC_{25} and IC_{50} respectively) could not be calculated. Therefore, responses to the different agents were compared by analyzing the area above the concentration-effect curves (Houston et al, 1985; Flavahan and Vanhoutte, 1986*b*). By this method, the x and y axes are considered to form two sides of a rectangle of area of $1000mm^2$ (Lotus 1-2-3 statistical program). As the concentration-effect curve is expressed as percent reduction of a contractile response, the area above the curve represents the amount of reduction of the contraction, i.e. the greater the area above the curve, the greater the relaxation. The absolute change in tension produced by the maximal concentration of each relaxing agent used, is presented in grams. Values are means $\pm$ SEM. For all experiments, n equals the number of

dogs from which tissues were taken. Within each group, statistical analysis was by Student's t-test for paired observations. For comparison between groups, analysis of variance (ANOVA) was used. When P was smaller than 0.05, differences were considered to be statistically significant.

RESULTS

General responsiveness of bronchial rings (Tables 6-10). There was no significant difference in the maximal responses to acetylcholine or 5-hydroxytryptamine between tissues with and without epithelium; the maximal responses to acetylcholine and 5-hydroxytryptamine were similar, [acetylcholine: tissues with epithelium, 5.7 $\pm$ 1.9g (n=46), tissues without epithelium, 5.5 $\pm$ 1.6g (n=46); 5-hydroxytryptamine: tissues with epithelium, 4.1 $\pm$ 1.9g (n=34), tissues without epithelium, 4.1 $\pm$ 1.6g (n=34)]. For all tissues, there was no significant difference in active force achieved between tissues with and without epithelium at either the EC_{40} or the EC_{80} level for either of the contractile agonists. Neither the resting tensions nor the EC_{40} and EC_{80} values for acetylcholine and 5-hydroxytryptamine were affected by the presence of either indomethacin or LY83583. There was no reduction in the response to either acetylcholine or 5-hydroxytryptamine during the course of the experiment.

Experiments were conducted in which the order of the relaxing agents was altered, to determine whether the sequence of administration of the drugs affected the

response of the tissues. There was no significant effect of altering the order of administration on the responses of the fourth order bronchi to the relaxing agents, in the presence of a contraction to either acetylcholine or 5-hydroxytryptamine (data not shown). Neither indomethacin nor LY83583 had any significant effect on the concentration-effect curves to acetylcholine or 5-hydroxytryptamine.

Isoproterenol

Isoproterenol evoked concentration-dependent relaxations in fourth order canine bronchi with and without epithelium. For bronchi contracted with the EC_{40} of acetylcholine, there was no significant difference in the response to isoproterenol between tissues with and without epithelium (Fig.18, Table 6). Raising the level of contraction to the EC_{80} level for acetylcholine reduced the percent relaxation to isoproterenol in bronchi with and without epithelium, as compared to values obtained with the EC_{40} (Fig.19, Table 6). However, this effect was significant only for tissues without epithelium. Thus, at the EC_{80} level for acetylcholine, bronchi with epithelium showed a significantly greater relaxation to isoproterenol than bronchi without epithelium (Fig.19, Table 6).

Isoproterenol evoked concentration-dependent relaxations during contractions to 5-hydroxytryptamine. There was no difference between tissues with and without epithelium. The relaxation to isoproterenol was not affected by the level of contraction to the monoamine (Figs. 18 and 19, Table 7).

At the ED_{80} level for the contractile agonists, all tissues were significantly more sensitive to isoproterenol during contractions to 5-hydroxytryptamine, compared to acetylcholine.

Indomethacin (10^{-5}M; Tables 6 and 7) and LY83583 (10^{-5}M; Tables 6 and 7) did not significantly affect responses to isoproterenol.

Sodium nitroprusside

Sodium nitroprusside induced concentration-dependent relaxations in fourth order canine bronchi with and without epithelium. During contractions to the EC_{40} of acetylcholine, sodium nitroprusside induced relaxations which were similar in tissues with and without epithelium (Table 8).

Raising the level of contraction to the EC_{80} value for acetylcholine did not significantly affect the response to sodium nitroprusside in tissues with epithelium; it significantly reduced the percent maximal relaxation to the drug in tissues without epithelium. Thus, bronchi with epithelium showed a significantly greater relaxation to sodium nitroprusside than bronchi without epithelium (Fig. 20, Table 8).

In the presence of 5-hydroxytryptamine, sodium nitroprusside induced concentration-dependent relaxations which were not affected by the level of contraction to 5-hydroxytryptamine (Table 9). There was no difference between tissues with and without epithelium. For bronchi with epithelium, the relaxation to sodium nitroprusside was similar to that obtained in the presence of acetylcholine,

at both the E_{40} and the E_{80} levels for the contractile agents (Tables 8 and 9). In tissues without epithelium, the relaxation to sodium nitroprusside was significantly greater during contractions to 5-hydroxytryptamine, as compared to acetylcholine, at both levels of contraction (Tables 8 and 9).

At the EC_{40} level for acetylcholine, indomethacin did not significantly affect the concentration-effect curve to sodium nitroprusside (Table 8). At the EC_{80} level for acetylcholine, indomethacin did not affect relaxations to sodium nitroprusside in bronchi with epithelium. However, for bronchi without epithelium, the relaxation to sodium nitroprusside showed a small augmentaion, such that there was no longer a difference in the response to the relaxing agent between tissues with and without epithelium (Table 8). Indomethacin had no significant effect on the concentration-effect curve to sodium nitroprusside in the presence of 5-hydroxytryptamine (Table 9).

LY83583 (10^{-5}M; Fig 21; Tables 8 and 9) did not significantly affect responses to sodium nitroprusside.

Forskolin

Forskolin evoked concentration-dependent relaxations of fourth order bronchi. There was no significant difference in relaxation obtained between tissues with and without epithelium. The response to forskolin was not affected by the level of contraction to acetylcholine (Table 10).

In the presence of a contraction induced by 5-hydroxytryptamine, forskolin evoked concentration-

dependent relaxations which were similar in bronchi with and without epithelium. The relaxation to forskolin was unaffected by the level of contraction to 5-hydroxytryptamine (Table 10). All tissues were significantly more sensitive to forskolin in the presence of 5-hydroxytryptamine, than acetylcholine (Table 10).

Nitric oxide

Over the concentration-range 3×10^{-9} - 10^{-4}M, nitric oxide did not induce relaxation in fourth order bronchi with or without epithelium, in the presence of a contraction to either acetylcholine or 5-hydroxytryptamine, at either the EC_{40} or the EC_{80} level for the contractile agonists (Fig 20).

DISCUSSION

The present study demonstrates that in canine fourth order bronchi: (a) at the EC_{80} level for the contractile agonists, the relaxing agonists were significantly more potent in the presence of a contraction to 5-hydroxytryptamine, as compared to responses obtained in the presence of acetylcholine. In addition, the percent relaxation obtained at the EC_{80} level for acetylcholine was attenuated in comparison to responses obtained at the EC_{40} level for the muscarinic agonist. These observations confirm previous findings in canine airways (Torphy et al, 1983; Russell, 1984; Torphy et al, 1985; Gunst et al, 1988; White et al, 1988); (b) in the presence of an EC_{40} level of contraction to acetylcholine, the relaxations to isoproterenol, sodium nitroprusside and forskolin are similar in bronchi with and without epithelium; (c) at the

EC_{80} level for the muscarinic agonist, removal of the airway epithelium causes a significant reduction of the relaxation to isoproterenol and sodium nitroprusside, but not of that to forskolin; (d) when active force is generated by 5-hydroxytryptamine, the relaxations obtained to isoproterenol, sodium nitroprusside and forskolin are not affected by removal of the airway epithelium; (e) indomethacin enchanced the response to sodium nitroprusside in tissues without epithelium at the EC_{80} level for acetylcholine. Neither indomethacin nor LY83583 had any other effects; (f) there was no relaxation to nitric oxide.

From results obtained previously in the dog (Flavahan et al, 1985; Chapter 1) and the pig (Chapter 2) it may be concluded that the threshold for the effect of epithelium-removal on the response to isoproterenol is at the EC_{50} level for acetylcholine. This effect may be due to stimulation of epithelial beta-adrenoceptors, causing release of an epithelium-derived relaxing factor (Flavahan et al, 1985; Chapter 1). Isoproterenol is thought to mediate relaxation of bronchial smooth muscle via stimulation of cyclic AMP (Torphy et al, 1983; Bulbring and Tomita, 1987; Torphy et al, 1987). Hence, it can be postulated that the airway epithelium may release a factor which augments cyclic AMP-dependent relaxations. To test this hypothesis, the effect of forskolin, a specific and potent stimulator of adenylate cyclase (Seamon and Daly, 1981; de Lanerolle, 1988) was examined. The relaxation to forskolin was considerably reduced in the presence of a contraction to acetylcholine, as compared to relaxations

obtained in the presence of 5-hydroxytryptamine. However, the response to forskolin was not affected by the level of contraction to acetylcholine, in contrast to earlier observations in the trachea of the dog (Torphy et al, 1985). Furthermore, bronchi with epithelium did not show an augmentation of the response to forskolin in the presence of a high level contraction to acetylcholine. These data suggest that the enhancing effect of the epithelium on relaxation of bronchial smooth muscle is not specific for cyclic-AMP-dependent relaxations.

Another possible mechanism for epithelium-dependent phenomena in canine airways is production of bronchodilator prostaglandins from the epithelial cells. Prostaglandin E_2 is thought to mediate epithelium-dependent relaxations to arachidonic acid in the airways of the rabbit (Butler et al, 1987), the guinea-pig (Tschirhart et al, 1987) and the dog, (Chapter 4). However, in the present study, indomethacin did not alter the effect of the epithelium on responses to isoproterenol. Indomethacin did not reduce the relaxation to sodium nitroprusside in tissues with epithelium. Overall, the data suggest that prostaglandins are not the mediators of the epithelial effects described in these experiments.

Sodium nitroprusside is thought to mediate relaxation via production of cyclic GMP (Schultz et al, 1977; Torphy et al, 1985; Torphy et al, 1987). In this study, sodium nitroprusside evoked concentration-dependent relaxations which were considerably reduced in the presence of acetylcholine, compared to 5-hydroxytryptamine. These

data demonstrate that the functional antagonism exerted by muscarinic agonists on bronchial smooth muscle is not confined to agents which mediate relaxation via cyclic AMP, as previously thought (Torphy et al, 1987). At the high level of contraction to acetylcholine, relaxations to sodium nitroprusside were reduced by epithelium-removal. As sodium nitroprusside is a putative stimulator of the production of cyclic GMP, relaxing factors derived from the airway epithelium may exert their effect via a cyclic GMP-dependent phenomenon. To investigate this possibility, the effect of nitric oxide, a potent stimulator of cyclic GMP (Palmer et al, 1987; Ignarro et al, 1988), was examined. Nitric oxide had no relaxing effect on fourth order canine bronchi in the presence of either contractile agent, whether or not epithelium was present. These data suggest that cyclic GMP may not be of major importance in the relaxation of canine bronchial smooth muscle, although this cyclic nucleotide does have a small relaxing effect in the canine trachea. Thus, sodium nitroprusside may be able to mediate relaxation of bronchial smooth muscle by mechanisms other than stimulation of guanylate cyclase. The data obtained with the compound LY83583, a potent inhibitor of the accumulation of cyclic GMP (Diamond and Chu, 1985; Schmidt et al, 1985) provide further evidence for these conclusions. LY83583 failed to inhibit relaxations to sodium nitroprusside in the presence of either acetylcholine or 5-hydroxytryptamine. In addition, this compound did not alter the enhancing effect of the epithelium on relaxations to either sodium

nitroprusside or isoproterenol at the high levels of contraction to acetylcholine, indicating that the epithelium-derived relaxing factor does not mediate its effects via cyclic GMP.

There is a considerable receptor reserve for acetylcholine in canine airway smooth muscle; 5-hydroxytryptamine, by contrast, has a very small receptor reserve (Gunst et al, 1987). The muscarinic receptor maybe more efficiently coupled to the contractile process than the receptor for 5-hydroxytryptamine (Flavahan and Vanhoutte, 1986a; Madison and Brown, 1988). In the context of the present study, the following model can be proposed. In the presence of a contraction to 5-hydroxytryptamine, relaxing agents can exert such a potent inhibitory effect that a modulatory relaxing signal (epithelium-derived relaxing factor) from the epithelium cannot be detected. When contraction is initiated by acetylcholine, the response to relaxing agents is attenuated because there is a very efficient mobilization of the contractile proteins (de Lanerolle, 1988). In this situation, an inhibitory signal from the epithelium, i.e. an epithelium-derived relaxing factor, becomes apparent. Release of the epithelium-derived relaxing factor might be stimulated via activation of epithelial beta-adrenoceptors (Flavahan et al, 1985; Chapter 1). Sodium nitroprusside might also act directly on the epithelial cells, or may mimic the effect of the epithelial factor on the smooth muscle.

Taken together, these data imply that the effect of the epithelium on relaxation of the airway smooth muscle depends on the contractile agent used to initiate contraction, and the level of active force achieved to that agonist. Therefore, when interpreting the effect of removal of the airway epithelium on the responsiveness of bronchial smooth muscle to bronchodilators, the experimental conditions under which the tissue is studied must be taken into account.

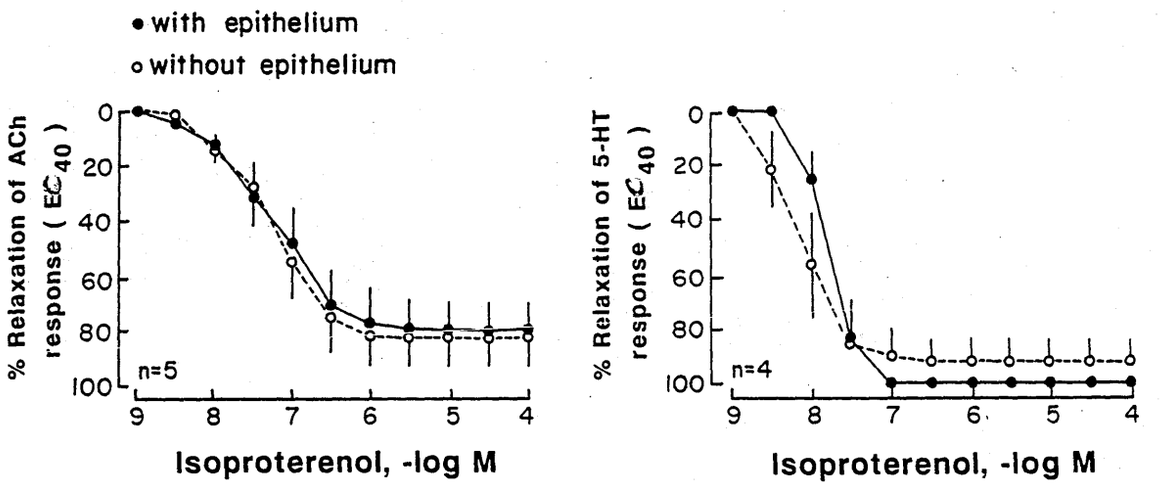

Figure 18: The effect of epithelium-removal on the concentration-effect curve for isoproterenol in canine bronchi. Left: in the presence of the EC_{40} for acetylcholine. Right: in the presence of EC_{40} for 5-hydroxytryptamine. Data (means $\pm$ SE) are expressed as percent relaxation of the response to the contractile agonist. ●, Rings with epithelium; O, Rings without epithelium.

