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The effects of alterations in electrogenic Na^+/K^+-pumping in guinea-pig isolated trachealis: their modulation by the epithelium

[1]David Raeburn & [2]Jeffrey S. Fedan

Physiology Section, Division of Respiratory Disease Studies, National Institute for Occupational Safety and Health, 944 Chestnut Ridge Road, Morgantown, WV 26505-2888 U.S.A.

1 An examination was made of the effect of epithelium removal on mechanical responses of guinea-pig isolated tracheal strips after inhibition or activation of electrogenic Na^+/K^+-pumping.

2 The Na^+/K^+-pump inhibitor ouabain (0.1–10 μM) evoked concentration-dependent contractions which were potentiated by epithelium removal.

3 K^+-free solution, which inhibits Na^+/K^+-pumping, produced a slow, sustained relaxation in intact preparations. In epithelium-free preparations the relaxation was transient and of lesser magnitude.

4 The addition of K^+ (10 or 30 mM), which activates Na^+/K^+-pumping, to preparations bathed in K^+-free solution caused a relaxation of preparations under spontaneous tone or contracted with methacholine; the magnitude and duration of relaxation was greater in the epithelium-free preparations. Ouabain (0.1 μM) attenuated the relaxation to K^+ in intact preparations and converted the response of epithelium-free preparations to a contraction. In the presence of a higher concentration of ouabain (1 μM), intact preparations contracted in response to K^+.

5 In normal K^+ solution, ouabain (0.1 μM) increased the sensitivity of intact preparations to methacholine but reduced their sensitivity to K^+. Ouabain was without these effects in epithelium-free preparations.

6 Thus, responses of intact preparations to perturbations which affect electrogenic Na^+/K^+-pumping in trachealis are influenced by an epithelium-derived factor. The production of the factor may be linked to an epithelial Na^+/K^+-pump, or the factor may modulate the activity of an electrogenic Na^+/K^+-pump in the muscle.

Introduction

An electrogenic Na^+/K^+-pump is present in airway smooth muscle (Souhrada *et al.*, 1981; Souhrada & Souhrada, 1981; 1983; Gunst & Stropp, 1988). Inhibition of the electrogenic Na^+/K^+-pump with ouabain or by reducing the extracellular K^+ concentration induces depolarization of airway smooth muscle (Souhrada *et al.*, 1981). Activation of the Na^+/K^+-pump by the addition of K^+ to K^+-free solution leads to membrane hyperpolarization (Souhrada *et al.*, 1981). In response to pump inhibition the smooth muscle may contract, or the response to other contractile agents may be augmented (Fleming, 1980; Souhrada & Souhrada, 1981). In contrast, the addition of K^+ to K^+-free medium results in hyperpolarization and relaxation which may be inhibited by ouabain (Fleming, 1980; Souhrada & Souhrada, 1981). The activity of the Na^+/K^+-pump is increased in ovalbumin-sensitized guinea-pigs. The resting membrane potential of the trachealis cells assumes a more negative value and the tissue exhibits enhanced reactivity to bronchoconstrictor agents such as histamine (Souhrada & Souhrada, 1984).

Cardiac glycosides cause contraction of canine airway smooth muscle *in vivo* (Marco *et al.*, 1968) and potentiate bronchoconstrictors acting on guinea-pig airway smooth muscle *in vitro* (Kolbeck *et al.*, 1981) and *in vivo* (Agrawal & Hyatt, 1986).

[1] Present address: Biological Research, Rhone-Poulenc, Rainham Road South, Dagenham, Essex RM10 7XS.
[2] Author for correspondence.

Ouabain can evoke bronchoconstriction in asthmatics (Agrawal *et al.*, 1986).

In several mammalian species the airway epithelium modulates the reactivity of the underlying airway smooth muscle (see reviews by Vanhoutte, 1987; Fedan *et al.*, 1988) by releasing an epithelium-derived relaxing factor (EpDRF) (Flavahan *et al.*, 1985; Hay *et al.*, 1986; 1987; Ilhan & Sahin, 1986; Tschirhart & Landry, 1986). The relevance of this modulation to bronchial asthma lies in the fact that epithelial cell loss or damage occurs in association with airway hyperreactivity (Laitinen *et al.*, 1985).

