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Relaxation of guinea-pig tracheal smooth muscle to arachidonate is converted to contraction following epithelium removal

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1 The effect of epithelium removal on responses of guinea-pig isolated trachealis to sodium arachidonate has been examined.

2 Arachidonate (100 μM) caused relaxation of epithelium-intact preparations, but following epithelium removal, the response to arachidonate was converted to contraction. In the presence of indomethacin (1 μM), arachidonate caused contraction in intact and denuded trachea.

3 Arachidonate also produced concentration-dependent effects, the qualitative nature of which varied with the presence or absence of the epithelium. In the presence of indomethacin, tracheal strips contracted in a concentration-dependent manner whether or not the epithelium had been removed.

4 Nordihydroguaiaretic acid (NDGA; 10 μM) markedly inhibited the contractile response of denuded strips to arachidonate. In intact tissues this lipoxygenase inhibitor converted the arachidonate-induced relaxation to a concentration-dependent contraction. The contraction to arachidonate, in the presence of NDGA, was epithelium-dependent. In the presence of both indomethacin and NDGA, responses to arachidonate were abolished.

5 It is concluded that the relaxation of guinea-pig trachea to arachidonic acid is epithelium-dependent and is mediated by an inhibitory product of the cyclo-oxygenase metabolic pathway. The contraction in denuded trachea, and trachea in the presence of indomethacin, may be mediated by lipoxygenase products of arachidonic acid metabolism, i.e. peptidoleukotrienes. The mediator of the epithelium-dependent contraction in NDGA-treated tissues is unknown.

Introduction

Mechanical removal of the epithelial cell layer from preparations of isolated airways has recently been shown to alter smooth muscle responsiveness to several bronchoconstrictors and bronchodilators (see references in Farmer, 1987). It has been proposed that the epithelium normally produces a factor which modulates smooth muscle responsiveness. This may be of particular significance in bronchial asthma, since the non-specific airway hyperreactivity in asthmatic patients is associated with damage to and loss of epithelial cells (Cutz *et al.*, 1978; Laitinen *et al.*, 1985). Indeed, it has been shown that epithelium removal increases the responsiveness of human trachealis to methacholine *in vitro* (Raeburn *et al.*, 1986). Since the effects of epithelium removal, in dog bronchi (Flavahan *et al.*, 1985) and in guinea-pig trachea (Hay *et al.*, 1986; Murlas, 1986), can be mimicked, to an extent, by indomethacin, the epithelium-dependent inhibitory effect may involve a cyclo-oxygenase product of arachidonic acid metabolism.

In a preliminary communication Butler *et al.* (1985) observed that arachidonate caused a relaxation in rabbit bronchus preparations with intact epithelium. Following epithelium removal arachidonate was without effect. In the present study we have examined the effect of epithelium removal on the nature of the response of guinea-pig trachea to arachidonate, since

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most studies have utilized this preparation (Farmer *et al.*, 1986; Goldie *et al.*, 1986; Hay *et al.*, 1986; Holroyde, 1986; Murlas, 1986).

Methods

Male, English short hair guinea-pigs (507 ± 16 g; Camm Research Institute) were used. Preparation of and measurement of isometric tension responses from tracheal strips, possessing or lacking an intact epithelium, have been described previously (Farmer *et al.*, 1986). Each strip was suspended in a 3 ml organ bath containing oxygenated, modified Krebs-Henseleit solution at 37°C. Tissues were equilibrated for at least 60 min, with washes every 15 min. Following equilibration, a reference contraction to methacholine (2 μM) was obtained. When examining the concentration-dependence of responses to arachidonate, cumulatively increasing concentrations were added to the bath. Only one concentration-response curve was obtained from each tissue. Concentration-response curves were drawn, and pD_2 values for each curve were calculated from regression analysis. Data are expressed as mean ± s.e.mean. Comparisons between groups were carried out using Student's paired *t* test or Duncan's Multiple Range Test as appropriate. Probability values of less than 0.05 were considered statistically significant.

Drugs

Indomethacin (1 μM) or nordihydroguaiaretic acid (NDGA, 10 μM) were added to the tissue bath at least 45 min before the addition of arachidonate. Indomethacin was prepared as a stock solution of 30 mM in 100 mM Na_2CO_3 and stored at −20°C, and NDGA was prepared as a stock solution of 100 mM in absolute ethanol. Sodium arachidonate was dissolved as a 153 mM stock solution (i.e. 50 mg ml^{-1}) in water and stored under N_2 at −20°C. Further drug dilutions were made in 0.9% saline. Indomethacin, NDGA, sodium arachidonate, methacholine chloride and atropine sulphate were purchased from Sigma Chemical Co.; diphenhydramine hydrochloride was obtained from Parke, Davis and Co.

