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Osmotic Regulation of Airway Reactivity by Epithelium[1]

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ABSTRACT

Inhalation of nonisotonic solutions can elicit pulmonary obstruction in asthmatic airways. We evaluated the hypothesis that the respiratory epithelium is involved in responses of the airways to nonisotonic solutions using the guinea pig isolated, perfused trachea preparation to restrict applied agents to the mucosal (intraluminal) or serosal (extraluminal) surface of the airway. In methacholine-contracted tracheae, intraluminally applied NaCl or KCl equipotently caused relaxation that was unaffected by the cyclo-oxygenase inhibitor, indomethacin, but was attenuated by removal of the epithelium and Na^+ and Cl^- channel blockers. Na^+-K^+-$2Cl^-$ cotransporter and nitric oxide synthase blockers caused a slight inhibition of relaxation, whereas Na^+,K^+-pump inhibition produced a small potentiation. Intraluminal hyperosmolar KCl and NaCl inhibited contractions in response to intra- or extraluminally applied methacholine, as well as neurogenic cholinergic contractions elicited with electric field stimulation (± indomethacin). Extraluminally applied NaCl and KCl elicited epithelium-dependent relaxation (which for KCl was followed by contraction). In contrast to the effects of hyperosmolarity, intraluminal hypo-osmolarity caused papaverine-inhibitable contractions (± epithelium). These findings suggest that the epithelium is an osmotic sensor which, through the release of epithelium-derived relaxing factor, can regulate airway diameter by modulating smooth muscle responsiveness and excitatory neurotransmission.

Exercise may cause airway obstruction in asthmatics. This has been thought to be initiated by water loss causing hyperosmolarity of the airway hypophase, as well as airway cooling and the release of bronchoactive mediators (McFadden et al., 1986). Inhaled hypo-osmolar, hyperosmolar, and isotonic aerosols also can elicit pulmonary obstruction in asthmatics and in laboratory animals (Osborne et al., 1987; Eichler et al., 1992; Fujimura et al., 1997) through the release of mediators such as histamine, leukotrienes and bradykinin (Finnerty et al., 1985; Umeno et al., 1990; Makhdum and Pearce, 1993). The precise mechanisms responsible for the obstructive responses are unclear. Circulation through the mucosal vasculature of the airways also is affected by hyperosmolar (vasodilation involving nitric oxide) and hypoosmolar (vasoconstriction) solutions applied to the mucosal surface (Smith et al., 1993; Prazma et al., 1994; Wells et al., 1994).

The airway epithelium is an important regulator of respiratory smooth muscle tone and reactivity (see Fedan et al., 1988 and Goldie and Hay, 1997 for review) because it is a diffusion barrier, a site of drug metabolism, and mediates the actions of some drugs. The epithelium also releases prostanoids and the nonprostanoid, nonnitric oxide inhibitory substance, epithelium-derived relaxing factor (EpDRF), which alters reactivity to contractile agonists, relaxant agonists, and allergen (Flavahan et al., 1985; Barnes et al., 1985; Hay et al., 1986a,b, 1987; Ilhan and Sahin, 1986; Grundström et al., 1992). Hyperosmolar solutions applied to the mucosal surface of guinea pig isolated, perfused trachea cause an epithelium-dependent relaxation of the smooth muscle via the release of EpDRF (Munakata et al., 1988; Fedan et al., 1990). The production of EpDRF and/or its inhibitory effects on the smooth muscle has been suggested to be linked to the Na^+,K^+-pump and Ca^{2+}-dependent K^+ channels (Raeburn and Fedan, 1989; Lamport and Fedan, 1990; Tamaoki et al., 1997).