Epithelium removal increases the reactivity of airway smooth muscle preparations to some agents but not to others (see Fedan *et al.*, 1988 for examples). This is a situation analogous to that which occurs in postjunctional supersensitivity, where increases in reactivity are sometimes associated with a reduction in electrogenic Na^+/K^+-pumping (Fleming, 1980). Procedures which inhibit the Na^+/K^+-pump prevent the endothelium-dependent relaxation of arterial smooth muscle to acetylcholine (De Mey & Vanhoutte, 1980; Rapoport *et al.*, 1985).

In view of the relationship between electrogenic Na^+/K^+-pumping, resting potential and reactivity in airway smooth muscle, we reasoned that the Na^+/K^+-pump could be involved in the regulation of smooth muscle reactivity by the epithelium. We examined the effects of epithelium removal on responses of airway smooth muscle after alterations in the activity of the Na^+/K^+-pump. A preliminary account of this study has been given (Raeburn *et al.*, 1987).

Methods

Preparation of tracheal strips

Male English short-hair guinea-pigs (340–500 g); Camm Research Institute, Inc.; Wayne, NJ) were killed by stunning and bleeding. The trachea was removed, placed in a modified Kreb-Henseleit (MKH) solution and cleaned. The trachea was opened by cutting in the longitudinal axis of the organ, diametrically opposite the trachealis muscle. Strips consisting of two adjacent cartilage rings were prepared. The strips were set up in organ baths containing MKH at 37°C for the isometric measurement of tension changes. The preparations were equilibrated for 1 h under an optimum resting load of 1 g, and washed every 15 min before the start of each experiment. The MKH contained (mM): NaCl 113, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 5.7. The solution was gassed with 95% O_2–5% CO_2 to give a pH of 7.4. K^+-free MKH was prepared by omitting KCl from the MKH and replacing KH_2PO_4 with NaH_2PO_4.

The epithelium was removed by gently rubbing the luminal surface with a cotton-tipped applicator. This procedure has been demonstrated histologically to remove the epithelium (Hay *et al.*, 1986). To minimize possible regional differences in reactivity, comparisons were made between adjacent strips from the same area of trachea, i.e. one intact and one rubbed. This permitted paired analysis between intact and epithelium-free preparations. The region of trachea used was randomized with respect to the particular protocol being studied.

Concentration-response curves

Cumulative concentration-response curves for ouabain, K^+ or methacholine were constructed by use of 1.6 fold (K^+), 3.2 fold (methacholine) or 10 fold (ouabain) concentration increments. The effect of ouabain on methacholine and K^+ concentration-response curves was assessed after 5 min incubation with ouabain.

K^+-free MKH and addition of K^+ to K^+-free MKH

After equilibration of the tracheal strips with MKH, the bathing solution was changed to K^+-free MKH. K^+-free MKH solution itself induced relaxant responses, which were monitored for up to 60 min. As no further changes in force occurred after 40 min, this time was chosen as the period of exposure to K^+-free MKH solution. The preparations were then either left under spontaneous tone, or tone was increased with methacholine. EC_{60} concentrations of methacholine (as observed in MKH) were used to raise the tone of intact or epithelium-free preparations, i.e. 2 μM and 1 μM, respectively. K^+ (10 or 30 mM) was then added. In some experiments, ouabain (0.1 or 1 μM) was added 5 min before the addition of K^+.

Effects of a mixture of inhibitors and indomethacin

Where indicated, the effects of a mixture of the following inhibitors were evaluated 15 min after it was added to the organ chambers: diphenhydramine (0.1 μM), atropine (0.1 μM), propranolol (1 μM) and tetrodotoxin (1 μM). These inhibitors were used to ascertain whether the effects of ouabain, K^+-free MKH, or the addition of K^+ to K^+-free MKH, were direct or involved the release of histamine, acetylcholine or catecholamines present in the preparations. The mixture of inhibitors had no effect on tone.

In some experiments, indomethacin (1 μM) was added to the baths, as indicated, to inhibit cyclo-oxygenase activity.

Vehicle controls (drug solvent only) were run in parallel; no vehicle effects were observed.