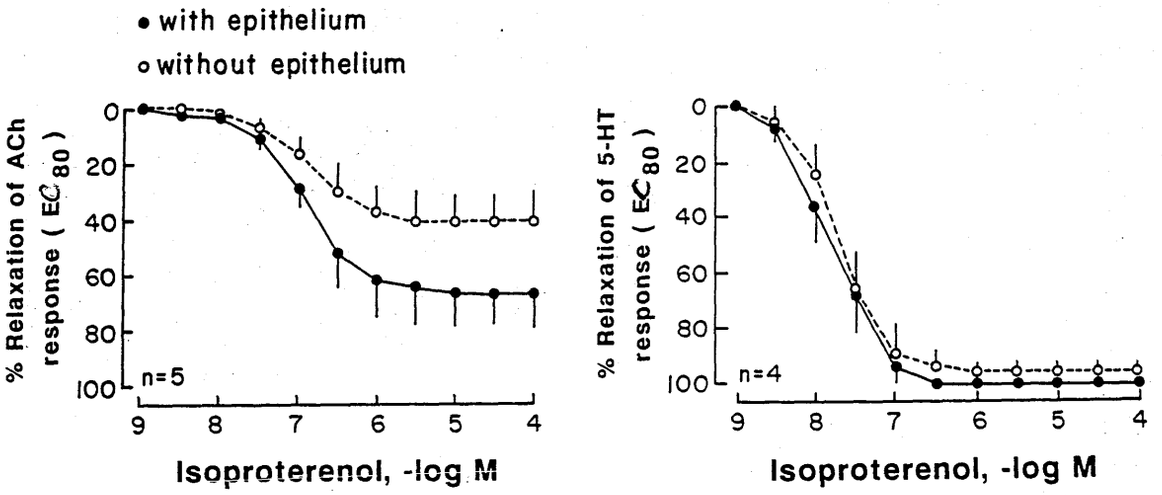

Figure 19: The effect of epithelium-removal on the concentration-effect curve for isoproterenol. Left: in the presence of the EC_{80} for acetylcholine. Right: in the presence of the EC_{80} for 5-hydroxytryptamine. Data (means ± SE) are expressed as percent relaxation in response to the contractile agonist. ●, Rings with epithelium; O, Rings without epithelium.

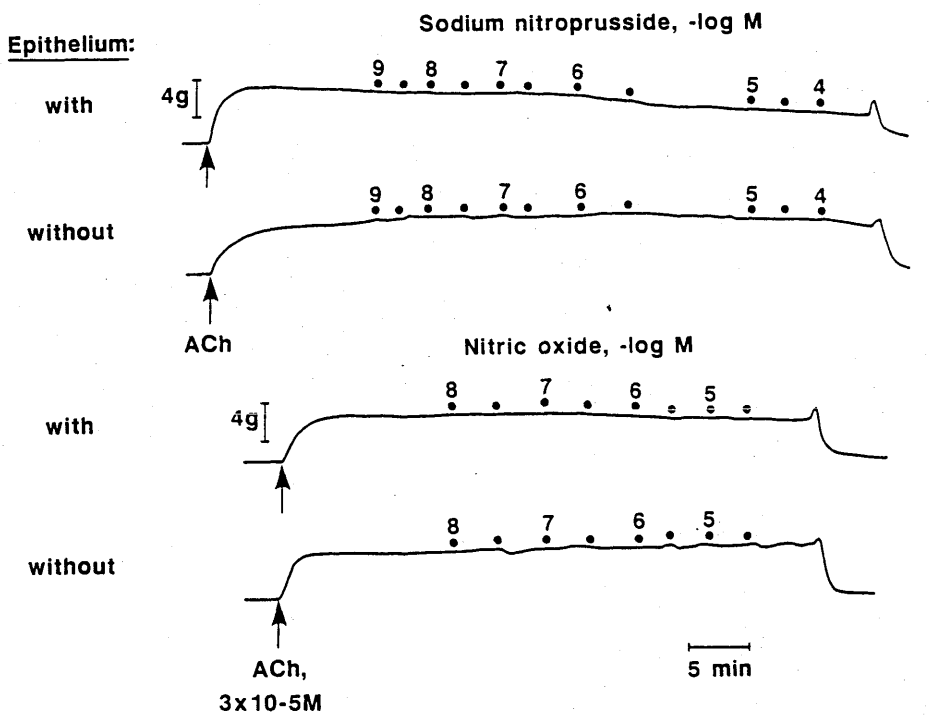

Figure 20: Representative tracing of the effect of epithelium-removal on the response to sodium nitroprusside and nitric oxide in canine bronchi. Top: response to sodium nitroprusside. Bottom: response to nitric oxide. Each tissue was contracted to the EC_{80} value for acetylcholine.

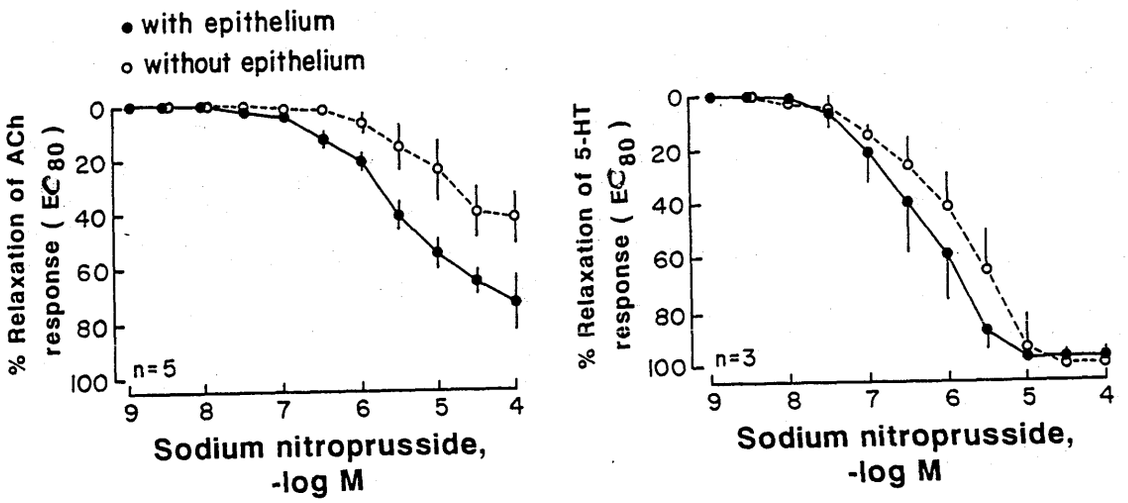

Figure 21: The effect of LY83583 on the concentration-effect curve to sodium nitroprusside. Left: in the presence of the EC_{80} for acetylcholine. Right: in the presence of the EC_{80} for 5-hydroxytryptamine. Data (means ± SE) are expressed as percent relaxation of the response to the contractile agonist. ●, tissues with epithelium, O, tissues without epithelium.

Table 6. Responses to Isoproterenol in Fourth Order Canine Bronchi

Contractile Agent		n	Level of Contraction (g)	Absolute Decrease in Tension (g)	Maximal Relaxation (%)	Area Above the Curve (mm^2)
Acetylcholine:	With Epithelium	5	EC_{40} (2.3±0.3)	1.8±0.3	80.3±23.6	524.2±189.1
	Without Epithelium	5	EC_{40} (2.1±0.2)	1.8±0.3	81.9±23.8	541.6±188.7
Acetylcholine:	With Epithelium	5	EC_{80} (4.1±0.7)	2.4±0.3	66.7±25.9	387.9±173.5
	Without Epithelium	5	EC_{80} (4.4±0.4)	1.7±0.4a	40.8±22.5a,b	235.2±141.1a,b
Acetylcholine: (Indomethacin)	With Epithelium	5	EC_{40} (2.5±0.2)	2.5±0.2	97.8±5.0	651.2±85.4
	Without Epithelium	5	EC_{40} (3.5±0.9)	2.7±0.6	85.1±17.6	567.7±192.1
Acetylcholine: (Indomethacin)	With Epithelium	5	EC_{80} (5.9±0.6)	4.3±0.4	76.8±21.1[b]	414.7±146.8[b]
	Without Epithelium	5	EC_{80} (4.7±1.0)	2.5±0.2a	59.1±15.9a,b	314.6±101.3a,b
Acetylcholine: (LY83583)	With Epithelium	5	EC_{40} (2.2±0.7)	1.8±0.6	77.9±7.2	525.1±67.6
	Without Epithelium	5	EC_{40} (2.1±0.3)	1.9±0.3	91.9±11.6	668.8±158.4
Acetylcholine: (LY83583)	With Epithelium	5	EC_{80} (3.4±0.6)	1.9±0,5	54.2±21.6[b]	314.13±136.7[b]
	Without Epithelium	5	EC_{80} (2.9±0.5)	1.0±0.4	35.2±26.3a,b	192.6±148.2a,b

Values are means + SEM. a Significantly differnet from bronchi with epithelium. [b] Significantly different from values obtained at the EI 40 level for acetylcholine. EC_{40} for acetylcholine 3×10^{-7} to 3×10^{-6}M. EC_{80} 10^{-5} to 3×10^{-5} to 3×10^{-5}M.

Table 7. Response to Isoproterenol in Fourth Order Canine Bronchi

Contractile Agent		n	Level of Contraction (g)	Absolute Decrease in Tension (g)	Maximal Relaxation (%)	Area Above the Curve (mm^2)
5-Hydroxytryptamine:	With Epithelium	4	EC_{40} (1.8±0.5)	1.8±0.5	100±0	762.2±31.4
	Without Epithelium	4	EC_{40} (1.8±0.3)	1.6±0.2	92±16	759.3±192.1
5-hydroxytryptamine:	With Epithelium	4	EC_{80} (2.6±0.2)	2.6±0.2	100±0a	757.1±67.6a
	Without Epithelium	4	EC_{80} (2.4±0.3)	2.3±0.2	96.1±7.8a	712.1±118.0a
5-hydroxytryptamine: (Indomethacin)	With Epithelium	3	EC_{40} (3.0±0.5)	3.5±0.6	100±0	737.8±144.0
	Without Epithelium	3	EC_{40} (2.6±0.6)	2.9±0.5	100±0	822.8±81.9
5-hydroxytryptamine: (Indomethacin)	With Epithelium	3	EC_{80} (4.1±1.5)	4.4±1.1	100±0a	699±132.2a
	Without Epithelium	3	EC_{80} (4.2±1.3)	3.8±1.0	100±0a	751.8±61.0a
5-hydroxytryptamine: (LY83583)	With Epithelium	3	EC_{40} (1.9±0.6)	1.9±0.7	95.7±8.7	772.8±91.6
	Without Epithelium	3	EC_{40} (2.3±0.4)	2.3±0.4	100±0	812±36.4
5-hydroxytryptamine: (LY83583)	With Epithelium	3	EC_{80} (2.6±1.1)	2.6±1.1	100±0a	823.8±32.0a
	Without Epithelium	3	EC_{80} (2.1±0.2)	2.2±0.2	100±0a	834±51.0a

Values are means ± SEM. a Significantly different from the values obtained for isoproterenol at the EC_{80} level for acetylcholine. EC_{40} for 5-hydroxytryptamine 10^{-7} to 10^{-6} M; EC_{80} 3 x 10^{-6} to 10^{-5} M.

Table 8. Response to Sodium Nitroprusside in Fourth Order Canine Bronchi

Contractile Agent		n	Level of Contraction (g)	Absolute Decrease in Tension (g)	Maximal Relaxation (%)	Area Above the Curve (mm^2)
Acetylcholine:	With Epithelium	5	EC_{40} (1.5±0.4)	1.0±0.3	68.7±11.5	224.95±113.1
	Without Epithelium	5	EC_{40} (2.1±0.5)	1.3±0.3	62.3±13.3	170.1±93.4
Acetylcholine:	With Epithelium	5	EC_{80} (3.1±0.7)	1.7±0.3	58.5±15.9	173.2±95.1
	Without Epithelium	5	EC_{80} (5.2±0.6)	1.5±0.2	33.8±20.5a[b]	83.8±109.0a[b]
Acetylcholine: (Indomethacin)	With Epithelium	5	EC_{40} (2.8±0.2)	2.3±0.1	84.1±11.1	295.3±113.0
	Without Epithelium	5	EC_{40} (3.2±0.8)	2.4±0.3	70.5±17.2	202.1±70.8
Acetylcholine: (Indomethacin)	With Epithelium	5	EC_{80} (6.1±0.6)	3.5±0.4	58.2±17.1	174.4±108.5
	Wihtout Epithelium	5	EC_{80} (4.6±0.7)	2.2±0.1a	50.8±12.2	141.6±64.3
Acetylcholine: (LY83583)	With Epithelium	5	EC_{40} (2.1±0.5)	1.8±0.4	87.7±8.6	370.5±130.8
	Without Epithelium	5	EC_{40} (2.6±0.5	2.1±0.3	85.4±11.2	332.7±114.8
Acetylcholine: (LY83583)	With Epithelium	5	EC_{80} (4.4±1.0)	3.1±0.6	73.0±7.8	234.6±44.4
	Without Epithelium	5	EC_{80} (3.4±0.7)	1.5±0.6a	41.6±21.2a[b]	109.0±83.2a[b]

Values are means + SEM. a Significantly different from bronchi with epithelium. [b] significantly different from values obtained at the EC_{40} level for acetylcholine. EC_{40} for acetylcholine 3×10^{-7} to 3×10^{-6} M; EC_{80}, 10^{-5} to 3×10^{-5} M.

Table 9. Response to Sodium Nitroprusside in Fourth Order Canine Bronchi

Contractile Agent		n	Level of Contraction (g)	Absolute Decrease in Tension (g)	Maximal Relaxation (%)	Area Above the Curve (mm^2)
5-hydroxytryptamine:	With Epithelium	4	EC_{40} (2.3±0.8)	2.0±0.6	84.4±13.7	306.8±126.0
	Without Epithelium	4	EC_{40} (1.2±0.3)	1.2±0.3	100±0a	462.2±177.1a
5-hydroxytryptamine:	With Epithelium	4	EC_{80} (2.7±0.9)	2.4±0.9	85.5±14.3	264.8±57.8
	Without Epithelium	4	EC_{80} (2.0±0.6)	2.0±0.5	98.0±4.0[b]	338.0±72.0[b]
5-hydroxytryptamine: (Indomethacin)	With Epithelium	4	EC_{40} (3.1±0.7)	3.8±0.8	95.8±9.3	355.0±128.9
	Without Epithelium	4	EC_{40} (3.2±0.8)	3.0±0.8	93.8±8.8	309.8±135.2
5-hydroxytryptamine: (Indomethacin)	With Epithelium	4	EC_{80} (4.8±0.8)	4.4±0.6	94.8±7.5[b]	365.3±154.2[b]
	Without Epithelium	4	EC_{80} (4.0±0.6)	3.6±0.4	90.8±13.1[b]	274.6±117.1[b]
5-hydroxytryptamine: (LY83583)	With Epithelium	3	EC_{40} (2.4±0.8)	2.3±0.9	95.2±8.3	420.5±54.3
	Without Epithelium	3	EC_{40} (2.4±0.8)	2.2±0.6	95.5±7.8	471.1±122.4
5-hydroxytryptamine:)LY83583)	With Epithelium	3	EC_{80} (3.1±1.0)	3.0±1.0	96.7±5.8[b]	449.9±106.9[b]
	Without Epithelium	3	EC_{80} (3.4±0.5)	3.4±0.5	99.2±1.4[b]	379.4±86.5[b]

Values are means ± SEM. a Significantly from values obtained for sodium nitroprusside at the EC_{40} level for acetylcholine. b Significantly different from values obtained for sodium nitroprusside at the ED_{80} level for acetylcholine. EC_{40} for 5-hydroxytryptamine 10^{-7} to 10^{-6} M; EC_{80}, 3 x 10^{-6} to 10^{-5} M.

Table 10. Responses to Forskolin in Fourth Order Canine Bronchi

Contractile Agent		n	Level of Contraction (g)	Absolute Increase in Tension (g)	Maximal Relaxation (%)	Area Above the Curve (mm^2)
Acetylcholine:	With Epithelium	5	EC_{40} (1.8±0.3)	1.0±0.3	65.0±32.3	375.0±170.3
	Without Epithelium	5	EC_{40} (1.9±0.1)	1.2±0.3	62.3±35.6	387±168.3
Acetylcholine:	With Epithelium	5	EC_{80} (4.0±0.6)	1.4±0.4	40.8±27.6	279.0±85.4
	Without Epithelium	5	EC_{80} (4.3±0.6)	1.2±0.7	30.3±41.1	282.4±107.3
5-Hydroxytryptamine:	With Epithelium	4	EC_{40} (3.0±0.5)	3.0±0.5[a]	100±0[a]	658.7±152.4[a]
	Without Epithelium	4	EC_{40} (3.1±0.4)	3.0±0.4[a]	100±0[a]	619.2±113.0[a]
5-Hydroxytryptamine:	With Epithelium	4	EC_{80} (4.1±0.9)	4.1±1.0[a]	100±0[a]	613.9±120.0[a]
	Without Epithelium	4	EC_{80} (3.5±0.9)	3.5±0.9[a]	100±0[a]	581.0±128.3[a]

Value are means ± SEM.
[a]Significantly different from values obtained for forskolin during contraction to acetylcholine.
EC_{40} for acetylcholine, 3×10^{-7} to 3×10^{-6} M; EC_{80}, 10^{-5} to 3×10^{-5} M.
EC_{40} for 5-hydroxytryptamine, 10^{-7} to 10^{-6} M; EC_{80}, 3×10^{-6} to 10^{-5} M.