In this study we hypothesized that the airway epithelium mediates or participates in responses of the airways to nonisotonic solutions and in the effects of nonisotonic solutions on airway reactivity to agonists. We employed the guinea pig isolated, perfused trachea preparation to examine the roles of epithelium in regulating smooth muscle diameter and reactivity to drugs because it allows separate delivery of agents to the mucosal (intraluminal) or serosal (extraluminal) surfaces (Munakata et al., 1989; Fedan et al., 1990; Fedan and Frazer, 1992; Kitano et al., 1992). Agents applied

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ABBREVIATIONS: DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulfonate; L-NAME, *N*ω-nitro-L-arginine methyl ester; EpDRF, epithelium-derived relaxing factor; C.I., confidence interval.

to the intraluminal perfusate affect the smooth muscle after having diffused across the epithelium, whereas agents applied to the serosal surface have direct access to the smooth muscle; consequently, reactivity to extraluminally applied contractile agonists is generally greater than after mucosal addition (Munakata et al., 1990; Fedan et al., 1990; Fedan and Frazer, 1992). With the regulatory role of the airway epithelium on smooth muscle reactivity in mind, the purposes of this study were to investigate 1) the relationship between luminal osmolarity and smooth muscle tone and reactivity to methacholine (MCh); 2) polarity across the epithelium in the effects of hyperosmolar solutions; 3) the effects of agents that inhibit prostanoid and nitric oxide formation, Na^+ and Cl^- channels, and ion pumping and transport mechanisms; and 4) the effects of raised intraluminal osmolarity on postganglionic nerve-mediated mechanical responses of the smooth muscle. The companion article following this one (Dortch-Carnes et al., 1999) describes the relationships between epithelial bioelectric responses triggered by nonisotonic solutions and the ensuing smooth muscle mechanical events.

Materials and Methods

Guinea Pig Isolated, Perfused Trachea Preparation. The experimental protocols were approved by the institutional Animal Care and Use Committee. Male English short-hair SPF guinea pigs (457–742 g; Camm Research Institute, Wayne, NJ and Harlan Sprague-Dawley, Inc., Indianapolis, IN) were anesthetized with sodium pentobarbital (65 mg/kg, i.p.). Four centimeters of the trachea was removed, placed in modified Krebs-Henseleit (MKH) solution, and cleaned. The segment was mounted onto a perfusion holder that contained indwelling side-hole catheters that were connected to the positive (inlet) and negative (outlet) sides of a differential pressure transducer, as described previously (Fedan and Frazer, 1992). The holder was placed into a 25-ml bath containing MKH solution (37°C), which is referred to as the serosal or extraluminal bath. The trachea was perfused (34 ml/min) with recirculating MKH solution (37°C) from a separate, 30-ml reservoir, which is referred to as the mucosal or intraluminal bath. Transmural pressure was adjusted to zero. Responses were measured as changes in the inlet minus outlet pressure difference (ΔP), in cm of H_2O. A 1-h equilibration period was allowed before the experiment while washing the preparations at 15-min intervals by changing the MKH solution in both baths.

MKH solution contained: 113.0 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$, and 5.7 mM glucose, pH 7.4 (37°C), and was gassed with 95% O_2-5% CO_2.

Epithelium Removal. To remove the epithelium from the trachea (>95%; Fedan and Frazer, 1992), before it was mounted to the perfusion apparatus, a 5- to 6-cm piece of trimmed pipe cleaner brush was advanced slowly into the lumen and withdrawn while rotating slowly.

MCh Concentration-Response Curves. MCh was added in stepwise-increasing, cumulative concentrations to the extra- or intraluminal baths. Two concentration-response curves were obtained, the first as a control and the second to examine the effect under study. The preparations were washed at 15-min intervals for 1.5 h between the concentration-response determinations. None of the effects on MCh concentration-response curves shown in *Results* are observed in two, consecutive control curves (Fedan and Frazer, 1992).

Concentration-Response Curves for Raised or Lowered Intraluminal Osmolarity. Concentration-response curves for relaxation responses to KCl or NaCl applied intraluminally to elevate osmolarity were generated after having obtained a stable contraction with extraluminally added MCh (3×10^{-7} M; ~EC_{50}). Two such curves were obtained, the first as a control and the second, obtained after 1.5 h of washing, in the presence of the agent under study. The concentrations of the salts shown in the figures refer to the molar concentrations added to the MKH solution. In normosmolar MKH solution, [Na^+], [K^+] and [Cl^-] are 138, 6, and 122.8 mM, respectively.