Statistical analysis

The results were expressed as % maximum response, force of contraction or relaxation (mg), or as the duration of the response(s) and are given as mean ± s.e.mean; n is the number of separate experiments. Geometric mean EC_{50} values were determined from linear regression analysis of probit-transformed data. The data were evaluated for differences by use of Student's t test for paired samples; $P < 0.05$ was considered significant. In the figures the s.e.mean is shown by the vertical bars, unless enclosed within the symbol.

Drugs

The following drugs (Sigma Chemical Co., St. Louis, MO, U.S.A.) were used: atropine sulphate, diphenhydramine hydrochloride, indomethacin, methacholine chloride, ouabain, (±)-propranolol and tetrodotoxin. All were dissolved in distilled water, with the exception of indomethacin (in 95% ethanol) and tetrodotoxin (in 0.9% NaCl). Drug solutions were prepared freshly each day.

Results

Ouabain concentration-response curves

Ouabain (0.1–10 μM) evoked contraction of intact and epithelium-free strips (Figure 1). The maximum responses of both preparations occurred at 10 μM; larger concentrations evoked no further contraction. EC_{50} values were obtained from probit analysis by use of two data points (as there is no probit value for 100%), and are, therefore, approximations. Epithelium removal increased significantly the sensitivity to ouabain ($-\log EC_{50}$ (M) values were 5.66 ± 0.18 in intact, and 6.30 ± 0.88 in denuded, preparations). The magnitude of responses, expressed both as % maximum response and force developed, also were significantly increased (maximum responses were 273 ± 89 mg in intact, and 560 ± 180 mg in denuded, preparations).

Similar results were obtained in the presence of the mixture of inhibitors. In denuded preparations the sensitivity to ouabain increased significantly, with $-\log EC_{50}$ (M) values decreasing from the control value of 5.77 ± 0.10 to 6.10 ± 0.30 in the absence of the epithelium. The maximum force of contraction again was increased after epithelium removal (from 356 ± 53 mg for intact preparations to 432 ± 108 mg for epithelium-free preparations), but the difference in the maximum force of contraction just failed to achieve significance.

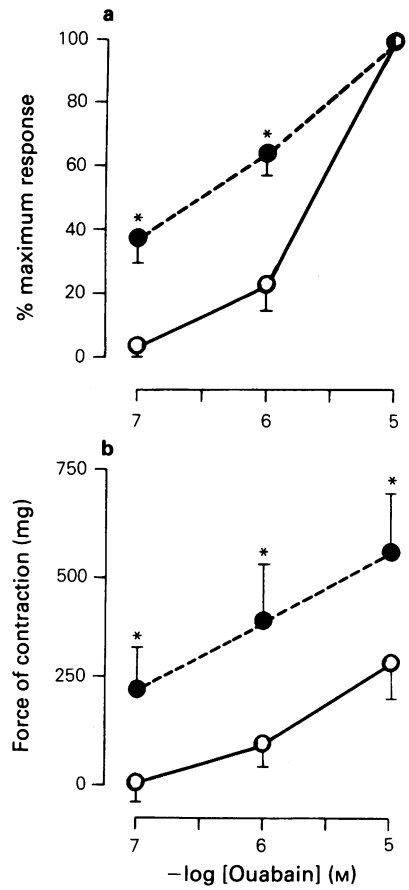

Figure 1 Guinea-pig isolated tracheal strips: effect of epithelium removal on the ouabain concentration-response curve. (○) Intact preparations; (●) epithelium-free preparations. In (a) the contractile responses to ouabain are given as % of the respective maximum responses; in (b) the responses to ouabain are expressed as the force of contraction. *Significantly different from epithelium-containing, paired preparations. $n = 5$.

The mixture of inhibitors did not significantly affect sensitivity to ouabain either in intact or denuded preparations.

Effect of K^+-free MKH

Upon exposure to K^+-free MKH, intact preparations relaxed, reaching a maximum relaxation of 533 ± 60 mg at approximately 40 min (Figures 2 and 3). Epithelium-free strips also relaxed during the first 10 min but then gained some tone. The peak tone achieved was less than the initial level, was well-maintained between 20 and 30 min after exposure to the K^+-free MKH but thereafter began to decline.