CHAPTER 4

Arachidonic Acid Evokes Epithelium-Dependent Relaxations in Canine Airways

INTRODUCTION

The lung is a major site of production of prostaglandins and leukotrienes (Bahkle and Ferreira, 1985). These substances play a major role in the asthmatic response; prostaglandin E_2 is a potent bronchodilator and leukotrienes C_4 and D_4, the components of slow-reacting substance of anaphylaxis, are bronchoconstricting agents. Arachidonic acid is metabolized by bronchial smooth muscle and airway epithelium to yield products of both cyclooxygenase and lipoxygenase (Orehek et al, 1973; Smith et al, 1982; Holtzman et al,1983; Bahkle and Ferreira, 1985; Leikauf et al, 1986; Samhoun and Piper, 1986). Preliminary experiments have suggested arachidonic acid induces a relaxation of canine bronchial smooth muscle which is dependent on the presence of respiratory epithelium (Flavahan et al, 1986). Epithelium-derived metabolites of arachidonic acid have also been implicated as the source of a relaxing factor in the rabbit (Butler et al, 1987) and the guinea-pig (Tschihart et al, 1987). The present study was designed to examine the epithelium-dependency of the response to arachidonic acid in three orders of canine bronchi, and to identify the mediators of this phenomenon.

METHODS

Experiments were performed on bronchi taken from mongrel dogs of either sex (15-20kg) anaesthetized with pentobarbital sodium (30 mg/kg intravenously) and exsanguinated. Tissues were taken from the lower lung lobes. Paired rings of second, third and fourth order bronchi, with and without epithelium, were placed in organ chambers as described previously (Chapter 1). After an equilibration period of one hour, the optimal resting tension was determined for each tissue. The bronchial rings were allowed to equilibrate for a further hour prior to the administration of drugs.

Experimental Protocols

Arachidonic acid. Responses to arachidonic acid were obtained during contractions evoked by 5-hydroxytryptamine. Each bronchial ring was contracted to the concentration of 5-hydroxytryptamine giving 30% of the maximal response (EC_{30}). The EC_{30} values were obtained from concentration-effect curves for 5-hydroxytryptamine obtained in earlier work (Chapter 1). When the contractile response to 5-hydroxytryptamine had stabilized (approximately ten minutes after the addition of the agonist) a concentration-effect curve to arachidonic acid was obtained by increasing the concentration in the organ chambers cumulatively in half-log increments in the concentration-range 10^{-9}M to 10^{-5}M (Van Rossum, 1963).

Preliminary studies indicated that the response to arachidonic acid in canine bronchi was tachyphylactic. Therefore, in each experiment, single cumulative concentration-effect curves were obtained in the three orders of bronchi. In the first series of experiments, the effect of arachidonic acid alone was determined. In subsequent series, the effect of a single inhibitor was examined. Inhibitors were added to the organ chambers 30 minutes before the response to arachidonic acid was tested, and remained in contact with the tissues throughout the experiment. The exception to this protocol was nordihydroguaiaretic acid (NDGA) which was present in the bath for 40 minutes and then washed out for 30 minutes prior to the experiment. NDGA has been shown to cause reversible depression of responses to contractile agonists (Rimele and Vanhoutte, 1983; Miller and Vanhoutte, 1985) as well as irreversible depression of lipoxygenase (Showell et al, 1980; Rimele and Vanhoutte, 1983). Therefore, this procedure ensures that responses to 5-hydroxytryptamine are not impaired, whereas the lipoxygenase enzyme remains inhibited.

Concentration-effect curves for prostaglandin E_2 and prostacyclin

The bronchial rings were incubated for thirty minutes with the inhibitor of cyclooxygenase indomethacin (10^{-5}M) [to prevent the action of endogenous prostanoids (Gardiner, 1986)], and the muscarinic antagonist atropine (10^{-6}M) to prevent any effect of prostaglandins on endogenous

acetylcholine release (Shore et al, 1987). These blockers remained in contact with the tissues throughout the experiment. The bronchi were then contracted to the EC_{30} value for 5-hydroxytryptamine. When the contraction had stabilized, a concentration-effect curve to prostaglandin E_2 was obtained (10^{-11}M to 10^{-6}M). The drugs were then washed out by changing the solution in the bath at least five times, and indomethacin and atropine were added again to the organ bath. After a thirty minute incubation period, the tissue was again contracted to the EC_{30} value for 5-hydroxytryptamine, and a concentration-effect curve to prostacyclin (10^{-10} - 10^{-5}M) was obtained.

Assay of prostaglandin E_2 and I_2 production by bronchial rings.

In this series of experiments, bronchial rings were first contracted to their EC_{30} value for 5-hydroxytryptamine. When the contraction had stabilized, arachidonic acid (10^{-5}M) was added to the bath. Three samples were drawn from the organ bath for assay of prostaglandins. The first sample was taken at baseline tension, before the addition of 5-hydroxytryptamine. The second was taken during the stable contraction to 5-hydroxytryptamine (approximately ten minutes after the addition of the monoamine). The third sample was taken when the response to arachidonic acid had reached its maximum (approximately five minutes after the addition of the agonist). The sample volume was 2ml in each case. After each experiment, the tissues were removed from the organ chambers, blotted on absorbent paper, and then

weighed (Shore et al, 1985).

Measurement of prostaglandins.

The 2ml samples were applied to ODS (Bond Elut) columns, prewashed with 2ml methanol and 2ml H_20 (pH3). Purification was achieved using sequentially 2ml of acidified water, 2ml of 5% methanol and 2ml of petroleum ether. Prostaglandins were then eluted with 2ml ethylacetate. After drying under N_2 samples were resuspended in 0.1M phosphate buffer. Radioimmunoassay was performed using specific antibodies (Erbil et al, 1984). Prostaglandin E_2 and the stable breakdown product of prostacyclin, 6-keto-Prostaglandin $F_{1\alpha}$ were measured. Results are expressed as pg/mg of tissue.

Drugs

The following drugs were used: arachidonic acid, sodium salt (Nu-Check Prep, Elysian, MN); atropine sulphate (Sigma Co., St. Louis, MO); BAY G6575 (nafazatrom; Miles Pharmaceutical, West Haven, CT); dimethyl sulphoxide (DMSO; Sigma); 5-hydroxytryptamine creatinine sulphate (Sigma); indomethacin (Sigma); sodium meclofenamate (Parke, Davis and Co., Detroit, MI); nordihydroguaiaretic acid (NDGA, Sigma); papaverine hydrochloride (Sigma); prostaglandin E_2 (Sigma); prostaglandin I_2 (prostacyclin; Sigma) and tetrodotoxin (Sigma). Unless otherwise stated, drugs were prepared daily in distilled water, and kept on ice. Indomethacin was prepared with an equimolar amount of Na_2CO_3. The stock solution of prostacyclin was prepared in $NaHCO_3$. Neither

Na_2CO_3 nor $NaHCO_3$ had significant effect on the pH of the organ chamber solution in the administered amounts. NDGA and BAY G6575 were dissolved in DMSO (1%). Preliminary experiments indicated that DMSO did not affect the response to arachidonic acid.

Data analysis

Responses to arachidonic acid, prostaglandin E_2 and prostacyclin are expressed as percent reduction of the response to 5-hydroxytryptamine. The results are expressed as means $\pm$ SEM. Statistical analysis was by Student's t-test for paired or unpaired observations. P smaller than 0.05 was considered to be statistically significant. In all experiments, n equals the number of dogs from which tissues were taken.

RESULTS

General responsiveness of bronchial rings. Within each order of bronchus, there was no significant difference in active force achieved to 5-hydroxytryptamine between tissues with and without epithelium (Table 11). The inhibitors of cyclooxygenase, indomethacin and meclofenamate, and the inhibitors of lipoxygenase, NDGA and BAY G6575, had no effect on basal tension in the three orders of bronchi, but significantly augmented the response to 5-hydroxytryptamine in some tissues (Table 11). However, preliminary experiments showed that an enhanced level of contraction to 5-hydroxytryptamine did not impair responses to arachidonic acid (data not shown). In all cases, there was no significant difference in active force

achieved between tissues with and without epithelium in each order in the presence of an individual inhibitor (Table 11).

Arachidonic Acid. Arachidonic acid evoked concentration-dependent relaxations in second, third and fourth order bronchi with epithelium (Figs. 22 and 23, Table 12). For fourth order bronchi with epithelium, the maximal response achieved was significantly less than that obtained in second and third order bronchi (Fig 23, Table 12). For the three orders, tissues without epithelium showed a minimal relaxation to arachidonic acid (Figs. 22 and 23, Table 12), but relaxed completely in response to papaverine (10^{-4}M) (Fig.22). In concentrations which abolish the contractions to electrical stimulation of the cholinergic nerves, neither atropine (10^{-6}M) nor tetrodotoxin (10^{-7}M) affected the response of second order bronchi with and without epithelium to arachidonic acid (n=2; data not shown).

Inhibitors of cyclooxygenase. The relaxation responses to arachidonic acid were blocked by indomethacin (10^{-5}M, 2nd, 3rd and 4th order; n=6) (Fig. 22) and meclofenamte (10^{-6} M, 2nd and 3rd order examined only; n=6), (data not shown). All tissues relaxed to baseline tension in response to papaverine (10^{-4}M).

Inhibitors of lipoxygenase. NDGA (10^{-5}M, n=4) and BAY G6575 (10^{-5}M, n=4; except 4th order, n=3) significantly inhibited the maximal relaxation to arachidonic acid in second order bronchi with epithelium (Figs. 22,24 and 25, Table 12). The relaxations in third and fourth order

bronchi with epithelium were not significantly affected by the presence of either inhibitor of lipoxygenase (Figs.24 and 25, Table 12). NDGA significantly enhanced the maximal relaxation to arachidonic acid in second, third and fourth order bronchi without epithelium (Fig. 24, Table 12); Bay G6575 caused a significant augmentation of the response in second and third order bronchi only (Fig.25, Table 12). The overall effect of the inhibitors of lipoxygenase was to abolish the difference in relaxation responses between tissues with and without epithelium in the three orders of canine bronchi (Figs. 24 and 25, Table 12). All tissues relaxed to baseline tension in the presence of papaverine (10^{-4}M).

Prostaglandins. Prostaglandin E_2 evoked concentration-dependent relaxations in second, third and fourth order bronchi (Fig. 26). There was no significant difference between tissues with and without epithelium in the three orders, as determined by ED_{50} values (Table 13). There was no significant difference in sensitivity to prostaglandin E_2 between the three orders (Fig.26, Table 13). Prostacyclin also evoked concentration-dependent relaxations in canine bronchi (Fig. 27). There was no significant difference in response to prostacyclin between tissues with and without epithelium, as determined by ED_{50} values, nor any difference in sensitivity between the three orders (Table 13). However, all bronchi were significantly less sensitive to prostacyclin than to prostaglandin E_2 (Table 13).

Assay of prostaglandin E_2 and 6-keto-$PGF_{1\alpha}$.

The following pairs of tissues were examined: second order with and without epithelium; fourth order with and without epithelium; second order with and without epithelium in the presence of indomethacin (10^{-5}M); and second order with and without epithelium, which had been exposed to NDGA (10^{-5}M) for 40 minutes prior to the experiment and then washed.

Prostaglandin E_2. Second order bronchi released prostaglandin E_2 spontaneously. There was no significant difference in basal release between tissues with and without epithelium (Fig.28, Table 14). In tissues without epithelium, the basal release was significantly depressed by the presence of indomethacin, but not of NDGA (Fig.28, Table 14). Fourth order bronchi also showed basal release of prostaglandin E_2, which was significantly greater than that of second order bronchi (Fig.31, Table 14). There was no difference between tissues with and without epithelium (Fig.31, Table 14). The contraction to 5 -hydroxytryptamine did not significantly affect the release of prostaglandin E_2 in any of the tissues studied (Figs 28 and 31, Table 14).

The addition of arachidonic acid caused a significant rise in prostaglandin E_2 release in second and fourth order bronchi with epithelium (Figs 28 and 31, Table 14). Fourth order bronchi without epithelium also showed a significant rise in prostaglandin E_2 release in the presence of arachidonic acid, although this rise was significantly smaller than that for fourth order with epithelium (Fig.

31,Table 14). Arachidonic acid did not significantly alter the release of prostaglandin E_2 from second order bronchi without epithelium (Fig 28, Table 14).

In order to examine the direct stimulatory effect of arachidonic acid on the release of prostaglandin E_2, the value obtained during the stable contraction to 5-hydroxytryptamine was subtracted from that during relaxation to arachidonic acid (stimulated release). Second order bronchi with epithelium showed a significantly greater stimulated release of prostaglandin E_2 than tissues without epithelium (Fig.29, Table 15). Indomethacin did not significantly reduce stimulated release of prostaglandin E_2 in second order tissues with or without epithelium. In contrast, NDGA did significantly reduce the release of prostaglandin E_2 in second order bronchi with epithelium, but had no effect in the absence of epithelium (Fig. 29, Table 15). Fourth order bronchi showed significantly greater stimulated release than second order bronchi, for both tissues with and without epithelium. Fourth order with epithelium showed a significantly greater release of prostaglandin E_2 than tissues without epithelium (Table 15).

Prostacyclin. Second order bronchi released 6-keto-$PGF_{1\alpha}$ spontaneously. There was no difference between tissues with and without epithelium (Fig.30, Table 16). The basal release was not significantly affected by either indomethacin or NDGA (Fig 30, Table 16). Fourth order bronchi also showed basal release of 6-keto-$PGF_{1\alpha}$. This release was significantly greater than that for second

order tissues (Fig. 31, Table 16). Contraction to 5-hydroxytryptamine did not affect the basal release of the stable breakdown product of prostacyclin in any of the tissues studied (Fig. 30, Table 16).

The addition of arachidonic acid caused a significant rise in 6-keto-$PGF_{1\alpha}$ production in second order bronchi with and without epithelium. There was no such rise in fourth order bronchi (Figs. 30 and 31, Table 16).

The direct stimulatory effect of arachidonic acid was then estimated by subtracting basal from stimulated release of 6-keto-$PGF_{1\alpha}$. Second order bronchi with epithelium had a significantly greater 6-keto-$PGF_{1\alpha}$ release than tissues without epithelium (Fig 29, Table 15). Indomethacin, but not NDGA, reduced the stimulated release of the stable breakdown product of prostacyclin in tissues with epithelium (Fig. 29, Table 15). There was no stimulated release in fourth order bronchi.

DISCUSSION

This study demonstrates epithelium-dependent relaxations to arachidonic acid in three orders of canine bronchi, thus confirming preliminary observations made in this tissue (Flavahan et al, 1986). These relaxations are greatest in second and third order bronchi, and are significantly reduced in fourth order bronchi. The response is not affected by tetrodotoxin and atropine, ruling out the involvement of cholinergic nerves. The effect of arachidonic acid is abolished in all tissues by the addition of two different inhibitors of cyclooxygenase, indomethacin and meclofenamate. This

implies the involvement of a cyclooxygenase product of the metabolism of arachidonic acid. If this were the only mechanism involved, inhibitors of the lipoxygenase pathway should have no effect on the relaxations. This is true in third and fourth order bronchi, where two different inhibitors of lipoxygenase, NDGA and BAY G6575, did not affect the relaxation in bronchi with epithelium. However, in second order bronchi with epithelium, the relaxation was significantly reduced by both NDGA and BAY G6575. Furthermore, the addition of the two lipoxygenase inhibitors revealed a relaxation to arachidonic acid in the three orders of bronchi without epithelium. The results suggest that if the arachidonic acid metabolite involved in the relaxing response is a cyclooxygenase product, then its effect is modulated by the lipoxygenase pathway in two situations: in second order bronchi with epithelium, and in the three orders of bronchi without epithelium.

The most likely mediator for the epithelium-dependent response to arachidonic acid is prostaglandin E_2. Indeed, prostaglandin E_2 is a potent bronchodilator (Orehek et al, 1973; Yamaguchi et al, 1976; Butler et al, 1987) and it is produced in significant quantities by the canine airway epithelium (Smith et al, 1982; Leikauf et al, 1986). The present study confirms these findings. Prostaglandin D_2, which may also be released from canine airway epithelium (Eling et al, 1986), is a potent bronchoconstrictor (Wasserman et al, 1980; Tamaoki et al, 1987). Prostacyclin, although it is released from canine

bronchial smooth muscle and has bronchodilator properties (Shore et al, 1985), has not been detected in canine airway epithelium. The present study demonstrates that canine airways are considerably less sensitive to prostacyclin than to prostaglandin E_2. Assay of 6-keto-$PGF_{1\alpha}$ release from canine bronchi shows a basal release of this substance, which is similar in tissues with and without epithelium, suggesting that is is derived from the bronchial smooth muscle. In second order bronchi with epithelium, there was a significant rise in 6-keto-$PGF_{1\alpha}$ release in response to arachidonic acid. However, the increase was considerably smaller than that for prostaglandin E_2. Thus, although prostacyclin is released from canine airways, it is not likely to be a major endogenous bronchodilator in this tissue.

