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Modulation of the reactivity of the guinea-pig isolated trachealis by respiratory epithelium: Effects of cooling

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1 Examination has been made of the effects of epithelium removal on the reactivity of guinea-pig trachealis to methacholine at 37°C and 22°C, and on responses to activation of the Na^+/K^+-pump by abrupt temperature increase from 22°C to 37°C.

2 At 37°C, epithelium removal increased the sensitivity of isolated tracheal strips to methacholine without affecting the maximum isometric contractile response. Epithelium removal resulted at 22°C in a decrease in sensitivity to methacholine, i.e. an effect opposite to that seen at 37°C. While the maximum response of intact strips to methacholine was enhanced at 22°C, the maximum response of denuded preparations was decreased.

3 The increase in sensitivity to methacholine at 37°C after epithelium removal was mimicked in intact preparations by indomethacin (1 μM). Indomethacin did not mimic the decrease in methacholine sensitivity and maximum response caused by epithelium removal at 22°C.

4 Following incubation at 22°C, abrupt increase in temperature to 37°C elicited relaxation in both epithelium-containing and epithelium-denuded tracheal strips. In epithelium-containing preparations the relaxation was more pronounced and followed by contraction. Ouabain (1 μM) converted the relaxation of denuded preparations to contraction, but was ineffective in intact strips. The relaxation of intact strips was, however, inhibited by a greater ouabain concentration (10 μM).

5 These findings indicate that the modulatory effect of the epithelium is temperature-dependent. In cooled preparations, the epithelium enhances reactivity. At 37°C, an epithelium-derived factor reduces reactivity, and this may partially be due to activation of the electrogenic Na^+/K^+-pump.

Introduction

Respiratory epithelial cell damage and loss accompany the late phase airway hyperreactivity response in asthmatics (Laitinen *et al.*, 1985). This finding has prompted investigation into the influence of epithelial cells on airway smooth muscle responsiveness. Mechanical removal of epithelial cells from airway smooth muscle preparations *in vitro* potentiates the response to many bronchoactive agents in several mammalian species (see Fedan *et al.*, 1988 for review). A number of investigators (Tschirhart & Landry, 1986; Ilhan & Sahin, 1986; Hay *et al.*, 1987) have demonstrated the existence of a diffusible, inhibitory factor liberated from the epithelium which modulates smooth muscle responsiveness. The loss of this epithelium-derived relaxing factor (EpDRF) could contribute to hyperreactivity in the asthmatic patient.

Since it occurs for some, but not all bronchoactive agents, the increase in reactivity after epithelium removal could involve the loss of an effect of EpDRF on a physiological process important to the responsiveness of the smooth muscle. Evidence has been obtained that EpDRF may modulate the activity of an electrogenic Na^+/K^+-pump (Raeburn & Fedan, 1989), which is electrogenic and contributes to the resting membrane potential in airway smooth muscle (Souhrada *et al.*, 1981; Souhrada & Souhrada, 1981). Alterations in electrogenic Na^+/K^+-pump activity may affect membrane potential and reactivity to excitatory agents (Fleming, 1980).

Several methods may be used to alter electrogenic Na^+/K^+-pump activity. The pump is inhibited when the extracellular fluid is rendered K^+-deficient, and stimulated upon the re-addition of K^+. Stimulation of the pump will relax a contracted smooth muscle, and this relaxation is blocked by ouabain. Likewise, Na^+/K^+-pump activity may be reduced by cooling (Fleming, 1980) and stimulated in a ouabain-sensitive fashion by warming (Taylor *et al.*, 1970; Souhrada *et al.*, 1981; Souhrada & Souhrada, 1981).

In the present study, responses to methacholine of intact and epithelium-denuded guinea-pig tracheal strips at 37°C and 22°C were compared. To evaluate further the possibility that electrogenic Na^+/K^+-pump activity is influenced by the epithelium, responses of intact and denuded preparations to abrupt increase in bath temperature from 22°C to 37°C were examined. A preliminary account of this work has been given (Lamport & Fedan, 1988).