Results

Effect of a single, high concentration of arachidonate

In 13 out of 15 tracheal strips with intact epithelium, arachidonate (100 μM) caused a biphasic response (Figure 1a). A small, transient contraction (40.8 ± 10.7 mg) was followed by a much larger, and sustained relaxation (202.1 ± 38.8 mg). In the other 2 intact strips the contractile response was followed by a relaxation which did not attain the original baseline. In contrast, all epithelium-denuded strips ($n = 15$) exhibited a sustained contraction of 322.9 ± 65.0 mg (Figure 1b). These responses to arachidonate were unaffected by prior addition of atropine (3 μM) or diphenydramine (3 μM) to the organ bath.

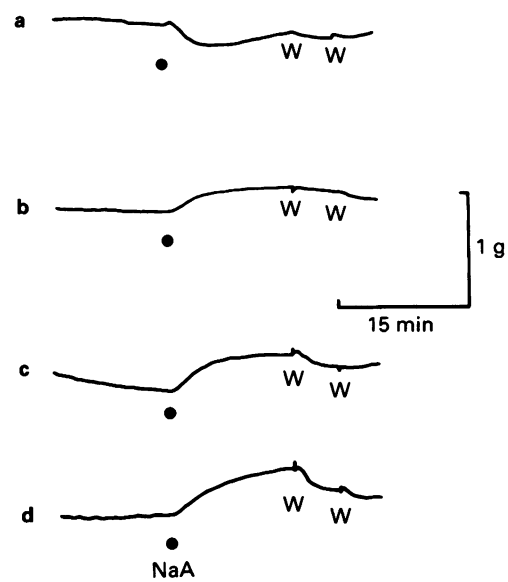

Figure 1 Effect of epithelium removal on the nature of the response of guinea-pig isolated trachealis to sodium arachidonate (NaA; 100 μM). (a) NaA relaxed intact preparations, and (b) contracted epithelium-denuded preparations. In the presence of indomethacin (1 μM), NaA elicited a contraction whether epithelium was present (c) or absent (d). W denotes wash.

Indomethacin (1 μM) induced a slowly developing relaxation which reached a stable level after 39.3 ± 3.5 min in intact strips, and after 15.4 ± 2.9 min in denuded strips. These values were significantly different ($P < 0.005$, Student's paired *t* test). The magnitude of the relaxation in intact strips was 700 ± 64 mg, and in denuded strips, 660 ± 97 mg. In the presence of indomethacin, arachidonate (100 μM) elicited a contraction regardless of epithelial integrity (Figure 1c and d); the magnitude of contraction in intact preparations was 300 ± 53 mg ($n = 9$) and in denuded preparations 422 ± 84 mg ($n = 9$).

Concentration-dependent effects

In tissues with intact epithelium cumulatively-added arachidonate produced qualitatively variable effects.

In 6 preparations arachidonate (0.1–300 μM) produced concentration-dependent relaxation, the maximal magnitude of which was 193 ± 49 mg. In another 3 preparations, low concentrations (0.1–1 μM) caused contraction, but as the concentration was progressively increased the response changed from contraction to concentration-dependent relaxation. In a further 3 tracheal strips arachidonate elicited concentration-dependent contractions only. Because of the qualitatively different nature of these responses to different concentrations it was not possible to calculate the mean response to any given concentration. In epithelium-denuded trachea arachidonate elicited concentration-dependent contractions (Figure 2) and relaxation of denuded strips was never observed. The maximum contractile response was 459 ± 78 mg and the pD_2 value was 4.47 ± 0.08 (n = 12).

In the presence of indomethacin (1 μM) arachidonate elicited concentration-dependent contractions irrespective of whether or not the epithelium was present (Figure 2). Although the threshold concentration of arachidonate was increased in the presence of indomethacin (Figure 2), the sensitivity was unaffected by the cyclo-oxygenase inhibitor. In the presence of indomethacin, the pD_2 for intact strips was 4.17 ± 0.05 (n = 6) and for denuded strips, 4.31 ± 0.14 (n = 6). Neither of these values was significantly different from each other, or from control. Therefore, in the presence of indomethacin, epithelium removal had no effect on responsiveness to arachidonate. In contrast, there was an apparent increase in maximum response to arachidonate in the presence of indomethacin (Figure 2). However, as discussed earlier, indomethacin was observed to lower baseline tension. Therefore, when calculating the maximum response to arachidonate, the reduction in resting tone of each tissue following incubation with indomethacin was taken into account: i.e., for each tissue the magnitude of the indomethacin-induced relaxation was subtracted from the total developed tension in response to arachidonate to yield the actual maximum developed response (from the original baseline). When the indomethacin-induced relaxation was taken into account, the maximum response to arachidonate was 273 ± 36 mg in intact tracheae, and 417 ± 96 mg in denuded tracheae. Neither of these values was significantly different from control (459 ± 73 mg).