The present results demonstrate basal release of prostaglandin E_2 from canine bronchi. Since the amount of prostaglandin E_2 released under resting conditions is similar in tissues with and without epithelium, this implies that under basal conditions the source of the prostaglandin E_2 is the bronchial smooth muscle. Contraction to 5-hydroxytryptamine did not alter the release of prostaglandin E_2, thus demonstrating that contraction of the tissue does not increase the release of prostaglandins from the smooth muscle or the epithelium, as has been suggested (Lazarus et al, 1984; Shore et al, 1985). When arachidonic acid was added to the tissues, second order bronchi with epithelium showed a significant rise in release of prostaglandin E_2. Bronchi without

epithelium showed no significant change from basal levels. Thus, for second order tissues, arachidonic acid appears to stimulate the release of prostaglandin E_2 mainly from the epithelium. This would then initiate relaxations of the bronchial smooth muscle. The data obtained in fourth order bronchi allow similar conclusions.

The stimulated release of prostaglandin E_2 in fourth order bronchi is significantly greater than that in second order, even though the relaxation to arachidonic acid is larger in the latter. This differential response cannot be due to a difference in sensitivity to prostaglandin E_2 between the two orders, to judge from the relaxations evoked by the prostaglandin in tissues without epithelium. A further complication is that although indomethacin, a potent and specific inhibitor of cyclooxygenase (Bahkle and Ferreira, 1985), abolished basal release of prostaglandin E_2 in second order bronchi, it did not affect stimulated release by arachidonic acid. However, the inhibitors of cyclooxygenase, indomethacin and meclofenamate, abolished the epithelium-dependent relaxing response to arachidonic acid. In cultured canine tracheal epithelial cells, indomethacin attenuates, but does not abolish, prostaglandin E_2 production in response to the calcium ionophore, A23187 (Welsh, 1987). Epithelium-dependent relaxations to arachidonic acid, which are abolished by indomethacin, have been demonstrated in the intrapulmonary bronchi of the rabbit (Butler et al, 1987). It was also demonstrated that, in rabbit airways, there is basal release of prostaglandin E_2 from the epithelium;

this release is abolished by indomethacin (Butler et al, 1987). These results are in agreement with the present study. Unfortunately, the effect of arachidonic acid on prostaglandin production, and the effect of indomethacin on stimulated release of prostaglandin E_2, were not determined. Thus, a comparison cannot be made between the airways of the rabbit and the dog. The present results suggest that although arachidonic acid stimulates the release of prostaglandin E_2 from canine bronchi, it is not the only factor responsible for the relaxation.

One factor which may be involved is the production of leukotriene C_4 and D_4 from the canine airway epithelium (Eling et al, 1986). These substances stimulate the release of prostaglandin E_2 from canine tracheal eptihelial cells (Leikauf et al, 1986). In second order tissues with epithelium (where the inhibitors of lipoxygenase NDGA and BAY G6975 reduced the relaxations to arachidonic acid) NDGA reduced the release of prostaglandin E_2. Thus, in the present experiments, exogenous arachidonic acid may be metabolized to leukotrienes via the lipoxygenase pathway. These substances (most likely leukotriene C_4 and leukotriene D_4) would then stimulate the release of prostaglandin E_2 and thus cause a relaxation. In the presence of an inhibitor of lipoxygenase, a major stimulus to the release of prostaglandin E_2 would be lost and the relaxation to arachidonic acid would be reduced correspondingly. The lack of effect of indomethacin on the release of prostaglandin E_2 then might be explained partially in the following way: inhibition of

cyclooxygenase would cause diversion of arachidonic acid to the lipoxygenase pathway, and thus stimulate an increased production of leukotrienes (Bahkle and Ferreira, 1985). This in turn would stimulate release of prostaglandin E_2, either by a direct effect on de novo synthesis of prostaglandin, or possibly by release of intracellular stores of cyclooxygenase, which cannot be reached by indomethacin. In addition, indomethacin may have a direct potentiating effect on lipoxygenase (Randall et al, 1980; Harvey and Osbourne,1983) which may further stimulate the production of leukotrienes. This mechanism, if present, probably only exists in the second order bronchi, as third and fourth order bronchi with epithelium show no significant effect of either lipoxygenase inhibitor on relaxing responses to arachidonic acid. In the case of the fourth order bronchi, leukotrienes generated in both the smooth muscle and the epithelium may have a direct contractile effect, thus counterbalancing the effect of prostaglandin E_2 and reducing the relaxation. Leukotrienes are known to have a more potent bronchoconstrictor effect in smaller airways (Samhoun and Piper,1986), although canine airways have been shown to be less responsive to the leukotrienes than bronchi of other species (Bahkle and Ferreira, 1985). This may explain why arachidonic acid does not induce a contraction in smaller airways in the presence of indomethacin.

In tissues without epithelium, exogenous arachidonic acid may stimulate the production of both leukotrienes and prostaglandins. If these two processes were in balance

there would be no apparent effect of arachidonic acid. When inhibitors of lipoxygenase are added, the major metabolite of arachidonic acid formed is prostaglandin E_2, and relaxation results.

Thus, metabolism of arachidonic acid by the airway epithelium has a profound and complex effect on bronchial smooth muscle. The exact mechanisms of this effect remain to be elucidated.

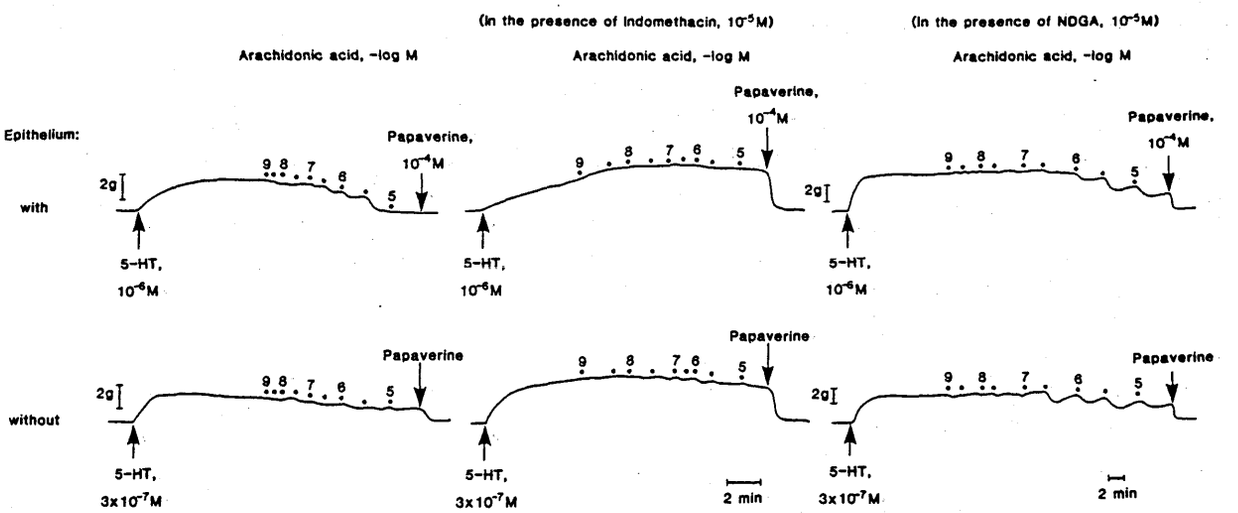

Figure 22: Representative tracings of the effect of arachidonic acid on paired rings of second-order bronchi. Each ring was contracted to its EC_{30} value for 5-hydroxytryptamine (5-HT). Top: tissues with epithelium. Bottom: tissues without epithelium; Left: response to arachidonic acid in control rings; Middle: effect of indomethacin (10^{-5}M) on responses to arachidonic acid; Right: effect of nordihydroguaiaretic acid (NDGA, 10^{-5}M) on responses to arachidonic acid.

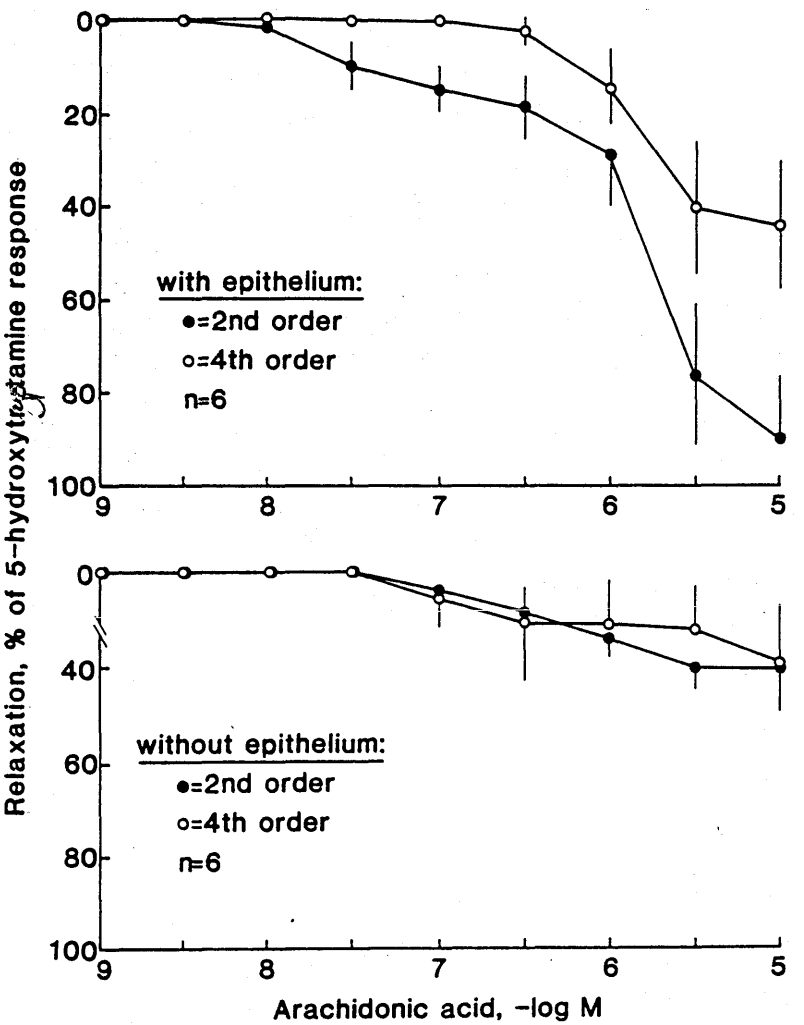

Figure 23: The effect of epithelium-removal on the concentration-effect curve to arachidonic acid in second-order and fourth-order canine bronchi. Rings were contracted using the EC_{30} value for 5-hydroxytryptamine. Data (means $\pm$ SE) are expressed as percent relaxation of the response to 5-hydroxytryptamine. Top: rings with epithelium. Bottom: rings without epithelium.

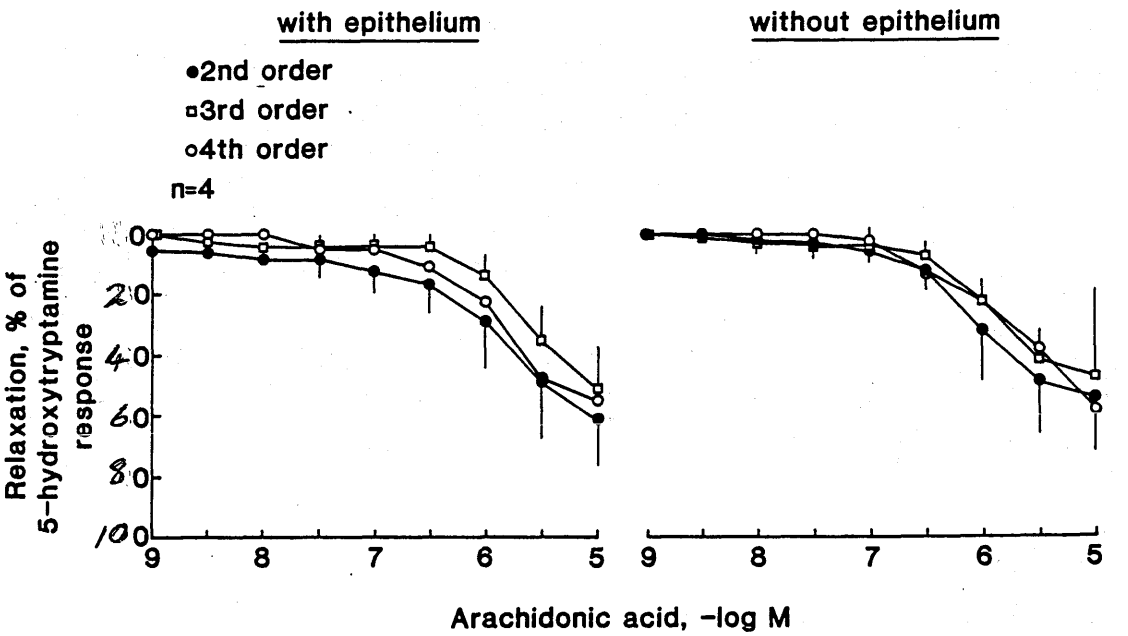

Figure 24: The effect of nordihydroguaiaretic acid (NDGA, 10^{-5}M) on the concentration-effect curve to arachidonic acid in second-, third-, and fourth-order bronchi. Data (means ± SE) are expressed as percent relaxation of the response to 5-hydroxytryptamine. Left: tissues with epithelium. Right: tissues without epithelium.

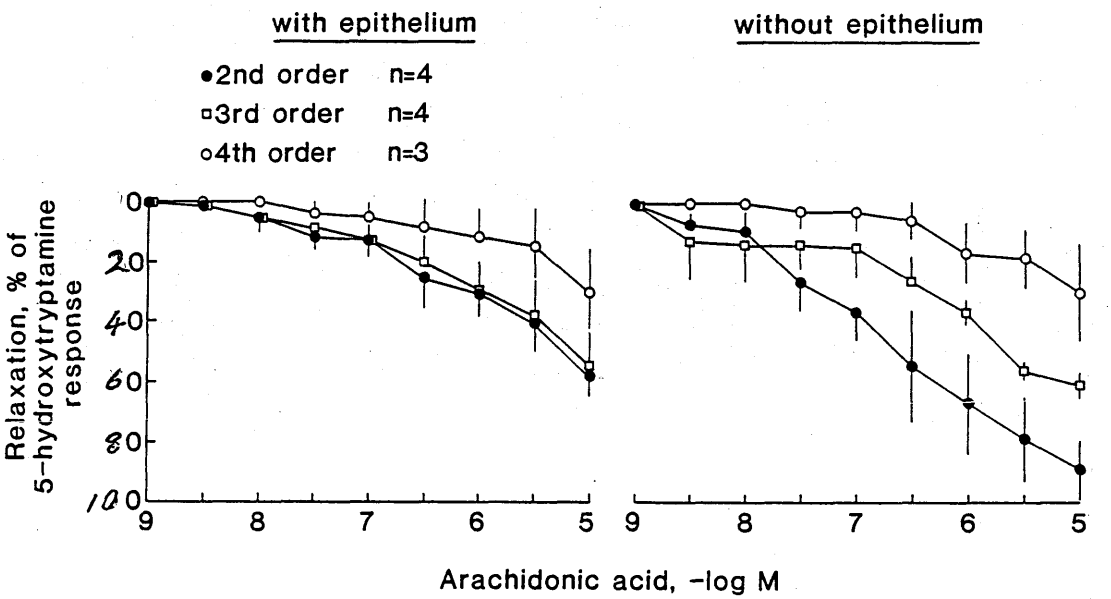

Figure 25: The effect of BAYG6575 (nafazatrom, 10^{-5}M), on the concentration-effect curve to arachidonic acid in second-, third-, and fourth-order bronchi. Data (means ± SE) are expressed as percent relaxation of the response to 5-hydroxytryptamine. Left: tissues with epithelium. Right: tissues without epithelium.