Methods

Preparation of guinea-pig isolated tracheal strips

Male English short hair guinea-pigs (300–700 g; Camm Research Institute, Wayne, NJ, U.S.A.) were killed by cervical dislocation and bled. The trachea was removed and placed in modified Krebs-Henseleit (MKH) solution (37°C) equilibrated with 5% CO_2 in O_2. The trachea was cleaned, cut along the longitudinal axis directly opposite the smooth muscle, and divided into segments two cartilage rings wide. The segments were tied at one end *via* silk suture to holders, placed in 3 ml water-jacketted organ baths containing gassed MKH, and attached at the other end to force displacement transducers for the isometric recording of tension changes. The MKH composition was (mM): NaCl 113.0, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 5.7.

Equilibration conditions

The strips were equilibrated in MKH at 37°C or 22°C for 1 h under an optimum (1 g) resting load and were washed at 15 min intervals. Preparations equilibrated at 37°C were then either held at 37°C during concentration-response determinations (see below), or used to examine the effect of abrupt

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temperature decrease to 22°C (see below). Preparations equilibrated at 22°C remained at 22°C during concentration-response determinations, or were subjected to rapid temperature increase to 37°C. The justification for not first incubating the latter preparations at 37°C was that spontaneous tone was negligibly and transiently affected when preparations which had been incubated at 37°C for 1 h were cooled rapidly to 22°C (see Results).

Epithelium removal

The epithelium was removed by gently rubbing the luminal surface of alternate segments with a cotton-tipped applicator. This procedure has been shown by histological analysis to remove the epithelium without damaging the smooth muscle (Hay *et al.*, 1986). To reduce possible regional differences in muscle reactivity, adjacent tissue segments comprised a test pair (i.e. one intact, one denuded). The region of trachea used was randomized with respect to the particular protocols employed.

Concentration-response curves

Methacholine concentration-response curves were obtained at 37°C and 22°C from responses to cumulative additions. Only one curve was constructed from each tracheal strip. In some experiments, the effect of indomethacin (1 μM) on the methacholine concentration-response curve was examined at 37°C and 22°C. The indomethacin was added at the end of the 1 h equilibration period. Methacholine was applied 40–45 min later, in the presence of indomethacin.

Comparison of force development in the absence and presence of indomethacin

Indomethacin (1 μM) had no effect on spontaneous tone at 22°C, but relaxed both the intact and denuded preparations under resting tension at 37°C, as observed previously (Hay *et al.*, 1986). When methacholine-induced contraction was measured from the indomethacin-lowered baseline, the apparent effect of indomethacin was to increase the magnitude of the tension increment evoked by each concentration step and therefore the total tension change between the foot and the summit of the concentration-response curve (the maximum response). Therefore, when indomethacin was present at 37°C, it was necessary to take this relaxation into account in order to compare methacholine-induced maximum responses with those obtained at 22°, and at 37°C in the absence of indomethacin. We have previously demonstrated (Hay *et al.*, 1986) that the peak tension developed by the preparations in response to methacholine (basal plus methacholine-induced) is not affected by indomethacin, either in the absence or presence of the epithelium. In the present study two parameters are used to report the response to the maximally-effective methacholine concentration. The first will be referred to as 'developed force', which is the tension increment measured from the resting tension prior to the addition of methacholine. Concentration-response curves in the figures are presented in terms of developed force, and Table 1 summarizes maximum developed forces obtained under several conditions. The second parameter will be referred to as 'corrected maximum developed force.' This parameter applies only when indomethacin was present at 37°C; it represents the maximum developed force observed in the presence of indomethacin less the reduction in basal tension induced by indomethacin. Corrected maximum developed force results are given in Table 1.