In epithelium-denuded preparations NDGA markedly inhibited the contractile response to arachidonate (Figure 3). Indeed, in half of the tissues NDGA abolished the response. Interestingly, NDGA, like indomethacin, converted the relaxant response of

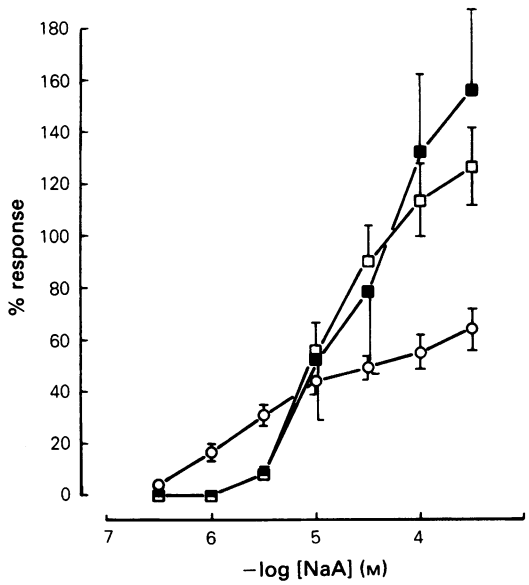

Figure 2 Concentration-response curves for the effect of sodium arachidonate (NaA) on guinea-pig isolated trachea and the influence of indomethacin (1 μM) and epithelium removal. (○) Epithelium-denuded controls; (□) intact, indomethacin-treated trachea; (■) epithelium-denuded, indomethacin-treated trachea. Increases in tension are expressed as a % of the response (reference contraction) to methacholine (2 μM). Each point is the mean of at least 6 observations and vertical lines indicate s.e.mean.

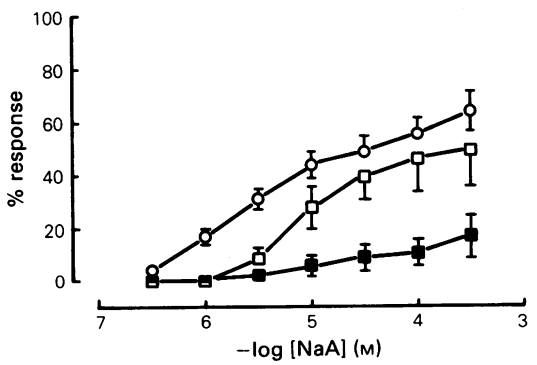

Figure 3 Concentration-response curves for the effect of sodium arachidonate (NaA) on guinea-pig isolated trachea and the influence of nordihydroguaiaretic acid (NDGA, 10 μM) and epithelium removal. (○) Epithelium-denuded controls; (□) intact, NDGA-treated trachea; (■) epithelium-denuded, NDGA-treated trachea. Increases in tension are expressed as a % of the response (reference contraction) to methacholine (2 μM). Each point is the mean of at least 6 observations and vertical lines indicate s.e.mean.

intact tissues to a contractile one, the pD_2 value for which was 4.46 ± 0.04 ($n = 6$). This value was not significantly different from denuded controls, although responses to low concentrations of arachidonate (i.e., $< 3\,\mu M$) were smaller in the presence of NDGA (Figure 3). Contractions to arachidonate, in the presence of NDGA, were epithelium-dependent (Figure 3).

Following incubation with both indomethacin and NDGA, contractile responses of intact and denuded strips to arachidonate were all but abolished (Figure 4). The qualitative effects of epithelium removal, indomethacin and NDGA on the nature of the response to arachidonate are summarized in Table 1.