To examine concentration-response relationships for contractile responses to intraluminal hypo-osmolarity, distilled water was added to the MKH solution in the intraluminal bath in volumes needed to achieve the desired reductions in osmolarity.

Comparison of Responses to Extra- versus Intraluminal Hyperosmolarity. Responses to elevated extra- and intraluminal osmolarity in the same trachea were compared using a paired design protocol. The preparation was contracted with extraluminally added MCh (3×10^{-7} M), after which 5.62, 13.3, or 80 mM NaCl was added to the extraluminal bath. After response stabilization, the same concentration of NaCl was applied to the intraluminal bath. The preparation was then washed repeatedly and allowed to equilibrate for 1.5 h. At the end of this period, MCh was added, and the procedure was repeated using KCl instead of NaCl. Only one concentration of NaCl and KCl was used in each experiment. These experiments were performed using intact and separate, epithelium-denuded tracheae.

Electric Field Stimulation of Perfused Trachea. After placement of the mounted trachea in the extraluminal bath, two platinum electrodes were aligned longitudinally on opposite sides of the trachea. The trachea was stimulated electrically with 10-s trains of square wave (120 V, 0.5 ms) pulses delivered at 7-min intervals. Two frequency-response curves, separated by 1.5 h of washing, were obtained; the first served as a control and the second was used to examine the effect under study. Neurogenic responses (contractile and relaxant phases, see *Results*) to electric field stimulation were blocked in the presence of the fast Na^+ channel blocker, tetrodotoxin (10^{-6} M; 30-min incubation), and contractions were antagonized by the muscarinic receptor blocker, atropine (10^{-6} M; 30-min incubation; not shown).

Inhibitors. When examining the effects of inhibitors, control preparations were always run simultaneously to monitor possible alterations in time that were independent of the test agent. There usually were no differences between the first and second curves in the controls, but in some cases changes in reactivity, although not significant, occurred that affected interpretation of the effect under study; these results will be shown as appropriate. The following agents were examined for their effects 30 min after addition to the extra- and intraluminal baths unless otherwise indicated in *Results*: the cyclo-oxygenase inhibitor, indomethacin (3×10^{-6} M); the Na^+ channel blocker, amiloride (10^{-4} M); the Cl^- channel blocker, 4,4′-diisothiocyano-2,2′-stilbene disulfonate (DIDS; 10^{-4} M); the Na^+-K^+-$2Cl^-$ cotransporter inhibitors, bumetanide and furosemide (10^{-4} M); the cAMP phosphodiesterase inhibitor papaverine; and the nitric oxide synthase inhibitor, *N*ω-nitro-L-arginine methyl ester (L-NAME; 10^{-4} M). The effects of the Na^+,K^+-pump inhibitor, ouabain (10^{-5} M), were examined 5 min after its addition to the extraluminal bath. This shorter incubation period was chosen to avoid the appearance of the slowly developing contraction that accompanies Na^+-loading of the muscle.

Analysis of Results. Responses were quantified as ΔP in centimeters of H_2O. Geometric mean EC_{50} values were derived from least-squares analysis of a four-parameter logit curve fit and are presented with 95% confidence intervals (C.I.) in parentheses. Statistical comparisons of EC_{50} values were done using normally distributed $-\log EC_{50}$ values. In *Results*, the EC_{50} values for KCl and NaCl are given in terms of molar concentration added to MKH solution. The results were analyzed for differences using one-way ANOVA, ANOVA on ranks, or Student's *t* test for paired or nonpaired samples, as appropriate. Other results are expressed as means ± S.E.M.; *n* is the number of separate experiments. $p < .05$ was considered significant.