Abrupt temperature change

Tracheal segments were incubated in MKH for 1 h either at 37°C or at 22°C. The bath temperature was then changed abruptly to the alternate temperature by changing the water-jacket fluid to a second circulator which had been set to the appropriate temperature. The large volume of the water jackets (ca. 46 ml) compared to the volume of the organ chambers (3 ml) allowed rapid temperature changes in the MKH. In some experiments, indomethacin (1 μM) was present during the hour-long incubation at 22°C and subsequent increase to 37°C. In other cases, ouabain (1 or 10 μM) was added 5 min before increasing the temperature to 37°C.

Statistical analysis

The results were quantified as developed force of contraction (g) and % maximum response, and are given as mean ± s.e.mean; n is the number of separate experiments. Geometric mean EC_{50} values (the concentration producing 50% of the maximum response) were determined from nonlinear least square curve fitting analysis (ALLFIT; DeLean *et al.*, 1978). The data were evaluated for differences by analysis of variance or Student's t test (where indicated). The 0.05 level of probability was considered significant. In the figures the s.e.mean is shown by the vertical bars, unless enclosed within the symbol.

Drugs

Methacholine (acetyl-β-methylcholine chloride), indomethacin, and ouabain were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. All drug solutions were prepared freshly each day in 0.9% w/v NaCl solution, with the exception of indomethacin, which was dissolved in 100 mM Na_2CO_3 solution and diluted with water.

Results

Indomethacin absent

The effects of epithelium removal on the reactivity of guinea-pig isolated trachealis to methacholine were temperature-dependent (Figure 1; Table 1). Epithelium removal resulted in a 3.6 fold increase in sensitivity at 37°C. Removal of the epithelium caused an opposite effect at 22°C, i.e. a 6 fold decrease in sensitivity to methacholine. There was, however, no effect of temperature on the sensitivities of intact preparations.

Epithelium removal had no effect at 37°C on the maximum developed force evoked by methacholine. At 22°C, the maximum developed force of intact strips was increased by 60%, but in denuded strips it was decreased by 39%.

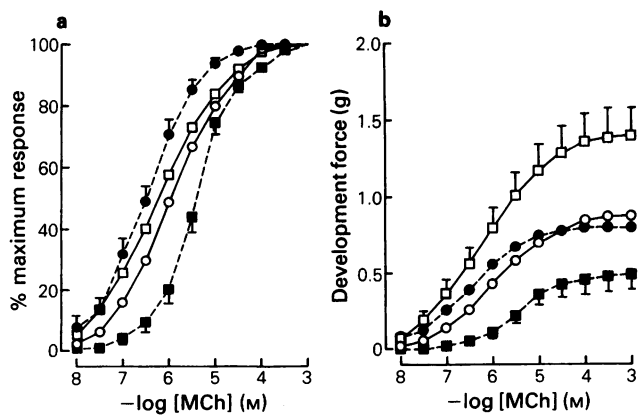

Figure 1 Guinea-pig isolated tracheal strips: effects of epithelium removal on reactivity to methacholine at 37°C and 22°C: (○) epithelium present, 37°C; (●) epithelium absent, 37°C; (□) epithelium present, 22°C; (■) epithelium absent, 22°C. Results are expressed as % of the maximum developed force of contraction to indicate alterations in sensitivity (a) and as g developed force to indicate alterations in maximum developed responses (b). $n = 5$.

Table 1 Effects of epithelium removal, temperature and indomethacin (1 μM) on reactivity to methacholine in guinea-pig isolated trachealis

Temperature	$-\log EC_{50}$ (M) Intact	$-\log EC_{50}$ (M) Denuded	Maximum developed force (g) Intact	Maximum developed force (g) Denuded
		Control ($n = 5$)		
37°C	5.97 ± 0.17	6.53 ± 0.13*†	0.88 ± 0.14	0.80 ± 0.18
22°C	6.22 ± 0.15	5.43 ± 0.09‡	1.40 ± 0.19§	0.49 ± 0.09‡
		Indomethacin ($n = 6$)		
37°C	6.47 ± 0.16¶	6.70 ± 0.11†	1.61 ± 0.30 (0.98 ± 0.14)**	1.24 ± 0.29 (0.85 ± 0.20)**
22°C	6.05 ± 0.18	5.57 ± 0.06‡	1.20 ± 0.23	0.60 ± 0.20‡