Discussion

Effect of epithelium removal

The epithelium-dependent relaxation to arachidonate may provide a useful method for assessing, during an experiment, the effectiveness of epithelium removal from guinea-pig trachea. Relaxation of intact preparations was converted to contraction following epithelium removal. Thus, arachidonate may be utilized in a manner similar to that of acetylcholine in determining vascular endothelial integrity (Furhgott, 1983). We and others (Goldie *et al.*, 1986; Hay *et al.*, 1986) have previously confirmed histologically that rubbing the luminal surface of guinea-pig trachea with a cotton swab consistently removes the epithelium completely, without damaging underlying tissue. Holroyde (1986), using a wooden stick, demonstrated that in four rubbed tracheae the procedure completely removed the epithelium, and in one, removed 80%. Nevertheless, caution should be exercised in assuming that contraction to arachidonate results only when the epithelial layer has been *completely* removed. Conversely, unless great care is taken during tissue preparation, it is possible that the epithelium may inadvertently be removed from 'intact' tissues. Indeed, this may explain the observation, in the present study, that 3 of the 27 intact preparations contracted in response to arachidonate. In studies of endothelial modulation of vascular smooth muscle, estimates of % endothelium removal are confirmed histologically using *en face* silver staining (Abrol *et al.*, 1984; Griffith *et al.*, 1984). This technique, which is based on the ability of silver to precipitate on the border of endothelial cells, may prove useful, in conjunction with arachidonic acid, in demonstrating tracheal epithelial integrity.

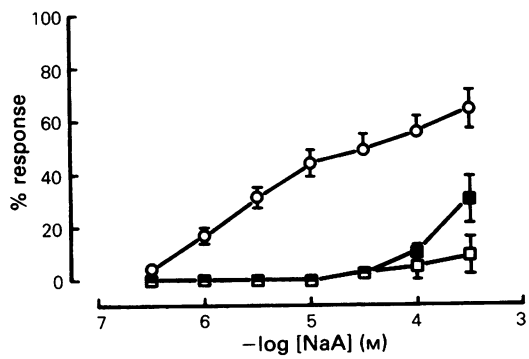

Figure 4 Concentration-response curves for the effect of sodium arachidonate (NaA) on guinea-pig isolated trachea and the influence of indomethacin (1 μM) plus nordihydroguaiaretic acid (NDGA, 10 μM), and epithelium removal. (○) Epithelium-denuded controls; (□) intact indomethacin- and NDGA-treated trachea; (■) epithelium-denuded indomethacin- and NDGA-treated trachea. Increases in tension are expressed as a % of the response (reference contraction) to methacholine (2 μM). Each point is the mean of at least 4 observations and vertical lines indicate s.e.mean.

Effect of indomethacin

In the presence of indomethacin, the qualitative nature of intact tracheal responses to arachidonate was altered in the same way as that produced by epithelium removal. This suggests that the epithelium-dependent relaxation is probably mediated by an inhibitory prostanoid (prostaglandin E_2), as proposed previously for rabbit airways (Butler *et al.*, 1985). Several investigators have demonstrated that, in guinea-pig trachea (presumably with the epithelium intact), exogenous arachidonic acid causes relaxation associated with an elevated release of prostaglandin E_2 (PGE_2) (Burka *et al.*, 1981; Spannhake *et al.*, 1981). In addition, low concentrations of PGE_2 are often excitatory in guinea-pig trachea (Gardiner & Collier, 1980). PGE_2, therefore, may also be the mediator responsible for the contractile response, of 3 of the intact strips, to low concentrations of arachidonate. The results of the present study indicate that exogenous arachidonic acid is metabolized by the epithelial cells to form a prostanoid which then acts to relax tracheal smooth muscle.

Arachidonate, either at a single, high concentration, or cumulatively added, elicited contractions in indomethacin-treated trachea irrespective of whether or not the epithelium was present. Previous studies (Burka, 1985; Mansour & Daniel, 1986) also showed that indomethacin converts arachidonic acid-induced tracheal relaxation into contraction. In indomethacin-treated guinea-pig trachea, Burka & Saad (1984) concluded that the mediators causing arachidonic acid-induced contraction are leukotrienes C_4 and D_4. This is probably also the case in the present study,

Table 1 Effect of epithelium removal and inhibitors of arachidonic acid metabolism on the nature of the response of guinea-pig trachea to arachidonate

	Control	*Indomethacin* (1 μM)	*NDGA* (10 μM)	*Indomethacin + NDGA*
+ Epithelium	↓[5]	↑	↑	○
− Epithelium	↑	↑	○	○

↑ Denotes contraction, ↓, relaxation and ○, no response
[5] Three out of 27 epithelium-intact, control tissues contracted in response to arachidonate. See text for explanation.
NDGA = nordihydroguaiaretic acid.

since the lipoxygenase inhibitor NDGA abolished tracheal contractions in indomethacin-treated trachea. It is interesting that in the presence of indomethacin alone, epithelium removal had no effect on tracheal responsiveness to arachidonate (as indicated by pD_2 values). This suggests that the mediators, probably one or more of the peptidoleukotrienes, are not released from the epithelium.