The magnitude of ΔP responses varies with the fifth power of the radius (Munakata et al., 1989). Small differences in tracheal internal diameter even in animals of similar body weight from the same shipment caused variability in the magnitude of the ΔP response (Fedan and Frazer, 1992). To offset this confounding variable, whenever possible all comparisons were assessed using a within-trachea paired design, or statistical analyses were performed on normalized data, e.g., EC_{50}, responses expressed with reference to the contraction induced by MCh, etc. Examination of the effects of epithelium removal involved comparing intact and denuded tracheae of different animals; nonpaired statistical analysis was used in these cases.

Results

Relaxation Responses to Hyperosmolar Intraluminal MKH Solutions. Addition of intraluminal KCl to MCh (3×10^{-7} M)-contracted tracheae elicited relaxation. As little as 4.2 mM added KCl evoked the response (Fig. 1). The EC_{50} was 15.5 (C.I., 12.6–19.2) mM added KCl. This value was not affected by the cyclo-oxygenase inhibitor, indomethacin (3×10^{-6} M; not shown). Contraction to intraluminally added KCl never occurred in intact, epithelium-containing tracheae. In unstimulated tracheae, KCl added extraluminally resulted only in contraction; in epithelium-denuded tracheae, KCl added intra- or extraluminally resulted only in contraction (not shown). These findings indicate that the relaxant response to elevated intraluminal KCl concentration was dependent upon and mediated by the epithelium.

Added intraluminal NaCl also relaxed the trachea [Fig.1; EC_{50}: 15.7 (C.I., 12.7–19.5) mM], being equipotent with KCl ($p > .05$). In the absence of the epithelium, relaxation to NaCl was inhibited significantly (Fig. 1); the EC_{50} of the rightward-shifted concentration-response curve was 65.9 (C.I., 57.9–75.0) mM ($p < .05$ compared with intact tracheae).

Effect of Inhibitors on Responses to Intraluminal Hyperosmolarity. To circumvent the potential problem of KCl-induced contraction, NaCl was most often used to elevate intraluminal osmolarity, because the two salts had been found to be equipotent intraluminal relaxants (+epithelium). L-NAME (10^{-4} M; Fig. 2) inhibited slightly the relaxation to intraluminal NaCl; this effect resembled the changes seen in the curves of control preparations examined in the absence of L-NAME (Fig. 2), but the effect of the inhibitor was significant, whereas the changes in the controls were not. Amiloride

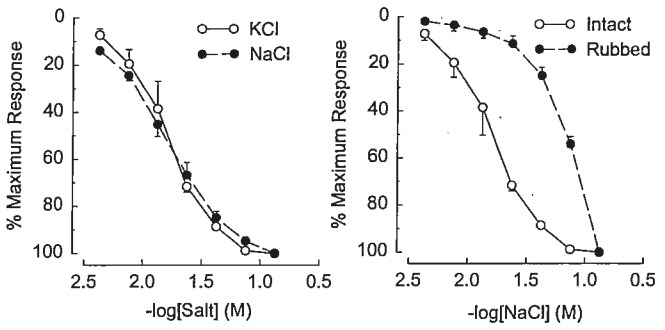

Fig. 1. Relaxation of intact tracheae by intraluminal hyperosmolarity; cumulative osmolar concentration-response curves for relaxation of MCh (3×10^{-7} M)-contracted tracheae. Concentrations on abscissa refer to added molar concentrations; additions in osmolar concentration terms are twice molar values. Left, comparison of intraluminal KCl ($n = 4$) and intraluminal NaCl ($n = 4$) concentration-response curves obtained from epithelium-containing, intact trachea. Right, comparison of intraluminal NaCl concentration-response curves obtained from epithelium-containing ("intact", $n = 4$) and epithelium-free ("rubbed", $n = 5$) trachea.