Data are means ± s.e.mean. The results were analysed for differences by ANOVA.
* Denuded significantly greater than intact.
† Significantly greater than at 22°C.
‡ Denuded significantly less than intact.
§ Significantly greater than at 37°C.
¶ Significantly greater than control (indomethacin absent) at 37°C.
** Indomethacin caused relaxation of the preparations at 37°C. Values in parentheses give the maximum forces developed from the pre-indomethacin baseline, i.e. the corrected maximum developed forces. These values were not significantly different, nor were they different from respective intact and denuded control values at 37°C.

Indomethacin present

To determine if the effects of epithelium removal at 37°C and 22°C could be due to differences in prostanoid production, concentration-response curves were constructed at both temperatures in the presence of the cyclo-oxygenase inhibitor indomethacin (1 μM) (Figure 2; Table 1). In agreement with earlier findings (Hay *et al.*, 1986), indomethacin reduced spontaneous tone at 37°C: intact strips relaxed by 0.55 ± 0.17 g and denuded preparations relaxed by 0.39 ± 0.12 g ($n = 6$; $P < 0.3$; Student's t test). At 22°C indomethacin had no effect on spontaneous tone.

Indomethacin increased the sensitivity of intact preparations to methacholine at 37°C. Sensitivity to methacholine was not increased further by epithelium removal at 37°C in the presence of indomethacin, i.e. the effect of indomethacin on intact strips mimicked epithelium removal. At 22°C the sensitivities of intact or denuded strips to methacholine were not affected by indomethacin.

At 37°C indomethacin increased the maximum developed force of contraction of both intact and denuded segments to methacholine. However, for both intact and denuded preparations, the corrected maximum developed force was not significantly different from the maximum developed force in the corresponding preparations not exposed to indomethacin. At 22°C indomethacin did not affect the maximum developed force of either control or denuded preparations.

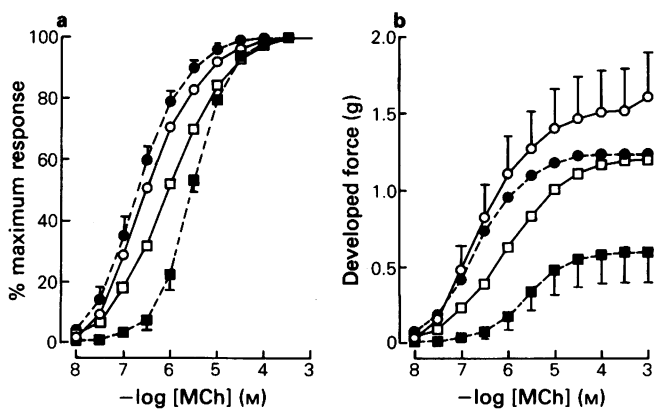

Figure 2 Guinea-pig isolated tracheal strips: effects of indomethacin (1 μM) on reactivity to methacholine in the presence and absence of the epithelium at 37°C and 22°C: (○) epithelium present, 37°C; (●) epithelium absent, 37°C; (□) epithelium present, 22°C; (■) epithelium absent, 22°C. Results are expressed as % of the maximum developed force of contraction (a) and as g developed force (b). $n = 6$.