The inflammation occurring in the airways of asthmatics is associated with sloughing of epithelial cells (Laitinen *et al.*, 1985). It is possible, therefore, that endogenous arachidonic acid, normally converted to smooth muscle inhibitory prostanoids by the epithelium, is converted (following epithelium loss) to contractile prostanoids such as $PGF_{2\alpha}$ or thromboxane A_2 (TxA_2), or to leukotrienes C_4 and D_4 These substances may then cause bronchospasm and contribute to the airway hyperreactivity associated with bronchial asthma.

Effect of nordihydroguaiaretic acid

It has previously been shown that in the presence of indomethacin the 5-lipoxygenase inhibitor, NDGA, reduces considerably arachidonic acid-induced contractions of guinea-pig trachea (Burka & Saad, 1984). As mentioned earlier, this was also a finding in the present study. Perhaps the most interesting observation with NDGA was that the arachidonate-induced tracheal relaxation was converted to a contraction which was dependent upon the presence of the epithelium. An explanation of the mechanisms involved in this contractile response is puzzling. In the presence of NDGA, arachidonic acid may be metabolized, via epithelial cell cyclo-oxygenase, to an excitatory prostanoid such as $PGF_{2\alpha}$ or TxA_2. This is supported by the finding that indomethacin abolished the arachidonate-induced contraction of NDGA-pretreated, intact trachealis. However, this explanation is difficult to reconcile with our earlier assertion that arachidonate is normally converted, by epithelial cyclo-oxygenase, to predominantly inhibitory prostanoids. It is possible that NDGA inhibits the formation of a lipoxygenase product, which in turn normally inhibits specifically the synthesis of an epithelium-derived smooth muscle-contracting prostanoid. Therefore, in the presence of NDGA, exogenous arachidonate is converted by the epithelium to a prostanoid which causes smooth muscle contraction. However, without more specific inhibitors of the myriad enzymes in the cascade of arachidonic acid metabolism and, given the various feedback mechanisms involved therein, the nature of the mediator(s) underlying this contraction can only be speculated upon. To our knowledge, the present study, of the effect of NDGA on responses to arachidonate, is the first time an epithelium-dependent contraction of airway smooth muscle has been demonstrated.

In a previous study Farmer *et al.* (1986) showed that epithelium removal augments the relaxant response of guinea-pig trachea to adenosine. It was speculated that adenosine may cause the secretion by epithelial cells of a factor which stimulates airway smooth muscle. Therefore, removal of the epithelium might cause the loss of this excitatory influence and augment the relaxation to adenosine. The postulated epithelium-derived excitatory factor may be the same mediator involved in the contractile response of NDGA-treated trachea to arachidonate. It would be interesting to determine whether NDGA, or indeed indomethacin, affects tracheal responsiveness to adenosine.

Since the original submission of the present study, another paper which presents very similar results, has been published elsewhere. Nijkamp & Folkerts (1986) examined the effect of epithelium removal on responses of guinea-pig tracheal spirals to a single concentration (22 μM) of arachidonic acid. Using indomethacin, NDGA or a leukotriene receptor antagonist these investigators concluded, in agreement with the present study, that the relaxation to arachidonic acid is mediated by epithelium-derived, inhibitory cyclo-oxygenase products. Further, it was also concluded that the arachidonate-induced contraction of denuded trachea is probably due to peptidoleukotrienes, which also concurs with the present study. However, Nijkamp & Folkerts (1986) did not examine the concentra-

tion-dependence of responses to arachidonic acid, nor did they comment on the effects of NDGA (singly or in combination with indomethacin) on the response of intact trachea to arachidonate. Nevertheless, the two studies are essentially in agreement.

In conclusion, the present study has demonstrated that the guinea-pig isolated tracheal relaxation to sodium arachidonate is epithelium-dependent, and probably mediated by an inhibitory prostanoid. Contractions of denuded preparations, and of intact and denuded preparations in the presence of indomethacin, are probably mediated by lipoxygenase products of arachidonate metabolism. However, in the presence of NDGA, arachidonate elicited an epithelium-dependent contraction. The mediator producing the latter response is unknown. These findings are important since, in the airways of asthmatics, there is considerable damage to the epithelium, associated with the non-specific bronchial hyperreactivity in these patients. Abnormal metabolism of endogenous arachidonic acid by the asthmatic airways may be a contributing factor to the bronchial hyperreactivity.

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