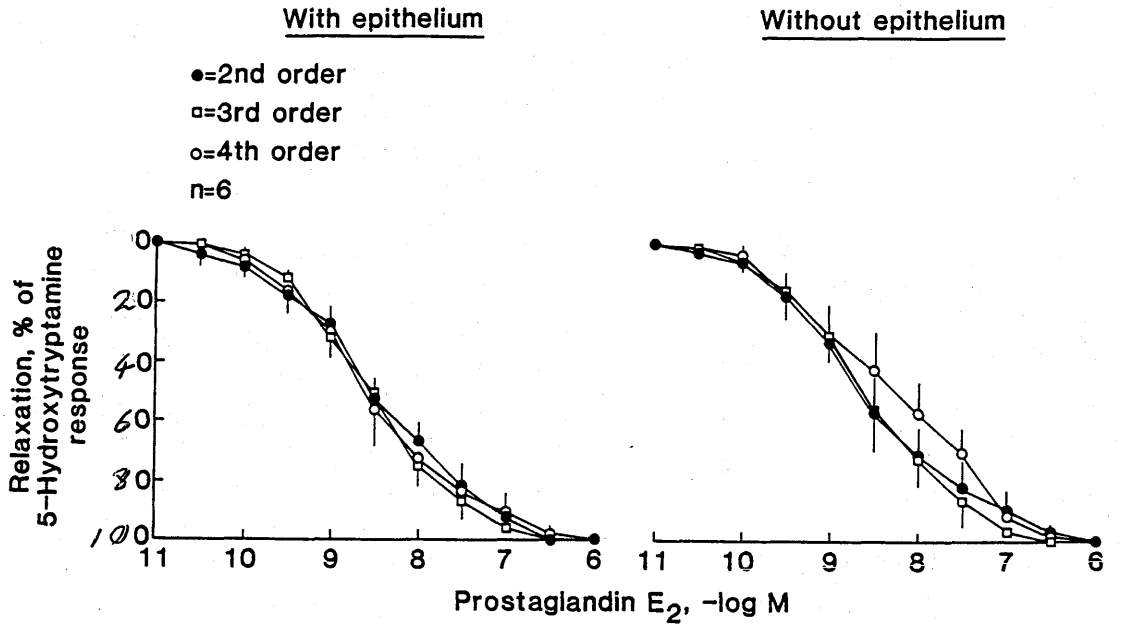

Figure 26: Responses to prostaglandin E_2 in second-, third-, and fourth-order canine bronchi. Each ring was contracted to its $E\hat{C}_{30}$ value for 5-hydroxytryptamine. Data (means ± SE) are expressed as percent relaxation of the response to 5-hydroxytryptamine. Left: tissues with epithelium. Right: tissues without epithelium.

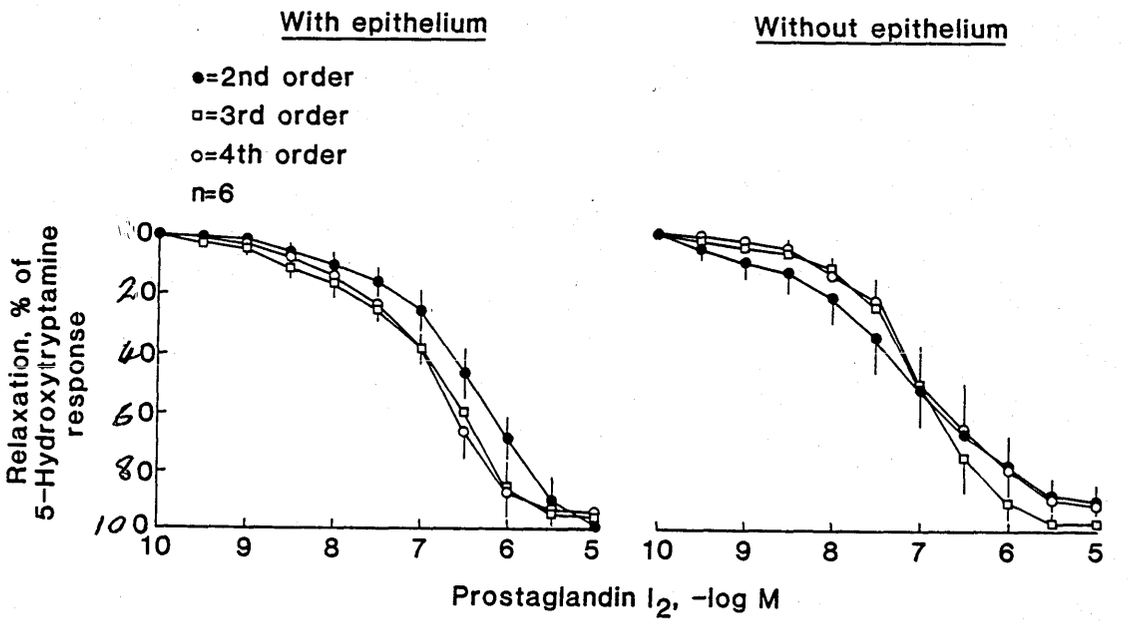

Figure 27: Responses to prostacyclin (prostaglandin I_2) in second-, third-, and fourth-order canine bronchi. Each ring was contracted to its EC_{30} value for 5-hydroxytryptamine. Data (means $\pm$ SE) are expressed as percent relaxation of the response to 5-hydroxytryptamine. Left: tissues with epithelium. Right: tissues without epithelium.

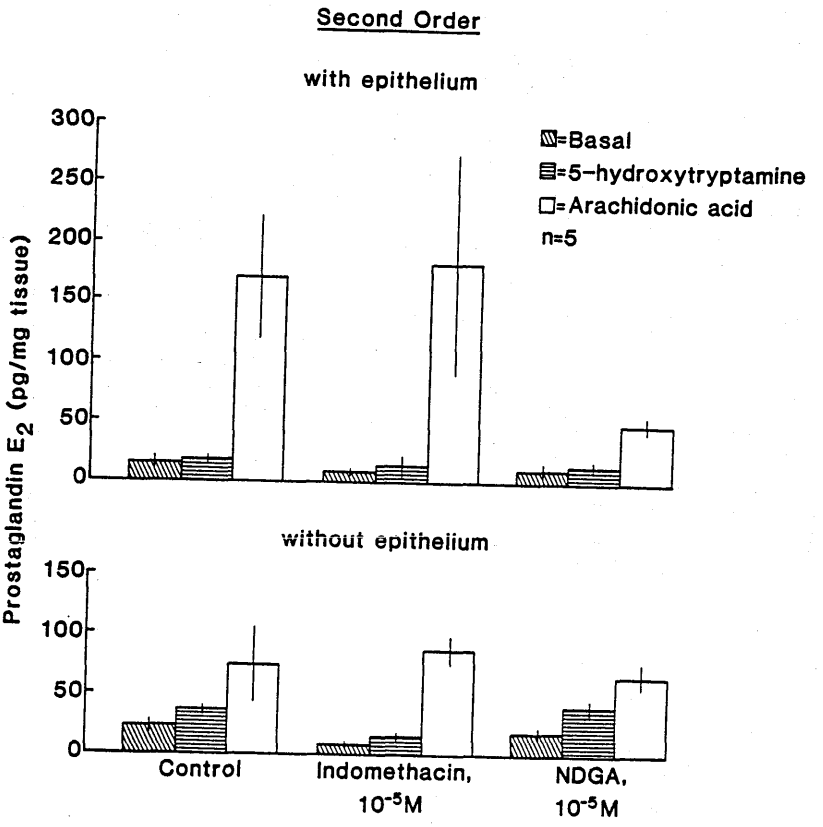

Figure 28: Release of prostaglandin E_2 from second-order canine bronchi under basal conditions, during contractions to 5-hydroxytryptamine (EC_{30}), and after addition of arachidonic acid (10^{-5}M). Data are means $\pm$ SE. Top: rings with epithelium. Bottom: rings without epithelium. NDGA, nordihydroguaiaretic acid.

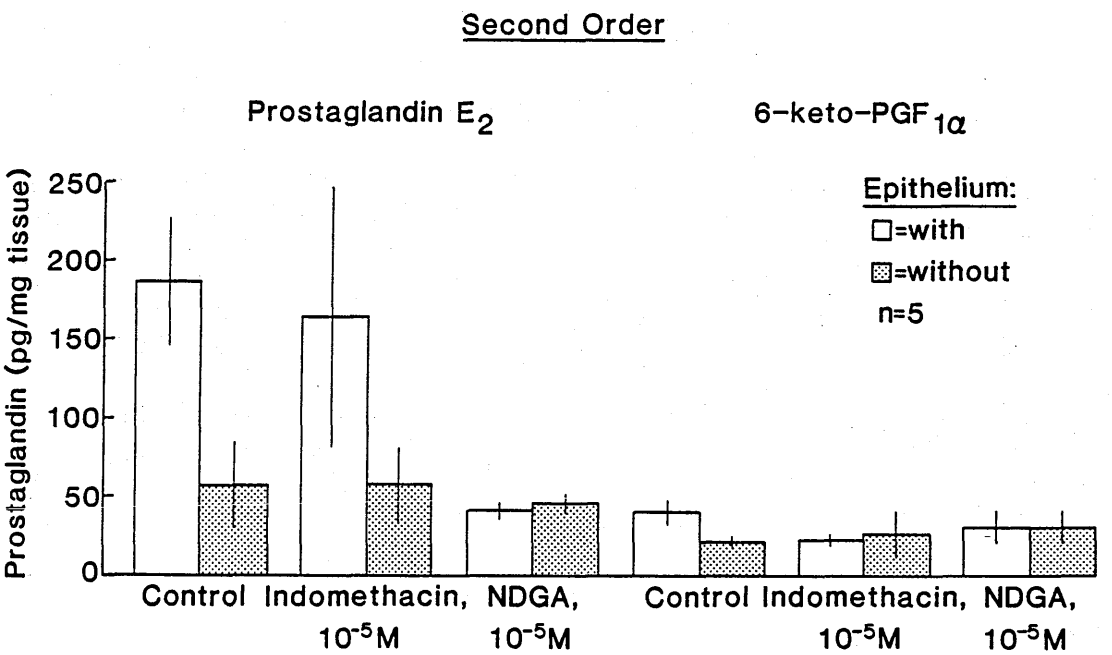

Figure 29: The effect of arachidonic acid (10^{-5}M) on prostaglandin release from second-order canine bronchi; and modification of this response by indomethacin (10^{-5}M) and nordihydroguaiaretic acid (NDGA, 10^{-5}M). Data are means ± SE. Left: prostaglandin E_2. Right: 6-keto-$PGF_{1\alpha}$ (the breakdown product of prostacyclin).

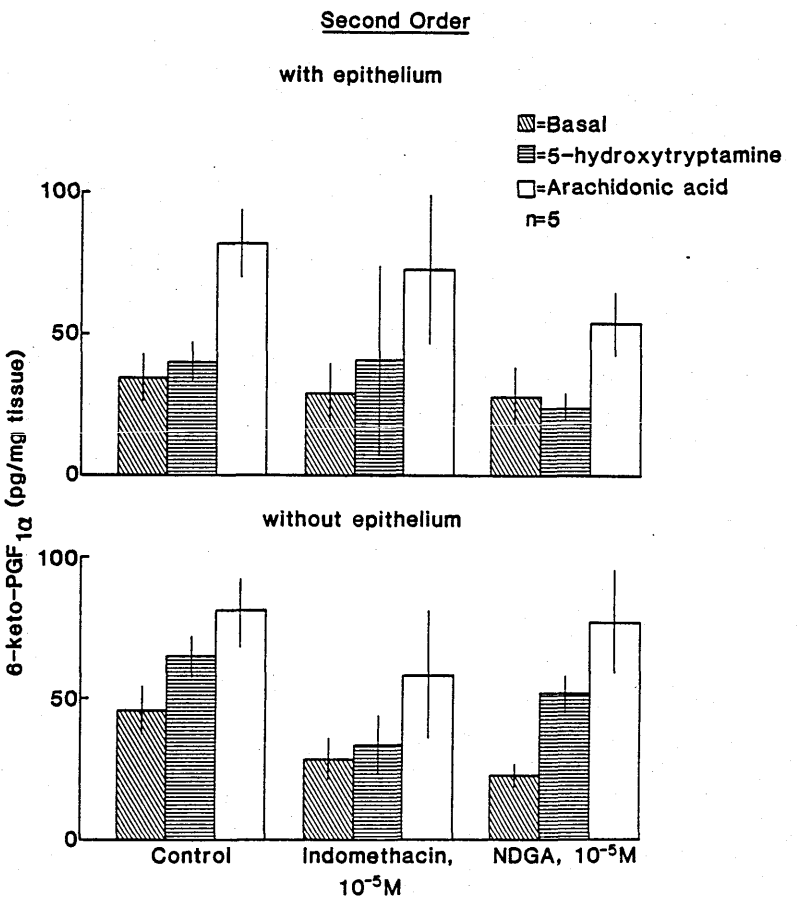

Figure 30: Release of 6-keto-$PGF_{1\alpha}$ from second-order canine bronchi under basal conditions, during contraction to 5-hydroxytryptamine (EC_{30}), and after addition of arachidonic acid (10^{-5}M). Data are means ± SE. Top: rings with epithelium. Bottom: rings without epithelium.

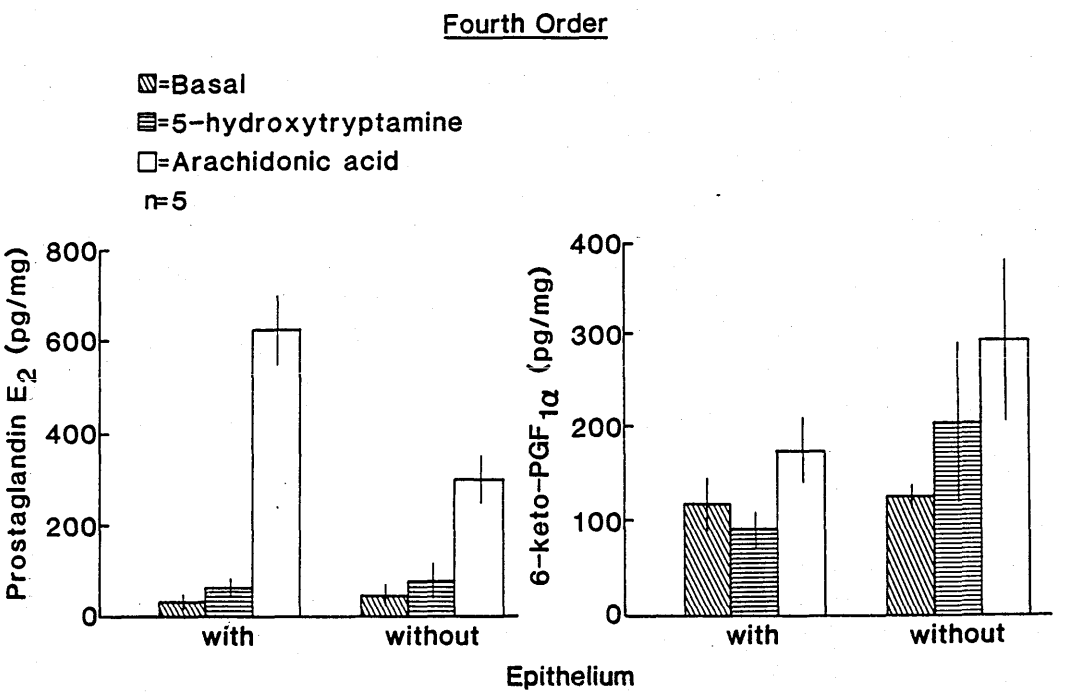

Figure 31: Release of prostaglandins from fourth-order canine bronchi under basal conditions, during contraction to 5-hydroxytryptamine (EC_{30}), and after addition of arachidonic acid. Left: prostaglandin E_2. Right: 6-keto-$PGF_1\alpha$. Data are means $\pm$ SE.

Table. 11. General Responsiveness of tissues: effect of inhibitors of arachidonic acid metabolism.