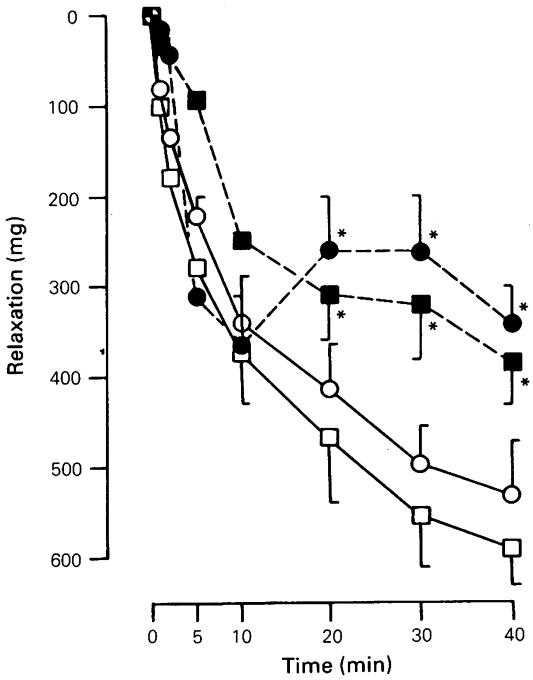

Figure 2 Guinea-pig isolated tracheal strips: time-course of the effect of K^+-free modified Krebs-Henseleit solution (MKH) on tone of intact and epithelium-free preparations in the absence or presence of the mixture of inhibitors. (□, ○) Intact preparations; (■, ●) epithelium-free preparations. (○, ●) Mixture of inhibitors absent; (□, ■) mixture of inhibitors present. Ordinate scale: the magnitude of relaxation from the level of force present in MKH solution before the solution was changed to K^+-free MKH. * Significantly different from epithelium-containing, paired preparations. $n = 9$, mixture of inhibitors absent or present.

Neither the time-course nor the magnitude of relaxation at 40 min (593 ± 56 mg) was affected in the presence of the mixture of inhibitors. In the absence or presence of the mixture of inhibitors, the relaxation at 40 min by epithelium-free strips was significantly less than that seen in intact preparations. No relaxation was observed during this time period when intact or epithelium-free strips were incubated in K^+-containing MKH.

Effects of K^+ added to preparations incubated in K^+-free MKH

K^+ (10 or 30 mM), added to preparations equilibrated with K^+-free MKH and in the absence or presence of methacholine, caused an initial relaxation (Figure 3; Table 1); this was followed by an increase in tone to a level greater than the pre-K^+ level. Relaxations produced by 10 mM K^+ (the EC_{25} for contraction in normal MKH) were larger and were of longer duration than relaxations to 30 mM K^+ (the EC_{50} for contraction in normal MKH). Relaxations to 30 mM K^+ in methacholine-contracted strips were larger than those obtained in the absence of methacholine. With one exception (not shown), the magnitude and duration of the K^+-induced relaxations were always greater in the epithelium-free preparations. When the mixture of inhibitors was present and the K^+ concentration was 30 mM, the relaxation to K^+ was attenuated and there was no difference in the magnitude of the relaxations between intact and epithelium-free preparations. However, the duration of relaxation was prolonged in denuded preparations.

In intact and rubbed preparations indomethacin (1 μM) alone was without effect on the response to the re-addition of K^+, i.e. results similar to those shown in Figure 3 were obtained ($n = 4$).

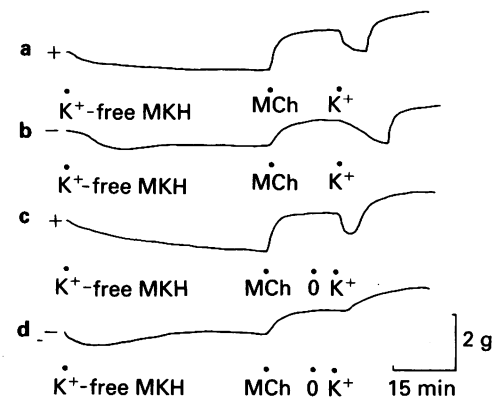

Figure 3 Guinea-pig isolated tracheal strips equilibrated with K^+-free modified Krebs-Henseleit solution (MKH) and methacholine: effects of epithelium removal and ouabain (0; 0.1 μM) on relaxant responses evoked by 30 mM K^+. (a and b) K^+-induced relaxation of intact (a) and epithelium-denuded (b) preparations in the absence of ouabain; (c and d) K^+-induced relaxation of intact (c) and epithelium-denuded (d) preparations in the presence of ouabain. The concentrations (EC_{60}) of methacholine (MCh) were 1 μM in denuded preparations and 2 μM in intact preparations. (+) and (–) refer to intact and rubbed preparations, respectively. After their addition (dots), methacholine, ouabain and K^+ remained present for the duration of the experiment. Note that in (a, b, c) the relaxation to K^+ was followed by an abrupt (a, b), gain in tone in the continued presence of K^+. The tracings shown are representative of identical results obtained in six separate experiments. Note that ouabain itself had no effect on methacholine-induced tone.