Responses to abrupt temperature change

Abrupt bath temperature change from 37°C to 22°C did not affect the tone of intact or denuded segments appreciably ($n = 5$; not shown); a slight tension increase was followed by a reduction in tone to the original level. However, in intact segments abrupt increase in temperature from 22°C to 37°C elicited an immediate and pronounced relaxation, followed by a contraction ($n = 17$; Figure 3). A few of the intact segments ($n = 5$) responded to temperature increase with a slow, weak relaxation, followed by a contraction. In some preparations ($n = 17$) tension returned to baseline, while in others ($n = 5$) it did not. In contrast, the denuded preparations relaxed slowly and weakly upon temperature elevation; a spontaneous contraction was not observed ($n = 20$; Figure 3). These experiments were repeated ($n = 20$) in the presence of indomethacin (1 μM) to evaluate whether the relaxation involved the production of an inhibitory prostanoid. The results obtained were not different from those shown in Figure 3. To investigate whether the relaxation responses to temperature increase involved activation of the Na^+/K^+-pump, the effect of ouabain was examined. Ouabain (1 μM) did not inhibit the

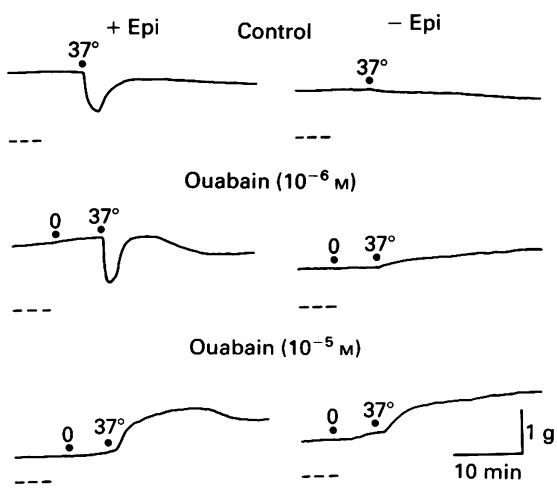

Figure 3 Guinea-pig isolated tracheal strips: representative tracings showing the effects of an abrupt temperature change from 22°C to 37°C in the presence (+Epi; left column of tracings) or absence (−Epi; right column of tracings) of the epithelium. (O) Addition of ouabain; (37°C) abrupt change from 22° to 37°C. The top row illustrates control tracings obtained in the absence of ouabain. Responses obtained in the presence of ouabain are shown in the middle (1 μM ouabain) and bottom (10 μM ouabain) rows. The dashed line below each tracing indicates zero force.

37°C-induced relaxation of intact preparations, but the relaxation of denuded segments was prevented and contraction was seen ($n = 4$; Figure 3). A ten fold higher concentration of ouabain (10 μM) was needed to inhibit the relaxation of intact segments ($n = 4$; Figure 3). Elevated temperature then evoked a contraction, as it had in denuded preparations (Figure 3).

Discussion

The modulatory effect of the epithelium on the sensitivity of guinea-pig trachealis to methacholine was dependent on the temperature of the physiological solution. As observed previously (Hay *et al.*, 1986), sensitivity to methacholine was increased at 37°C by epithelium removal. Although the sensitivity of intact segments at 22°C was the same as at 37°C, the sensitivity of denuded preparations at 22°C was reduced substantially in the absence of the epithelium. The effect of epithelium removal on maximum developed force also was temperature-dependent. There was no effect of the epithelium on the maximum force developed to methacholine at 37°C. However, at 22°C, denuded preparations developed smaller responses to methacholine while intact segments generated greater tension. The present findings indicate that the presence of epithelial cells at 22°C causes an increase in sensitivity and contractility to methacholine, i.e. an excitatory influence on reactivity.

While the reduced responsiveness of denuded segments at 22°C is surprising, potentiation of contractile agents at low temperatures has been observed previously in intact airway preparations of the ox (Souhrada & Souhrada, 1981), guinea-pig (Souhrada & Souhrada, 1981; Kolbeck *et al.*, 1988), man (Black *et al.*, 1984), rat (Ishii & Shimo, 1985) and rabbit (Bratton *et al.*, 1987). As was reported by Kolbeck *et al.* (1988) for guinea-pig airways, but unlike the observations in human airways made by Black *et al.* (1984), in the present study there were no substantial changes in tone upon abrupt temperature reduction from 37°C to 22°C. The alterations in sensitivity and maximum response observed at 22°C in both intact and denuded preparations were not, therefore, likely to be due to sustained change in basal tone.