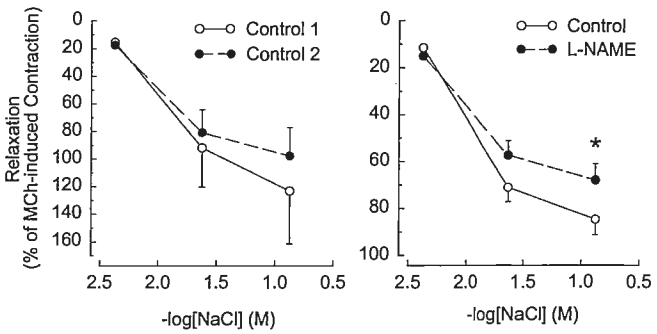

Fig. 2. Effect of L-NAME (10^{-4} M) on relaxation responses of MCh (3×10^{-7} M)-contracted, intact tracheae to intraluminally applied hyperosmolar NaCl. Left, NaCl concentration-response curves, the second obtained in the absence of L-NAME ($n = 6$). Right, NaCl concentration-response curves, the second obtained in the presence of L-NAME ($n = 6$; separate tracheae). *Maximum relaxation response significantly less than control. Note that, although not significant, a similar difference was seen between the two control curves.

(10^{-4} M) and DIDS (10^{-4} M), alone and in combination (Fig. 3), inhibited significantly intraluminal NaCl-induced relaxation. Amiloride also inhibited intraluminal KCl-induced relaxation responses ($p < .05$, $n = 7$; not shown), whereas DIDS alone ($n = 6$, not shown) and amiloride together with DIDS ($n = 6$; not shown) inhibited relaxation at a nearly significant level ($p < .06$).

Bumetanide (10^{-4} M) produced a modest inhibition ($p < .07$) only at the higher NaCl concentrations (Fig. 4), whereas when intraluminal KCl was used the effect of bumetanide was significant at the highest KCl concentration. These effects were not observed in the control intraluminal NaCl concentration-response curves ($n = 6$–8 for each protocol; not shown).

Ouabain (10^{-5} M) added to the extraluminal bath did not inhibit relaxation responses to intraluminally added NaCl, but caused a nearly significant ($p < .07$; Fig. 5) potentiation; such changes were not seen in control preparations.

The question of whether the effects of the inhibitors are polarized across the epithelium in relation to the apical or basolateral location of the ion transporters and channels (Fedan et al., 1994) was examined on a limited basis using amiloride. When amiloride was present only in the extraluminal bath, the drug had no effect on intraluminal NaCl-induced relaxation responses ($n = 4$; not shown); when amiloride was present in both the extra- and intraluminal baths,

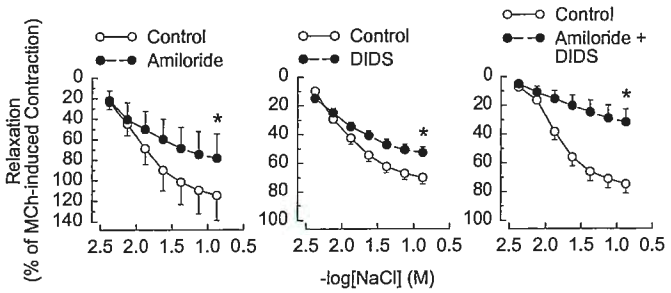

Fig. 3. Inhibitory effects of amiloride and DIDS, alone and together, on relaxation responses of MCh (3×10^{-7} M)-contracted, intact tracheae to intraluminally applied hyperosmolar NaCl. The second NaCl concentration-response curves were obtained in the presence of amiloride alone (left, 10^{-4} M; $n = 4$), DIDS alone (middle, 10^{-4} M; $n = 6$), or both agents (right, $n = 6$). *Maximum relaxation response significantly less than control.