Table 1 Effect of K^+ (10 or 30 mM) on intact (+Epi) and epithelium-free (−Epi) tracheal preparations incubated for 40 min in K^+-free Krebs-Henseleit solution

Treatment	n	*Relaxation* (mg) +*Epi*	*Relaxation* (mg) −*Epi*	*Duration of relaxation* (s) +*Epi*	*Duration of relaxation* (s) −*Epi*
Spontaneous tone					
10 mM K^+	4	825 ± 87*	1038 ± 80*‡	550 ± 79	1150 ± 120‡
30 mM K^+	6	180 ± 77*	345 ± 89*‡	323 ± 6	413 ± 26‡
Methacholine-contracted					
30 mM K^+	6	718 ± 138*	1125 ± 193*‡	405 ± 12	615 ± 43‡
30 mM K^+, mixture of inhibitors present	6	388 ± 53*	344 ± 91*	324 ± 53	442 ± 72‡
10 mM K^+, 0.1 μM ouabain present	4	210 ± 56*	†	130 ± 18	ND
30 mM K^+, 0.1 μM ouabain present	6	375 ± 102*	†	142 ± 19	ND

* Transient relaxation followed by sustained contraction (see Figure 3).
† Only contractions were obtained.
‡ Significantly different from +Epi.
ND, not determined; response stable > 2 h.

Effect of ouabain on K^+-induced relaxation responses

The effect of ouabain (0.1 μM) on the relaxation response of methacholine-contracted strips to 30 mM K^+ depended on whether the epithelium was present or not (Figure 3; Table 1). In intact preparations, the K^+-induced relaxation was still evident in the presence of ouabain (Figure 3c); the relaxation, which had an altered profile, was followed by a sustained contraction. In the absence of the epithelium, however, ouabain (0.1 μM) inhibited the K^+-induced relaxation (Figure 3c); the response was converted to a contraction. Similar results were obtained with 10 mM K^+, and in tissues under 'basal' tone (not shown). In intact and rubbed control preparations incubated only in normal MKH and pre-contracted with methacholine, K^+ (10 and 30 mM) elicited only contractile responses (not shown; $n = 3$). The K^+-induced relaxations were therefore obtained only in preparations which had been incubated in K^+-free MKH. Identical results were obtained in the presence of the mixture of inhibitors ('basal' tone preparations). At a higher concentration of ouabain (1 μM), the relaxation of intact preparations was fully inhibited, and K^+ elicited a contraction as it did in the epithelium-free preparations, i.e. results comparable to Figure 3d were obtained ($n = 5$).

Effect of ouabain on methacholine and K^+ concentration-response curves

In intact preparations ouabain (0.1 μM) produced a significant (ca. 4 fold) leftward shift in the methacholine concentration-response curve, and a significant (ca. 1.6 fold) rightward shift in the K^+ concentration-response curve (Figure 4; Table 2). In the absence of the epithelium, ouabain had no effect on the sensitivities of the preparations to meth-