	Response to EC_{30} 5-Hydroxytryptamine, g				
	Control	Indomethacin (10^{-5}M)	Meclofenamate (10^{-6}M)	NDGA (10^{-5}M)	BAY G6575 (10^{-5}M)
Second Order					
With Epithelium	2.5±0.4	6.7±1.2[a]	5.8±0.9[a]	5.9±1.4[a]	3.2±0.5
Without Epithelium	2.1±0.4	6.5±0.8[a]	4.7±0.9[a]	4.7±0.8[a]	2.3±0.6
Third Order					
With Epithelium	2.6±0.6	5.0±0.9	3.9±0.8	4.3±0.9	2.5±0.6
Without Epithelium	2.1±0.4	4.2±0.7[a]	3.8±0.8[a]	3.8±0.5[a]	2.0±0.8
Fourth Order					
With Epithelium	1.7±0.3	2.3±0.6		1.9±0.2	2.9±0.4[a]
Without Epithelium	1.3±0.3	2.5±0.3[a]		1.8±0.5	3.1±0.7[a]

Values are means + SE; n = 6 for control, indomethacin, and meclofenamate; n = 4 for nordihydroguaiaretic acid (NDGA); n = 4 for second and third-order BAY G6575; and n = 3 for fourth-order BAY G6575. EC_{30}, 30% effective dose. [a]Significantly different from tissues without inhibitors of arachidonic acid metabolism.

Table 12. Effect of epithelium removal and inhibitors of lipoxygenase on responses to arachidonic acid in canine bronchi

	Relaxation of Response to 5-Hydroxytryptamine, %		
	Control	NDGA (10^{-5} M)	BAY G6575 (10^{-5} M)
Second Order			
With Epithelium	90.0±10.4	61.2±14.3[b]	57.9±6.4[b]
Without Epithelium	20.4±8.0[a]	54.6±15.6[b]	8.8.8±9.5[b]
Third Order			
With Epithelium	78.6±11.9	51.0±14.6	54.2±10.8
Without Epithelium	23.9±11.9[a]	64.6±14.6[b]	60.7±4.2[b]
Fourth Order			
With Epithelium	44.4±13.4[c]	55.0±17.5	30.0±10.7
With Epithelium	19.5±12.5[a]	58.4±13.1[b]	29.5±16.4

Values are means ±SE; n = 6 for control, n = 4 for nordihydroguaiaretic acid (NDGA), n = 4 for second- and third order BAY G6575, and n = 3 for fourth-order BAY G6575. [a] Significantly different from tissues with epithelium. [b] Significantly different from control tissues. [c] Significantly different from second- and third-order bronchi with epithelium.

Table 13. Prostaglandin E_2 and prostacyclin in canine bronchi with and without epithelium

	Log EC_{50} Values for Prostaglandin E_2		Log EC_{50} Values for Prostacylin	
	With Epithelium	Without Epithelium	With Epithelium	Without Epithelium
Second Order	8.4±0.2	8.5±0.2	6.4±0.2[a]	7.0±0.4[a]
Third Order	8.6±0.1	8.4±0.3	6.7±0.2[a]	6.9±0.2[a]
Fourth Order	8.5±0.3	8.2±0.3	6.8±0.2[a]	7.0±0.3[a]

Values are means ± SE; n = 6. [a] Significantly different from 50% effective dose (EC_{50}) value for prostaglandin E_2.

Table. 14. Prostaglandin E_2 release

	Basal	During Contractions to 5-Hydroxytryptamine	During Relaxation to Arachidonic Acid
	Second Order		
Control			
With Epithelium	12.8±3.3	13.9±3.1	171.5±42.0[a]
Without Epithelium	24.0±5.9	36.1±2.5	75.7±33.7[b]
Indomethacin			
With Epithelium	8.8±8.2	15.4±13.7	181.6±79.3[a]
Without Epithelium	6.2±3.0[d]	13.2±4.7	89.8±24.8[b]
NDGA			
With Epithelium	8.5±2.7	12.9±2.7	47.8±8.4[a,c]
Without Epithelium	19.1±6.8	40.5±8.7	66.3±11.6
	Fourth Order		
With Epithelium	39.3±11.9[e]	51.2±3.8[e]	626.7±124.0[a,e]
Without Epithelium	41.3±21.5[e]	77.3±34.7[e]	306.06±50.2[a,b,e]

Values are means + SE in pg/ml; n = 5, NDGA, nordihydroguaiaretic acid. [a] Significantly different from values before arachidonic acid. [b] Significantly different from tissues with epithelium. [c] Significantly different from control and indomethacin-treated tissues with epithelium. [d] Significantly different from control and NDGA-treated tissues without epithelium. [e] Significantly different from second-order bronchi.

Table. 15. Prostaglandin release stimulated by arachidonic acid.

	Second Order		Fourth Order
	PGE_2	6-keto-$PGF_{1\alpha}$	PGE_2
Control			
With Epithelium	157.6±41.6	41.5±7.1	573.5±123.2[b]
Without Epithelium	55.3±27.8[a]	19.5±6.7[a]	229.0±31.5[a,b]
Indomethacin			
With Epithelium	161.6±82.0	21.6±9.2[d]	
Without Epithelium	80.9±27.1[a]	25.2±19.6	
NDGA			
With Epithelium	38.1±6.4[c]	31.5±12.7	
Without Epithelium	40.2±6.9	32.0±11.9	

Values are means ± SE in pg/ml; n=5. PGE_2, prostaglandin E_2; 6-keto-$PGF_{1\alpha}$ 6-ketoprostaglandin $F_{1\alpha}$; NDGA, nordihydroguaiaretic acid. [a]Significantly different from tissues with epithelium. [b]Significantly different from second-order bronchi. [c]Significantly different from control and indomethacin-treated tissues with epithelium. [d]Significantly different from control and NDGA-treated tissues.

Table. 16. 6-keto-$PGF_{1\alpha}$ release

	Basal	During Contraction to 5-Hydroxytryptamine	During Relaxation to Arachidonic Acid
	Second Order		
Control			
With Epithelium	33.9±8.8	39.2±7.5	80.7±13.5[a]
Without Epithelium	45.1±9.0	64.3±7.8	81.0±11.2[a]
Indomethacin			
With Epithelium	29.7±10.2	50.7±34.5	73.7±39.2
Without Epithelium	28.7±6.6	33.2±10.0	57.2±22.7
NDGA			
With Epithelium	28.2±9.11	23.3±5.8	54.2±12.0
Without Epithelium	22.0±4.0	51.6±6.8	75.9±19.4
	Fourth Order		
Control			
With Epithelium	119.8±32.2[b]	94.2±22.4[b]	175.5±35.7[b]
Without Epithelium	125.2±16.3[b]	211.8±87.3[b]	297.4±98.2[b]

Values are means ± SE in pg/ml; n = 5. See Table 15 footnote for definition of abbreviations. [a]Significantly different from values before arachidonic acid. [b]Significantly different from second-order bronchi.

GENERAL DISCUSSION AND CONCLUSIONS

These studies demonstrate that the airway epithelium modifies the responses of bronchial smooth muscle to various pharmacological agents. The effect of epithelium -removal shows variation along the respiratory tree. There is also some interspecies variation. It is proposed that the epithelium releases various relaxing and contracting factors, which can exert a profound influence on airway tone.

Heterogeneity in the effects of epithelium-removal may reflect heterogeneity in the production and release of these factors. However, several other hypotheses may be put forward to explain the results of this study. These hypotheses, and the evidence for the existence of epithelium-derived factors, will be discussed. Theories concerning their release and mechanism of action will be presented. The clinical implications of these theories, and future directions for this field of research, will then be examined.

Evidence for the existence of epithelium-derived relaxing factors.

1. Damage to the smooth muscle

One possible explanation for the increased sensitivity of the bronchus to contractile agents, and reduced response to relaxing agents, upon epithelium-removal, might be that mechanical rubbing of the tissue has damaged the smooth muscle. Evidence against this explanation is that, for canine bronchi, the response to potassium chloride is not affected by epithelium-removal

(Chapter 1). In addition, the maximal response to contractile agents is similar in tissues with and without epithelium (Flavahan et al, 1985; Gao and Vanhoutte, 1988; Chapter 1). For the smallest canine airways, where rubbing of the tissue might be expected to cause the greatest damage, there was no significant effect of epithelium-removal on the response to contractile agonists (Chapter 1). In the bronchi of the pig, the maximal responses to electrical stimulation, and histamine, were not affected by the presence or absence of the epithelium (Chapter 2). For both species, histological examination of the bronchial rings at the end of each experiment confirmed that the smooth muscle layer was undisturbed (Fig 3, Chapter 1). Taken together, these data strongly imply that the increased sensitivity of bronchial smooth muscle in the absence of the epithelium cannot be due to damage.

Further confirmation of this view comes from electrophysiological studies (Gao and Vanhoutte, 1988). These demonstrate that, in canine bronchial smooth muscle cells, the resting membrance potential is unaltered by removal of the epithelium (Gao and Vanhoutte 1988). Similarly, the depolarisation of these cells in response to potassium is not affected by the presence or absence of the epithelium (Gao and Vanhoutte, 1988). This is a further indication that mechanical rubbing of the tissue does not affect the integrity of the cell membrane, or cause damage to the contractile mechanisms.

2. The loss of a diffusion barrier

A second explanation for these findings might be that removing the epithelium has revealed a larger area of smooth muscle for interaction with agonists. This mechanism has been suggested for guinea-pig trachea (Holroyde 1986). The results of the present study argue against such an interpretation.

The evidence is as follows:-

a) The heterogeneity in the effect of epithelium-removal on the response to contractile agents. If the results could be explained by the removal of a diffusion barrier, then the leftward shift of the concentration-effect curve to contractile agonists would occur for all agents in all preparations where the epithelium was removed.

However, there is no leftward shift of the concentration-effect curve to potassium chloride in the dog (Chapter 1), the pig (Chapter 2), the cow (Barnes et al, 1985) or the guinea-pig (Holroyde 1986). For the guinea-pig trachea, epithelium-removal enhances the contraction to leukotrienes C_4 and D_4 but not leukotriene E_4 (Hay et al, 1987a). The fourth order bronchi of the dog show no shift of the concentration-effect curve to any contractile agent (Chapter 1). The influence of the epithelium on airway smooth muscle contraction shows considerable inter-species variation. In the dog, the effect of epithelium-removal on contraction diminishes with airway diameter (Chapter 1). This is not so in the pig (Chapter 2). For the rabbit, epithelium-removal enhances the response to methacholine and histamine in second order bronchus, but

has no effect on the trachea, i.e., in this species, the influence of the epithelium on contraction increases as airway size is reduced (Raeburn et al, 1986). Such complex heterogeneity cannot be explained by the loss of a diffusion barrier.

(b) The response to isoproterenol. In the bronchi of the dog and the pig, the relaxation to isoproterenol is considerably reduced in the absence of the epithelium (Chapters 1,2 and 3). Clearly, this effect cannot be due to loss of a diffusion barrier. Furthermore, the contribution of the epithelium to isoproterenol-induced relaxation shows considerable heterogeneity. In the dog, the epithelial component of the response is greatest in fourth order bronchi; in the pig, the distribution is more uniform (Chapters 1 and 2). The results obtained in fourth order bronchi demonstrate that the influence of the epithelium is very specific (Chapter 3). In the presence of a contraction to 5-hydroxytryptamine, or in the presence of a low concentration of acetylcholine, epithelium-removal does not affect the response to isoproterenol. When a high concentration of acetylcholine is used to induce tone, the relaxation to the beta-adrenergic agonist is maintained in tissues with epithelium, and is markedly attenuated in tissues without epithelium (Chapter 3). The data suggest that the epithelium modulates beta-adrenergic relaxation in specific circumstances, probably via the release of a relaxing factor(s). Indeed, the heterogeneity of the modulatory effects of the epithelium between

pharmacological agents, between orders of bronchi, and between species, argues against a simple diffusion barrier.

3. Enzymatic breakdown of pharmacological agents by the epithelium

It has been suggested that the airway epithelium contains enzymes which break down exogenously added substances, thereby reducing their effect on the bronchial smooth muscle (Farmer et al, 1986; Advenier et al, 1988; Frossard et al, 1988). For example, contractions of the guinea-pig trachea to the tachykinins, substance P and neurokinin A, are augmented by removal of the epithelium, or by addition of phosphoramidon, an inhibitor of the enzyme, metalloendopeptidase (Frossard et al, 1988). This enzyme degrades tachykinins (Shore and Drazen, 1988), and is thought to be present in airway epithelial cells (Frossard et al, 1988). This implies that the epithelium functions as a site of enzymatic breakdown of the neuropeptides, which are potent contractile agents in the airways of several species (Lundberg et al, 1982; Barnes, 1986; Sekizawa et al, 1987). Removal of the epithelium therefore augments the response to these substances, at least in the guinea-pig trachea.

Enzymatic degradation of administered drugs cannot explain the present results, however. In canine airways, the acetylcholinesterase inhibitor ecothiopate causes a large leftward shift of the concentration-effect curve for acetylcholine. Nevertheless, the difference between tissues with and without epithelium remains unaltered

(Flavahan et al, 1985). Similar results are obtained for the monoamine oxidase inhibitor pargyline in the case of 5-hydroxytryptamine (Flavahan and Vanhoutte, 1985). Thus the canine airway epithelium is not acting as a site of drug metabolism for these contractile agents.

The role of the epithelium in the degradation of relaxing agents is more controversial. Some workers have demonstrated that, for the guinea-pig trachea, the relaxation to adenosine shows a significant leftward shift when the epithelium is removed (Holroyde, 1986; Advenier et al, 1988). In the presence of dipyridamole, the inhibitor of adenosine uptake, tissues with epithelium show a considerably augmented reponse to adenosine, such that there is no longer a difference between tissues with and without epithelium (Advenier et al, 1988). This means that the epithelium is acting as a site of uptake and metabolism of this drug, thereby reducing its action on the smooth muscle (Advenier et al, 1988). However, other workers have reported that there is no difference between tissues with and without epithelium in the response to adenosine, and that in the presence of dipyridamole and erythro-9-2-hydroxy-3-nonyl adenine (EHNA, the inhibitor of adenosine deaminase), epithelium-removal augments the relaxation (Farmer et al, 1986). These authors interpret their results to mean that adenosine releases an epithelium-derived contracting factor (Farmer et al, 1986). Thus, using the same drugs in apparently similar circumstances has produced

conflicting results and led to very different conclusions.

A similar problem arises with regard to isoproterenol. It has been reported that in the guinea-pig trachea, removal of the epithelium augments the relaxation to the beta-adrenergic agonist (Holroyde, 1986; Farmer et al, 1986). Farmer et al found that corticosterone, an inhibitor of extraneuronal uptake of catecholamines, abolished the difference between tissues with and without epithelium. They proposed that the epithelium represents a site of extraneuronal uptake for isoproterenol, thus reducing its action on the smooth muscle (Farmer et al, 1986). However, in canine and porcine airways, relaxations to the beta-adrenergic agonist were obtained in the presence of cocaine and hydrocortisone, inhibitors of neuronal and extraneuronal uptake respectively (Chapters 1,2 and 3). In the dog, responses to isoproterenol are unaffected by the presence or absence of these inhibitors (Chapter 3). This means that epithelial metabolism of isoproterenol does not play a role in the relaxation to this agonist in these airways. Furthermore, Goldie et al, also using guinea-pig trachea, have found that epithelium-removal reduces responses to isoproterenol (Goldie et al, 1986). This contradicts the findings of other workers in this tissue (Farmer et al, 1986; Holroyde, 1986).

The interpretation of the results obtained by Farmer et al in the guinea-pig trachea are complicated by the fact that responses to isoproterenol were obtained either

on basal tone, or at comparatively modest levels of cholinergic activation (Farmer et al, 1986). Goldie et al studied the effects of isoproterenol in tissues which had been contracted with a supra-maximal concentration of carbamylcholine chloride (Goldie et al, 1986). The present results demonstrate that the level of contraction achieved to muscarinic agonists is crucial when considering the influence of the epithelium on the relaxation of smooth muscle (Chapter 3). The same may be true of the guinea-pig. Hence Goldie et al, using a maximal concentration of a muscarinic agonist, obtained results similar to those in the bronchi of the dog and the pig (Chapter 1,2 and 3), whereas Farmer et al may have used concentrations of methacholine which were too low to observe epithelium-related phenomena (Farmer et al, 1986). From this evidence, it is not clear what contribution epithelial metabolism of isoproterenol may make to the relaxation to this agonist.

Differences in contraction level cannot explain the conflicting observations for adenosine, however, as the responses were obtained on basal tone (Farmer et al, 1986; Advenier et al, 1988). The cause of this discrepancy remains unclear. The guinea-pig trachea may be an unpredictable tissue, as it has a high degree of inherent tone, due to the production of prostaglandin D_2 (Gardiner, 1986). Individual preparations vary in their degree of basal contraction, and this hampers the study of epithelium related phenomena (Goldie et al, 1986). Overall the case for enzymatic breakdown of relaxing agents by the epithelium remains unproven.