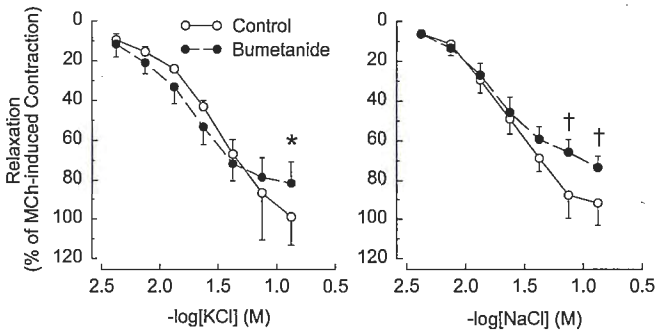

Fig. 4. Inhibitory effects of bumetanide (10^{-4} M) on relaxation responses of MCh (3×10^{-7} M)-contracted, intact tracheae to intraluminally applied hyperosmolar KCl (left, $n = 6$) and intraluminally applied NaCl (right, $n = 4$). *Maximum relaxation response significantly less than control. †Absence versus presence of bumetanide, $p < .07$.

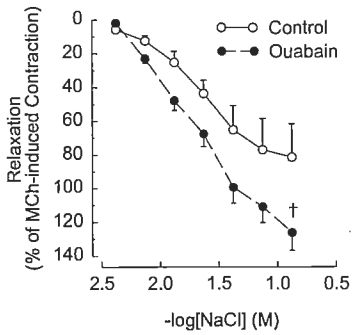

Fig. 5. Potentiating effect of ouabain (10^{-5} M, 5-min incubation) on relaxation response of MCh (3×10^{-7} M)-contracted, intact tracheae to intraluminally applied hyperosmolar NaCl ($n = 4$). †Maximum relaxation response in the absence versus presence of ouabain, $p < .07$.

however, relaxant responses to intraluminally added NaCl were inhibited in the manner depicted in Fig. 3 ($n = 8$). Thus, the polar inhibitory effect of amiloride was in agreement with the apical localization of Na^+ channels in respiratory epithelium.

Effects of Hyperosmolar Intraluminal MKH Solutions on Reactivity to MCh. Under control conditions, extraluminally applied MCh was appreciably and significantly more potent than intraluminally applied MCh (Table 1 and Fig. 6). Intraluminal hyperosmolarity was used to evoke EpDRF release, and the effects of released EpDRF on

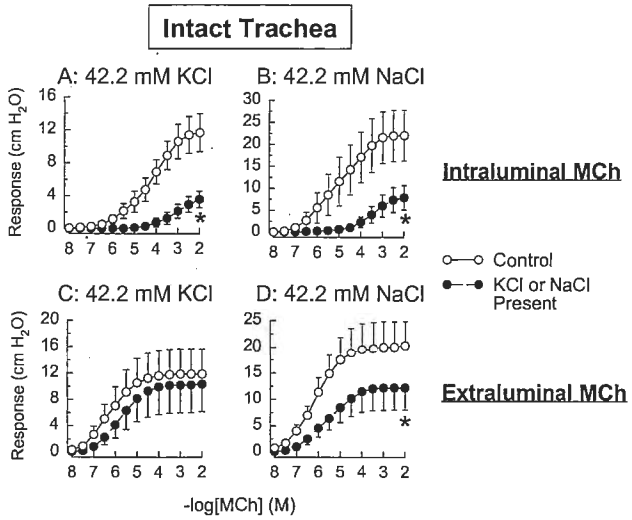

Fig. 6. Inhibitory effects of hyperosmolar intraluminal NaCl (A, C) or NaCl (B, D) on intraluminal (A, B) and extraluminal (C, D) MCh concentration-response curves of intact tracheae. n values were 4 to 7 for each group. *Maximum response significantly less than control. Note that maximum responses were reduced in all cases except C.

MCh concentration-response curves were assessed. Added KCl or NaCl concentrations were employed that approximated the EC_{50} and EC_{90} values for relaxation responses of intact trachea (i.e., 13.3 and 42.2 mM, respectively; Fig. 1). In both intact and rubbed preparations, the administration of intraluminal KCl or NaCl slightly decreased baseline in a few preparations, reflective of a small variable amount of spontaneous basal tone in the preparations. In the vast majority of tracheae there was no effect of the intraluminally administered salts on basal tone. The results presented in this section are, therefore, not attributable to effects of the salts on basal ΔP. When added to the intraluminal bath, both concentrations of both salts decreased reactivity to intraluminal MCh (Fig. 6 and Tables 1 and 2). Intraluminal KCl and NaCl were less effective inhibitors of responses to extraluminal MCh, compared with those elicited by intraluminally added MCh. NaCl produced a greater antagonism of these responses than did KCl, irrespective of the bath to which MCh was added. The same inhibitory effects of hyperosmolar intraluminal KCl on intra- and extraluminal reactivity to