The possibility that the different reactivities of intact and denuded segments to methacholine at 37°C and 22°C could be due to the influence of a prostanoid was examined. Methacholine concentration-response curves obtained at 22°C were not affected by indomethacin. However, at 37°C the increase in sensitivity observed after epithelium removal was mimicked in intact preparations by indomethacin, as has been reported previously (Hay *et al.*, 1986). At 37°C, the change in reactivity to many bronchoactive substances upon epithelium removal is not attributable solely to the loss of a source of prostanoids, as the cyclo-oxygenase inhibitor does not mimic epithelium removal under all circumstances (Fedan *et al.*, 1988). The failure of indomethacin to alter the reactivity-enhancing effect of the epithelium at 22°C suggests that the effect is entirely independent of prostanoids. The reason for the ineffectiveness of indomethacin at 22°C is of interest but is unexplained at present.

Reduced temperature inhibits electrogenic Na^+/K^+-pumping in guinea-pig trachealis (Souhrada *et al.*, 1981; Souhrada & Souhrada, 1981). The present study indicates that the epithelium also modulates the response of the muscle when the Na^+/K^+-pump is activated by raising the temperature rapidly from 22°C to 37°C. The relaxation of denuded preparations was attributable to the stimulation of the electrogenic pump, in so far as the response was inhibited by ouabain. A tenfold greater concentration of ouabain (10 μM) was required to inhibit the relaxation of intact segments. This suggests that EpDRF production is temperature-dependent, and that, once released, it stimulates Na^+/K^+-pump activity. The factor, in a sense, competes functionally with ouabain, and confers a degree of ouabain-resistance. A similar phenomenon was observed at 37°C in the effect of the epithelium on the K^+-induced, ouabain-sensitive relaxation response (Raeburn & Fedan, 1989). EpDRF, by stimulating electrogenic pump activity, may induce membrane hyperpolarization of guinea-pig trachealis and decrease reactivity to depolarizing agents.

Two procedures which activate electrogenic Na^+/K^+-pumping, i.e. the addition of K^+ to K^+-free solution (Raeburn & Fedan, 1989) and rapid warming (present study), induce relaxation responses which are sensitive to ouabain and yet modified by the epithelium. Nevertheless, the effects of ouabain at 37°C and reduced temperature on reactivity to methacholine are not equivalent. At 37°C ouabain increased sensitivity to methacholine but did not affect the maximum response, and mimicked epithelium removal in intact preparations (Raeburn & Fedan, 1989). If the effect of reduced temperature on reactivity to methacholine were to result solely from inhibition of the Na^+/K^+-pump, then sensitivity of the preparations at 22°C also should have been increased, but this was not observed even though evidence was obtained that the pump had been inhibited (Figure 3). A difference between the effect of ouabain at 37°C and reduced temperature is that a reduction in temperature probably interferes with other processes in the smooth muscle cell related to reactivity. It has been suggested that alterations in the release of intracellular Ca^{2+} and Ca^{2+} extrusion mechanisms may be involved in cooling-induced potentiation of responses of (presumably) epithelium-containing tissues (Ishii & Shimo, 1985; 1987). In view of the profound effects of epithelium removal on the sensitivity and maximum responses to methacholine at 22°C, the possible involvement of epithelium-derived factor(s) in these mechanisms might be worthy of investigation.

In summary, the modulatory influence of the epithelium on reactivity of guinea-pig trachealis is changed at reduced temperature, i.e. the epithelium decreases reactivity at 37°C and increases reactivity at 22°C. The epithelium influences the relaxation response of the cooled smooth muscle to abrupt warming, which involves stimulation of electrogenic Na^+/K^+-pumping. The modulation of reactivity at 37°C may involve, at least in part, stimulation of electrogenic Na^+/K^+-pump activity.

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