4. The Response to Arachidonic Acid.

Chapter 4 demonstrates that canine bronchi exhibit relaxations to arachidonic acid which are dependent on the presence of the epithelium. Similar observations have been made in other species [the guinea pig (Farmer et al, 1987; Nijkamp and Folkerts, 1987; Tschirhart et al, 1987; and Braunstein et al, 1988;) and the rabbit (Butler et al, 1987)]. Prostaglandin E_2, derived from the airway epithelial cells, is the most likely mediator of this phenomenon (Chapter 4; Butler et al, 1987; Braunstein et al, 1988). These observations reveal two fundamental points. Firstly, they establish that substances released from the airway epithelium may diffuse towards the bronchial smooth muscle and alter its contractile tone. Indeed, prostaglandin E_2 is released preferentially in a submucosal direction in canine tracheal epithelium (Welsh, 1987). From this evidence, it might be suggested that prostaglandin E_2 is the epithelium-derived relaxing factor (e.g. Butler et al, 1987; Braunstein et al, 1988). However, the inhibitor of cyclooxygenase, indomethacin, does not affect the epithelial modulation of the response to contractile agents in the cow (Barnes et al, 1985), the guinea-pig (Holroyde, 1986) or the dog (Flavahan et al, 1985). The response to isoproterenol is also unaffected by indomethacin (Chapter 3). Thus prostaglandin E_2 is not involved in these responses. This illustrates the second and most important point, which is that although prostaglandin E_2 is an inhibitory substance derived from

the epithelium, it is not the epithelium-derived relaxing factor.

It appears from the foregoing discussions, that the data could be best explained if the epithelium were to release a relaxing factor, which diffuses towards the bronchial smooth muscle, and profoundly affects the response of the airways to pharmacological agents. Variations in the release, or the effect of these factors occur along the respiratory tree.

The Release and Mechanism of action of the epithelium-derived relaxing factor

1. Basal Release

The evidence presented in Chapters 1 and 2 suggest that there is a continuous basal release of the epithelium-derived relaxing factor, which exerts a tonic restraint on the bronchial smooth muscle, thus attenuating the response to contractile agents. In the dog, this basal release is seen only in the larger airways; fourth order bronchi are unaffected. The pig also shows basal release of a relaxing factor. However, although there are variations in the release or effect of the factor along the porcine respiratory tree, the heterogeneity is not as marked as in the dog. For both species, the basal release of relaxing factor is non-

specific in nature, as the degree of shift of the concentration-effect curve is similar for the various contractile agents. It is not clear how such a factor may exert its effect. It is interesting that, in all species studied so far, the contractile response to potassium is not affected by epithelium-removal [the dog (Chapter 1); the pig (Chapter 2); the cow (Barnes et al, 1985) or the guinea-pig (Goldie et al, 1986)]. Electrophysiological studies in canine tracheal smooth muscle show that potassium induces a graded depolarisation of the smooth muscle cell, which is independent of the presence or absence of the epithelium (Gao and Vanhoutte, 1988). By contrast, the degree of depolarisation achieved for a given concentration of acetylcholine is significantly greater when the epithelium is removed (Gao and Vanhoutte, 1988). Taken together, these data suggest that the epithelium-derived factor exerts its effect in the presence of substances which act via receptor-operated mechanisms.

This observation may provide clues to the mechanism of action of the basally-released relaxing factor. Potassium induces contraction of canine tracheal smooth muscle via electromechanical coupling, i.e. depolarisation of the cell is directly related to the influx of extracellular calcium, and the consequent activation of the contractile proteins (Giembycz and Rodger, 1987). However, acetylcholine, 5-hydroxytryptamine (and probably histamine) induce

contraction via pharmacomechanical coupling, i.e. stimulation of the receptor activates a second messenger system which ultimately results in contraction (Giembycz and Rodger, 1987; Torphy, 1987). Unlike potassium, these events can be independent of membrane potential and seem to depend on the mobilisation of intracellular calcium stores (Giembycz and Rodger, 1987; Torphy, 1987). The second messengers modulating the response to contractile agents are most likely the inositol phosphates (Torphy, 1987). These substances accumulate intracellularly following receptor activation, and mobilize cell calcium stores (Nahorski and Potter, 1989). It may be speculated that an epithelium-derived relaxing factor may interfere with either the accumulation or the action of inositol phosophates. This would reduce intracellular calcium mobilisation, and hence reduce the level of contraction. In the guinea-pig trachea, methacholine and histamine stimulate a greater accumulation of inositol phosphates in the smooth muscle when the epithelium is absent; this parallels the potentiating effect of epithelium-removal on the contraction to these agents (Hay et al, 1988).

Such a model for the action of the basally-released epithelium-derived relaxing factor may help to explain the effects of epithelium-removal on membrane potential in canine bronchi (Gao and Vanhoutte, 1988). In this study,

removal of the epithelium did not affect resting membrane potential. However, tissues without epithelium showed an enhanced depolarisation in response to acetylcholine. If there is greater mobilisation of calcium in smooth muscle which lacks the epithelium, then there might be greater activity of the Na/Ca exchange pump at the smooth muscle membrane. This could cause an increase in sodium entry into the cell, and thus augment the depolarisation induced by the cholinergic agent. In this context, the effect of epithelium-removal on membrane potential becomes a secondary phenomenon, and not the primary event, as has been suggested (Gao and Vanhoutte, 1988). However, there is little information currently available on the metabolism of the inositol phosphates in canine airway smooth muscle. In consequence, this model of the mechanism of action of basally-released epithelium-derived relaxing factor must remain purely speculative.

2. Stimulated Release

Release of an epithelium-derived relaxing factor may be stimulated by beta-adrenergic agonists. Beta-adrenoceptors are present in large numbers throughout the airways of all species studied so far. It is said that the number of beta-adrenoceptors in the bronchial smooth muscle increases progressively from bronchi to bronchioles (Nadel and Barnes, 1984), although

this detailed information is available only for one species, the ferret (Barnes et al, 1982). Observations in the dog suggest that the number of smooth muscle beta-adrenoceptors may actually decrease in the peripheral airways of this animal (Russell, 1980). This is borne out in the present study, where tissues without epithelium showed decreasing sensitivity to isoproterenol with decreasing airway diameter (Chapter 1). Data obtained in the pig point to a more uniform distribution of smooth muscle beta-adrenoceptors in this species (Goldie et al, 1982). This is also confirmed in the present study (Chapter 2). In both animals, the maximal relaxation achieved to isoproterenol was greater in tissues with than without epithelium (Chapters 1,2 and 3). There is considerable evidence that beta-adrenoceptors can modulate secretory functions of airway epithelial cells. In the canine tracheal epithelium, stimulation of beta-adrenoceptors enhances transepithelial chloride and water transport (Davis et al, 1979; Smith et al, 1982; Welsh, 1986; Welsh, 1987). Epithelial beta-adrenoceptors have also been demonstrated in the airway epithelium of the pig (Goldie et al, 1986) as well as several other species [the rat (Jones and Reid, 1978; Xue et al, 1983), the rabbit (Rugg et al, 1978) and the ferret (Barnes et al, 1982)]. It is postulated that isoproterenol stimulates beta-adrenoceptors on the epithelial cells to release a relaxing factor which augments the effect of the beta-adrenergic agonist on the

smooth muscle. In the bronchi of the dog, the influence of the epithelium on the beta-adrenoceptor-mediated relaxations increased with decreasing diameter, such that in the smallest bronchi; the relaxation was virtually dependent on the presence of the epithelium (Chapter 1). Thus, the release of an epithelium-derived relaxing factor would be most important in the smallest canine bronchi, where isoproterenol-induced relaxation of the smooth muscle is minimal. Pig bronchi do not show a progressive diminution in the relaxation of the smooth muscle, and the contribution of the epithelium to the response is similar in all three orders studied (Chapter 2). It appears that if isoproterenol stimulates release of an epithelium-derived relaxing factor, the distribution or the effect of this factor may vary in proportion to the local sensitivity of the bronchial smooth muscle to beta-adrenergic agents.

The influence of the stimulated release is seen in very specific circumstances, namely, when there is a high degree of cholinergic activation of the bronchial smooth muscle. Under these conditions, tissues without epithelium show a considerably attenuated response to isoproterenol (Chapter 3). This phenomenon is well-documented (Torphy et al, 1983; Russell, 1984; Torphy et al, 1985). The present study demonstrates that, when the epithelium is intact, there is no diminution in the response to the beta-adrenergic agent (Chapter 3). By contrast, raising the level of contraction to 5-hydroxytryptamine does not alter the relaxation nor does the presence or absence of the

epithelium affect the response (Chapter 3). It appears that the epithelium-derived relaxing factor exerts its effect, or is most prominent, in situations where beta-adrenoceptor function in the smooth muscle is inhibited. This parallels the heterogeneity of stimulated release; the influence of the epithelium is more prominent in airways which have fewer beta-adrenoceptors (Chapter 1).

It is not clear how the epithelium-derived relaxing factor might exert such an effect. Proposed mechanisms are discussed in detail in Chapter 3. Since removal of the epithelium has no effect on the response to either forskolin or nitric oxide, it may be concluded that the influence of the epithelium is not related specifically to modulation of either cyclic AMP or cyclic GMP. In addition, the lack of effect of indomethacin excludes a role for inhibitory prostaglandins. However, the relaxation to sodium nitroprusside exhibits an epithelial component (Chapter 3). This is not a cyclic GMP-mediated phenomenon, as the inhibitor of guanylate cyclase does not affect the response. It is possible that sodium nitroprusside elicits release of the epithelium-derived relaxing factor in a manner which by-passes the beta-receptors on the epithelial cells. Alternatively, the drug may act on the smooth muscle in such a way as to render it more sensitive to the effects of a relaxing factor. The role of sodium nitroprusside in the stimulated release of the epithelium-derived relaxing factor merits further investigation.

Evidence for an epithelium-derived contracting factor

1. The response to electrical stimulation

In the bronchi of the dog and the pig, the contractile response to transmural electrical field stimulation is divided into two components. At the start of the the stimulation period there is a rapid contraction, which reaches a peak within approximately 30 seconds, followed by a gradual 'fade' to reach a plateau level. The fade response is frequency-dependent, becoming more prominent with increasing frequency of stimulation. This pattern of response was first described in canine bronchi (Russell, 1978; Flavahan et al, 1985). The present results extend these observations to the pig (Chapter 2). In the dog, the fade response is greater in tissues with epithelium, reaching a lower plateau level. The greater fade has been attributed to the release of a relaxing factor in response to electrical stimulation (Flavahan et al, 1985). Only the smallest airways of the pig show a greater fade response in the presence of the epithelium. In this latter species, the major effect of epithelium-removal is to cause a significant rightward shift of the peak response to nerve stimulation (Chapter 2). Transmural field stimulation of porcine airways may cause release of a contracting factor, which enhances the contractile response to endogenous acetylcholine. An epithelium-dependent response to electrical stimulation has also been described in human airways (de Jongste et al, 1987). As yet there is no evidence for an epithelium-derived contracting factor in the dog.

2. The maximal response to contractile agents

Removal of the airway epithelium of the pig reduces the maximal response to potassium chloride in third and fourth order bronchi, and that to acetylcholine in fifth order bronchi. As discussed in Chapter 2, these data may imply that removing the epithelium removes the source of a contracting factor, thus attenuating the maximal contraction. The response to electrical stimulation may reflect stimulated release of a contracting factor, whereas the effect of the epithelium on the maximal contractile response could represent basal release. If this is so, there is heterogeneity in the release or effect of the contracting factor along the porcine respiratory tree.

The role of the epithelium-derived contracting factor may be to counterbalance any relaxing effect of the epithelium, and thereby maintain a constant airway calibre. The nature of such a factor remains unknown.

The mechanism of epithelium-dependent relaxations to arachidonic acid

Arachidonic acid evokes epithelium-dependent relaxations in canine bronchi: the most likely mediator of this relaxation is prostaglandin E_2. The results, which are fully discussed in Chapter 4, indicate that the regulation of prostaglandin E_2 production in the airway epithelium is extremely complex. A schematic representation of the proposed mechanism of arachidonic acid-induced relaxation is shown in figure 32.

In tissues without epithelium, the arachidonic acid is broken down by cyclooxygenase and lipoxygenase enzymes in the smooth muscle to produce prostaglandins and leukotrienes, respectively. Under normal circumstances these two processes must be almost in balance, as the net effect of arachidonic acid administration is minimal relaxation. In the presence of the inhibitors of lipoxygenase, nordihydroguaiaretic acid (NDGA) and BAY G6575 (nafazatrom), leukotriene production would be reduced. This removes the stimulus to contraction, allowing the prostaglandins (most likely E_2), to initiate a relaxation. The inhibitors of cyclooxygenase, indomethacin and meclofenamate, might be expected to produce contraction, since prostaglandin synthesis is blocked and leukotriene synthesis continues. However, the leukotrienes are poor contractile agents in canine airways (Hirshman et al, 1983; Bakhle and Ferreira, 1985). Thus, the net result of inhibition of cyclooxygenase is that the arachidonic acid has no apparent effect on the tissues.

In bronchi with epithelium, arachidonic acid is also metabolised by the epithelial cells. The metabolism proceeds via the cyclooxygenase pathway to yield prostaglandin E_2 (Smith et al, 1982; Leikauf et al, 1985; Leikauf et al, 1986). This substance is secreted from the epithelium and causes relaxation of the bronchial smooth muscle. Activation of the lipoxygenase pathway produces leukotrienes (Holtzman et al, 1983; Eling et al, 1986). Leukotriene B_4 is secreted from the epithelium,

probably into submucosal blood vessels, where it acts as an inflammatory signal, recruiting mast cells and neutrophils. (O'Byrne et al, 1985; Burka, 1987). It has no direct bronchoconstrictor effect, and so is probably inactive in this in vitro model. Leukotrienes C_4 and D_4 are also produced. These substances are contractile agents, but have little direct effect in the canine preparation (Hirshman, et al 1983). The major effect of the leukotrienes seems to be to stimulate prostaglandin E_2 production in the epithelium (Leikauf et al, 1986). In the presence of inhibitors of lipoxygenase, a major stimulus to prostaglandin synthesis is lost. Prostaglandin E_2 production is reduced, and relaxation of the smooth muscle is attenuated. The cyclooxygenase inhibitor indomethacin would tend to reduce prostaglandin synthesis. However, indomethacin may stimulate leukotriene production indirectly, by diverting arachidonic acid to the lipoxygenase pathway (Bahkle and Ferreira, 1985), and directly, by stimulating the lipoxygenase enzyme (Randall et al, 1980; Harvey and Osborne, 1983). The result would be enhanced leukotriene synthesis, and thus stimulation of prostaglandins.

However, this hypothesis cannot explain why indomethacin and meclofenamate block the relaxation of the bronchial smooth muscle, in the face of apparently continuing prostaglandin E_2 production by the epithelium. In this study, prostaglandin was detected by a specific radioimmunoassay technique. It may be that in the presence of inhibitors of cyclooxygenase, a substance is being produced through an alternative metabolic pathway which is sufficiently similar to prostaglandin E_2 to be

detected as such in a radioimmunoassay system, but which is so altered as to be inactive at receptors on the muscle. To examine this possibility, chemical analysis of the arachidonic acid metabolites produced by the epithelium and smooth muscle is required. At present, any hypothesis to explain the current findings must be purely speculative.

Clinical Implications

Asthma is characterised physiologically by bronchial hyperresponsiveness to contractile agents, and histologically by acute and chronic damage to the airway epithelium (Alexander and Paddock, 1921; Huber and Koessler, 1922; Curry, 1946; Boushey et al, 1980; Barnes, 1983; Nadel, 1983; Hogg and Eggleston, 1984; Laitinen et al, 1985; Beasley et al, 1989; James et al, 1989). This study demonstrates that bronchial hyperreactivity can be mimicked in vitro by removal of the airway epithelium. The implication is that normal function of the airway smooth muscle is dependent, at least in part, on the functional integrity of the epithelium.