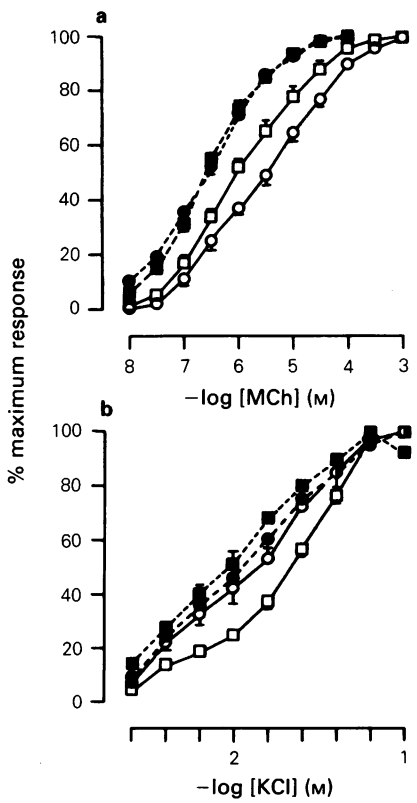

Figure 4 The effect of ouabain (0.1 μM) on cumulative concentration-response curves for methacholine (a; $n = 6$) and K^+ (b; $n = 5$) in guinea-pig isolated tracheal strips. (□, ○) Intact preparations; (■, ●) epithelium-free preparations. (○, ●) Ouabain absent (control); (□, ■) ouabain present. Responses are plotted as a % of the preparations' respective maximum responses.

Table 2 Effect of ouabain (0.1 μM) on the sensitivities and maximum responses to methacholine and K^+ of intact (+Epi) and epithelium-free (−Epi) tracheal strips

		+Epi		−Epi	
Agent	n	$-log\ EC_{50}$ (M)	*Maximum* (mg)	$-log\ EC_{50}$ (M)	*Maximum* (mg)
Methacholine					
Control	6	5.32 ± 0.14	743 ± 108	6.70 ± 0.01*	590 ± 53
Ouabain	6	5.89 ± 0.12†	600 ± 95	6.70 ± 0.01*	643 ± 141
K^+					
Control	5	1.87 ± 0.10	1285 ± 330	1.92 ± 0.08	1120 ± 181
Ouabain	5	1.67 ± 0.01†	920 ± 133	1.99 ± 0.10*	910 ± 180

* Significantly different from intact preparations.
† Significantly different from control.

acholine or K^+ (Figure 4; Table 2). Ouabain (0.1 μM) did not affect the maximum responses to methacholine or K^+ in strips containing or denuded of epithelium (Table 2).

Discussion

This study indicates that the epithelium influences the response of airway smooth muscle to alterations in electrogenic Na^+/K^+-pumping activity caused by ouabain, K^+-free MKH and the addition of K^+ to preparations in K^+-free MKH. The results indicate a direct action of ouabain, K^+-free MKH and added K^+ on the muscle, and an indirect component involving the epithelium. Responses to these manipulations do not appear to involve non-epithelial, indirect components to any appreciable extent, since similar results were obtained in the presence of the mixture of inhibitors. Chideckel *et al.* (1987) also found no evidence for an indirect component in the responses of airway smooth muscles of several species to ouabain.

The reactivities of intact guinea-pig tracheal preparations to methacholine and K^+ were altered by ouabain in an epithelium-dependent manner. Ouabain decreased the reactivity to K^+ only in the presence of the epithelium, and had no effect in epithelium-free preparations. Ouabain increased the responsiveness of intact preparations to methacholine, and, as such, partially mimicked the effect of epithelium removal. However, ouabain had no effect on the reactivity to methacholine in denuded preparations. The effects of ouabain and epithelium removal were not additive, and could, therefore, involve the same mechanism, i.e. the electrogenic Na^+/K^+-pump.

Both intact and epithelium-free preparations incubated in K^+-free MKH responded to added K^+ with relaxation; this presumably occurs in association with membrane hyperpolarization due to activation of electrogenic Na^+/K^+-pumping (Souhrada *et al.*, 1981). In the presence of ouabain (0.1 μM) to block activation of the Na^+/K^+-pump, the addition of K^+ to epithelium-free preparations caused only a contraction. As the pump was inhibited, this response presumably reflected membrane depolarization. Our results obtained with denuded strips are identical to those obtained by Souhrada *et al.* (1981), who also removed the epithelium but used a higher concentration of ouabain (10 μM).

An explanation must be sought for our observation that the addition of K^+ to intact preparations, equilibrated with K^+-free MKH and with 0.1 μM ouabain, induced relaxation while contraction was observed under similar circumstances for the epithelium-denuded preparations. A satisfactory explanation should also take into account our observation that 1 μM ouabain abolished the relaxant responses to K^+ in both intact and epithelium-free preparations. Two possibilities, either alone or in combination, may account for our findings. The first is that the production and/or release of an inhibitory factor is linked to the activity of the epithelial Na^+/K^+-pump. The basolaterally-located Na^+/K^+-pump in epithelial cells (reviewed by Nadel *et al.*, 1985; Widdicombe *et al.*, 1987), which is involved in glandular secretory activity (Marin, 1986), should be affected, although not necessarily identically, by the perturbations which affect the corresponding pump in the trachealis, i.e. it would be inhibited in K^+-free MKH, and activated upon the addition of K^+. Our findings can be explained if the epithelial pump differs from the muscle pump in other aspects, such as sensitivity to ouabain and K^+.