It is proposed that, under normal conditions, in healthy individuals, the airway epithelium secretes epithelium-derived relaxing factors, which regulate the tone of the airway smooth muscle. Basal release of these factors attenuates the response to contractile agents. Stimulated release, either via activation of beta-adrenoceptors on the epithelial cells, or by stimulation of the arachidonic acid cascade, augments the

responsiveness of the smooth muscle to relaxing agents. Disruption of epithelial cell function, either by the inflammatory process associated with extrinsic atopic asthma (Laitinen et al, 1985; Befus, 1987; Burka, 1987; Leff, 1988; Wardlaw et al, 1988; James et al, 1989) or viral infection (Empey et al, 1976), or toxic injury due to ozone (Fabbri et al, 1984; Seltzer et al, 1986; Leikauf et al, 1988), sulphur dioxide (Welsh, 1987; Leff, 1988) or various industrial chemicals (Nadel, 1983), reduces or abolishes secretion of epithelium-derived relaxing factors. The loss of basal release then induces hyperresponsiveness to contractile agents such as histamine, 5-hydroxytryptamine and acetylcholine. The loss of stimulated release would reduce the bronchodilator response to inhaled adrenergic agonists.

The loss of basal release would mean that, in the presence of a given contractile agent, there would be an augmented contraction of the airway smooth muscle. The degree of this augmentation would be small, as the shift in the concentration-effect curve caused by epithelium-removal in vitro is small (Chapters 1 and 2). However, in asthmatic individuals, the walls of the airways exhibit considerable thickening, secondary to chronic inflammation and oedema (Beasley et al, 1989; James et al, 1989). In such circumstances, a small amount of smooth muscle contraction causes excessive narrowing of the bronchus, and thus a very large rise in airway resistance. For example, James et al have demonstrated that, for normal human bronchi, smooth muscle shortening

of 40% causes a 15-fold rise in airway resistance. By contrast, in a diseased bronchus taken from an asthmatic patient, a similar degree of muscle shortening causes a 290-fold rise in airway resistance (James et al, 1989). If this thickening of the airway wall were associated with an enhanced sensitivity to contractile agents, the result would be a marked rise in airway resistance, and thus severe respiratory embarassment.

The situation is exacerbated by the loss of stimulated release of the epithelium-derived relaxing factors. The loss of stimulated release may occur by two mechanisms. Firstly, actual disruption of the epithelium by chronic inflammation and infection, and secondly, by reduction of the numbers of beta-adrenoceptors on the epithelial cells. Patients with chronic obstructive airways disease show a marked reduction in the density of beta-adrenoceptors on the bronchiolar epithelium (Raaijmakers et al, 1987). The loss of stimulated release may be unimportant at low levels of vagal cholinergic tone, or during contraction induced by mediators such as 5-hydroxytryptamine, because beta-adrenoceptors are able to induce significant relaxation under these circumstances (Chapter 3). This correlates with the generally accepted view that there is no dysfunction of the smooth muscle beta-adrenoceptor in asthma, and that asthmatic individuals may require a degree of beta-adrenergic stimulation to maintain normal airway tone (Boushey et al, 1980; Barnes, 1983; Nadel, 1983). However, when there is a high level of cholinergic activation (a situation made more likely by

the loss of basal release), the smooth muscle becomes refractory to beta-agonists (Chapter 3). This is a well-documented phenomenon in severe acute asthma (Nadel and Barnes, 1984; Torphy, 1987).

One of the major sources of epithelial damage in asthma is the eosinophil, which releases several proteins which are cytotoxic to the airway epithelium (Gleich et al, 1988; Motojima et al, 1989). Eosinophils are present in large numbers in the broncho-alveolar lavage fluid of asthmatic subjects, in association with mast cells and exfoliated epithelial cells (Wardlaw et al, 1988; Beasley et al, 1989). These changes are apparent even in mild clinical disease (Beasley et al, 1989). Similar results can be obtained in animal studies. In the allergic sheep model of asthma, antigen challenge results in bronchial hyperresponsiveness and a concomitant rise in eosinophils obtained in the broncho-alveolar lavage fluid (Abraham et al, 1988). In vitro studies performed in the trachea of the guinea-pig have demonstrated that eosinophil major basic protein induces hyperresponsiveness of the smooth muscle similar to that seen on epithelium-removal (Flavahan et al, 1988). This phenomenon occurs at concentrations of major basic protein which are too low to cause detectable damage to the epithelial cells (Flavahan et al, 1988). Cell culture studies in canine tracheal epithelium have shown that the secretory properties of epithelial cells exposed to major basic protein are altered substantially, even in the absence of overt histological signs of cell damage (Jacoby et al, 1988). Other eosinophil-derived proteins also caused damage to airway epithelia, but their

role in airway hyperresponsiveness has not yet been assessed (Motojima et al, 1989). Eosinophil major basic protein is detected in significant quantities in the sputum of patients suffering from acute asthma (Frigas et al, 1981; Wardlaw et al, 1988). Thus it seems very likely that loss of epithelial function in asthma is due, in part, to toxic proteins derived from the eosinophil component of the acute allergic response.

Damage to the airway epithelium may also account for the phenomenon of exercise-induced broncoconstriction (Hogg and Eggleston, 1984; Freed et al, 1985; Freed et al, 1987a; Barbet et al, 1988). In the guinea-pig, inhalation of dry air causes extensive damage to the airway epithelium (Barbet et al, 1988). In the dog, an isolated segment of the lung may be studied using a wedged-bronchoscope technique (Freed et al, 1985). In this in vivo model, installation of dry air into the segmental bronchus causes a substantial rise in airway resistance in the isolated segment (Freed et al, 1985). The response is associated with large numbers of exfoliated epithelial cells in the broncho-alveolar lavage fluid (Freed et al, 1987a). Since the lavage fluid also contains significant quantities of the contractile agents prostaglandin D_2 and thromboxane B_2 (Freed et al, 1987a) and since the dry air-induced bronchoconstriction is partially inhibited by indomethacin (Freed et al, 1987a; Freed et al, 1987b), it may be postulated that cyclooxygenase products released from desquamated epithelial cells are responsible for the rise in airway resistance. However,

there is no direct correlation between the concentration of eicosanoids in the lavage fluid and the degree of bronchoconstriction (Freed et al, 1987a). Furthermore, indomethacin only causes a 50% reduction in the response to dry air (Freed et al, 1987a; Freed et al, 1987b). It is known that one of the effects of dry air is to render the surface of the airway mucosa hyperosmolar, due to evaporative water loss (Hogg and Eggleston, 1984). Hyperosmolarity inhibits the secretory function of canine tracheal epithelial cells in culture (Yankaskas et al, 1987). It is proposed that, in the presence of dry air, there is diminished secretion of the epithelium-derived relaxing factors. The reduction in secretion might occur via direct damage to the epithelium, and via inhibition of the secretory functions of the remaining epithelial cells. It is possible that loss of basal release of the epithelium-derived relaxing factor, combined with release of contractile eicosanoids, may contribute to the bronchial hyperresponsiveness seen in the presence of dry air, and may explain, in part, the phenomenon of exercise-induced asthma.

In the case of arachidonic acid metabolites, the following may occur. Under normal conditions, stimulation of arachidonic acid metabolism (e.g. via attachment of immunoglobulins (Morris, 1985)) causes production of prostaglandin E_2, and relaxation of the smooth muscle. Cells which release leukotrienes C_4 and D_4 e.g. mast cells (Befus, 1987), would tend to enhance prostaglandin E_2 release, via stimulation of the cyclooxygenase pathway (Chapter 4). In disease states,

where epithelial function and/or morphology are altered, leukotrienes released from inflammatory cells would act directly on the smooth muscle to cause contraction. Human airways are quite sensitive to the contractile effects of leukotrienes C_4 and D_4 (Dahlen et al, 1980; Ghelani et al, 1980). Histamine and 5-hydroxytryptamine would have an augmented effect due to impaired release of epithelium-derived relaxing factors. Thus the total effect of damage to the airway epithelium would be a considerably increased tendency to bronchoconstriction and thereby facilitation of the asthmatic attack.

Future Directions

Bioassay of an epithelium-derived relaxing factor.

The in vitro techniques employed in this study provide considerable circumstantial evidence for epithelial modulation of bronchial smooth muscle tone. However, these methods cannot prove conclusively that factors released from the epithelium diffuse to the smooth muscle and initiate a response. Such evidence can only be obtained from a bioassay system, in which the fluid from an isolated bronchus with epithelium is allowed to come into contact with a tissue without epithelium (the bioassay tissue). Any epithelium-related phenomena observed in the bioassay tissue must then be due to diffusion of substances from the preparation with intact epithelium. In the first such study, utilising a superfusion cascade system, perfusate from the bronchial epithelium of the dog was shown to cause direct relaxation of bronchial smooth muscle (Flavahan and

Vanhoutte, 1985). Unfortunately these results could not be repeated by other workers (Holroyde, 1986), or by the original authors (Vanhoutte, 1988). The great disadvantage of a superfusion system is that a small epithelial signal is affected by the relatively long transit time between the two preparations, fluctuations in the temperature of the perfusate, and dilution of the epithelium-derived relaxing factor itself. In addition, this system depends on perfusion of the luminal surface of the epithelium. It is possible that there is polar release of epithelium-derived factors in an abluminal direction, i.e. towards the smooth muscle. Obviously, a superfusion system would not detect such unidirectional release of epithelial substances.

Greater success has been achieved using systems in which the airway epithelium and the bioassay tissue are present in the same organ chamber. Ilhan and Sahin (1986) employed a so-called "sandwich" preparation in which a segment of the aorta of the rabbit, without its endothelium, was placed inside a ring of guinea-pig trachea, which was either intact, or denuded of epithelium. Such an arrangement is termed a co-axial bioassay system. In the presence of airway tissue with epithelium, the aorta relaxed in response to acetylcholine. When the airway epithelium was removed, the bioassay tissue showed no responses to the cholinergic agonist (Ilhan and Sahin, 1986). Other workers have obtained similar results using the thoracic aorta of the rat as a bioassay tissue (Goldie et al, 1988). In the presence of guinea-pig trachea with

epithelium, the aorta relaxed in response to histamine and methacholine. These studies demonstrate several interesting points. Firstly, the epithelium must be producing a diffusible substance. Secondly, this substance is not a prostaglandin, as the effect was not inhibited by indomethacin (Ilhan and Sahin, 1986). Lastly, it is interesting that the airway epithelium can apparently influence vascular smooth muscle. This point may have considerable implications for the local control of pulmonary vasculature, and merits much further study.

In another co-axial system, using airway smooth muscle for the bioassay, a strip of guinea-pig trachea without epithelium was placed inside a ring of tracheal tissue with or without epithelium, and the response of the denuded strip to ovalbumin was recorded (Hay et al, 1987). There was an eight-fold increase in the sensitivity of the denuded strip to ovalbumin when it was placed inside a ring without epithelium, as compared to results obtained in the presence of an intact ring (Hay et al, 1987b). Using a 'sandwich protocol', it can be shown that the hyperresponsiveness to substance P seen in guinea-pig trachea without epithelium is abolished by applying the luminal suface of an intact airway to the luminal surface of the denuded tissue (Tschirhart and Landry, 1986). In a novel preparation, the hyperresponsiveness of de-epithelialized canine trachealis to acetylcholine and histamine is abolished when a strip of canine mucosa is placed close to, but not in contact with the

airway smooth muscle (Manning et al, 1988).

These results demonstrate that the airway epithelium releases a diffusable, non-prostanoid factor, which influences the responses of both vascular and bronchial smooth muscle. The next step will be the identification of pharmacological agents or environmental conditions which inhibit the diffusion of this epithelial substance.

Novel in vitro techniques. Recently, Munakata and his colleagues have developed an in vitro perfusion system which monitors alterations in airway tone, using the intact trachea of the guinea-pig (Munakata et al, 1988). In this system, the trachea is suspended in an organ chamber, and fluid passes separately through the lumen and over the outer surface of the tissue. The two perfusing fluids do not come into contact with each other. Using this technique, either the serosal or the epithelial surface of the airway may be stimulated independently: changes in tracheal tension are measured as the drop in pressure between the inlet and outlet of the trachea under constant flow. Tracheae with and without epithelium may be studied. When the contractile agonists histamine and acetylcholine are placed in the outer serosal fluid, there is no difference in contraction between tissues with and without epithelium. However, when these substances are perfused through the lumen, epithelium-removal causes a significant leftward shift of the concentration-effect curves for the agonists (Munakata et al, 1989). Further experiments have shown that application of a hypertonic solution of potassium chloride to the serosal surface of the trachea evokes a

concentration-dependent contraction. If the hypertonic solution is then administered to the inner (epithelial) surface, a concentration-dependent relaxation results (Munakata et al, 1988). In airways which have been contracted with carbachol, the addition of a hypertonic solution of potassium chloride, urea or mannitol to the epithelial side of the preparation causes profound relaxation. This demonstrates that the guinea-pig trachea exhibits epithelium-dependent relaxations to osmotic stimuli. These responses are not affected by either tetrodotoxin, propanolol or indomethacin, implying the presence of an epithelium-derived relaxing factor whose action is independent of nerve stimulation or prostaglandin release (Munakata et al, 1988).

The potential of this system is considerable. For example, it is possible that it could form part of a superfusion system, in which fluid from either the serosal or the epithelial side comes into contact with a bioassay tissue without epithelium. It seems likely that the osmotic stimulus could generate a more powerful epithelial signal than the other superfusion systems which have been discussed. The ability to perfuse the inner and outer surfaces of the airway separately would allow pharmacological inhibitors to be examined, so that inferences could be made about the mechanism of action, and perhaps even the nature, of the epithelium-derived relaxing factor itself.

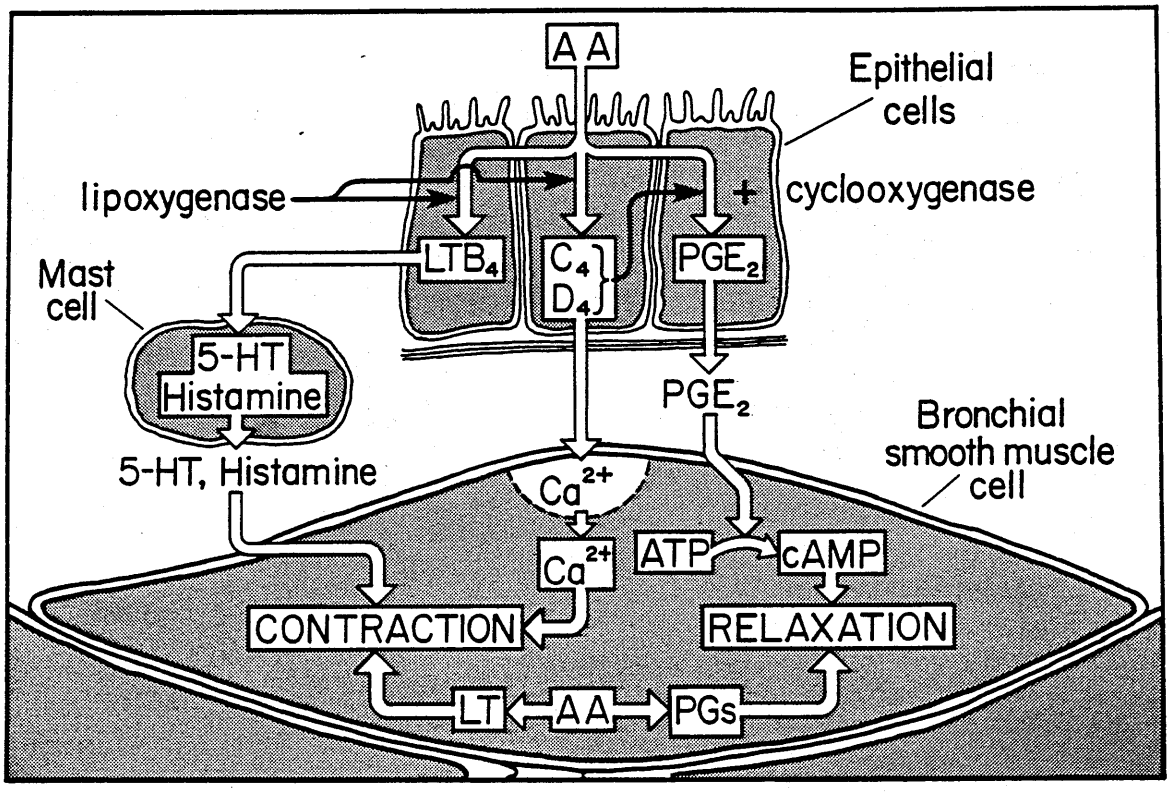

Figure 32: Proposed mechanism for the relaxation induced by arachidonic acid in canine bronchi with and without epithelium. A full explanation is given in the text. AA = arachidonic acid. LTB_4, C_4 and D_4 = leukotrienes B_4, C_4 and D_4, respectively. Ca^{2+} = calcium. 5-HT = 5-hydroxytryptamine.

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