The alternative hypothesis is that EpDRF stimulates the activity of the muscle electrogenic Na^+/K^+-pump. The K^+-induced relaxation of intact preparations in the presence of 0.1 μM ouabain might reflect the release of EpDRF from the epithelium, such as has been described by Munakata *et al.* (1988). However, the inhibitory effect of 1 μM ouabain on K^+-induced relaxation of intact preparations must also be explained, and it is convenient

to consider a muscle Na^+/K^+-pump site to explain why this higher concentration is effective. The effects of ouabain and of the presence of the epithelium are, in a sense, opposite. EpDRF possibly facilitates muscle pump turnover when K^+ is added, even when the pump is partially inhibited (by 0.1 μM ouabain), or EpDRF reduces the binding of 0.1 μM ouabain, allowing K^+ to promote pump cycling. However, the amount of EpDRF is insufficient to prevent the effect of 1 μM ouabain, and the response to K^+ is abolished.

Inhibition of electrogenic Na^+/K^+-pumping with ouabain or reduced extracellular K^+ results in a partial membrane depolarization and alters airway smooth muscle reactivity to several agents (Souhrada & Souhrada, 1981; Souhrada *et al.*, 1981; Gunst & Stropp, 1988). The loss of an epithelium-derived factor, which stimulates the muscle Na^+/K^+-pump and causes membrane hyperpolarization, could explain the increases in reactivity after epithelium removal. However, epithelium removal does not significantly change the resting membrane potential in canine bronchial smooth muscle, but nevertheless potentiates the contraction and depolarisation induced by acetylcholine (Gao & Vanhoutte, 1988). It must be admitted that these findings in canine preparations are difficult to reconcile with the hypothesis that a change in activity of the Na^+/K^+-pump in the smooth muscle rather than in the epithelium is involved in the effects observed in the present study.

In the absence of ouabain the relaxation of intact preparations to K^+ was faster in onset compared to the epithelium-free strips, but its duration was shorter (Figure 3). It is probable that a linkage of EpDRF production to the epithelial Na^+/K^+-pump, or a stimulating action of EpDRF on the smooth muscle pump, explains the difference in the relaxation profiles of intact and epithelium-free strips. It is unlikely that K^+-induced relaxation involved a direct action of EpDRF, inasmuch as it was produced by 10 mM K^+. The hyperosmotic challenge-induced release of the factor (Munakata *et al.*, 1988) at 10 mM K^+ would have led to very small relaxation responses compared to the large ones seen in the present study.

Why the preparations relaxed when exposed to K^+-free MKH is unclear. This effect, not observed by Souhrada & Souhrada (1981), was seen by Chideckel *et al.* (1987). It is possible that, in our experiments, hyperpolarization in K^+-free MKH has a greater initial effect on membrane potential than depolarization resulting from inhibition of electrogenic pump current (Fleming, 1980). Indeed, Souhrada *et al.* (1981) observed hyperpolarization to K^+-free solution at times similar to those where we saw relaxation.

When placed in K^+-free solution, denuded preparations first lost and then gained some tone, whereas intact preparations only exhibited relaxation (Figures 2 and 3). The difference in the profiles of this response appears to be related to the presence of the epithelium, but the mechanism involved is unknown.

It is likewise difficult to explain the reduced sensitivity of intact strips to K^+ in the presence of ouabain under conditions that increased sensitivity to methacholine. This finding argues against the hypothesis that the potentiation of responses to methacholine by ouabain or by epithelium removal results from reduced electrogenic Na^+/K^+-pumping, since responses to K^+, being triggered electromechanically, should in theory have been potentiated (Fleming, 1980). Nevertheless, the findings that only ouabain reduced sensitivity to K^+, and that this effect occurred only in intact preparations, favours the general conclusion that the epithelium affects reactivity of the smooth muscle by a mechanism involving the Na^+/K^+-pump.

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