TABLE 1
Effect of intraluminal KCl on reactivity to intra- and extraluminal MCh

	Intraluminal MCh		Extraluminal MCh	
	EC_{50}	Maximum response	EC_{50}	Maximum response
	M		*M*	
13.3 mM KCl				
Control	1.12×10^{-4} (0.85–1.48)	13.8 ± 1.8	1.12×10^{-6} (0.55–2.29)	13.0 ± 5.5
KCl present	3.98×10^{-4}* (2.04–7.76)	6.2 ± 1.4**	1.66×10^{-6} (1.20–2.29)	14.2 ± 5.4
n	4		5	
42.2 mM KCl				
Control	0.51×10^{-4} (0.22–1.20)	11.7 ± 2.3	0.93×10^{-6} (0.5–1.74)	11.8 ± 3.8
KCl present	7.94×10^{-4}* (3.72–17.0)	3.6 ± 1.0**	2.82×10^{-6}* (1.70–4.68)	10.3 ± 4.2
n	5		7	

Maximum response (ΔP) is given in centimeters H_2O; 95% C.I. in parentheses.
* Significantly larger than control.
** Significantly smaller than control.

TABLE 2
Effect of intraluminal NaCl on reactivity to intra- and extraluminal MCh

	Intraluminal MCh		Extraluminal MCh	
	EC_{50}	Maximum response	EC_{50}	Maximum response
	M		*M*	
13.3 mM NaCl				
Control	0.45×10^{-4} (0.20–1.00)	16.2 ± 11.2	4.27×10^{-6} (0.54–33.6)	14.0 ± 1.8
NaCl present	3.72×10^{-4}* (2.00–6.92)	8.1 ± 5.7**	8.59×10^{-6}* (2.96–24.91)	18.1 ± 3.2
n	4		4	
42.2 mM NaCl				
Control	0.15×10^{-4} (0.04–0.69)	22.0 ± 5.8	9.89×10^{-6} (4.19–23.37)	20.1 ± 4.6
NaCl present	3.66×10^{-4}* (1.91–6.88)	7.8 ± 2.8**	39.54×10^{-6}* (10.79–144.85)	12.2 ± 4.2**
n	4		4	

Maximum Response (△P) is given in centimeters H_2O; 95% C.I. in parentheses.
* Significantly larger than control.
** Significantly smaller than control.

MCh were observed in the presence of indomethacin (3×10^{-6} M; Fig. 7 and Table 3), in support of previous observations that EpDRF is not a prostanoid.

To determine whether the inhibitory effects of intraluminal hyperosmolarity on reactivity to MCh involved the epithelium, experiments were conducted with tracheae from which the epithelium was removed. NaCl was used in these studies rather than KCl because intraluminal KCl contracts the denuded trachea. A maximal (120 mM) concentration of added NaCl was used in these experiments to provide a stronger test of the hypothesis. As shown in Fig. 8, there were no effects of intraluminal hyperosmolar solution on intra- or extraluminal MCh concentration-response curves in the absence of the epithelium.

Responses to Hypo-Osmolar Intraluminal MKH Solutions. Because obstruction in human airways may be produced by hypo-osmolar as well as hyperosmolar aerosols (see *Introduction*), we reasoned that hypo-osmolar solutions might also affect airway diameter. Figure 9 illustrates that as little as a 1% reduction in the osmolarity of the perfusing Krebs' solution resulted in a measurable increase in ΔP, and the response increased as tonicity decreased.

To determine whether the increase in ΔP in response to intraluminal hypo-osmolarity involved swelling of the epithelium, the release of a contractile mediator (Lamport and Fedan, 1990), and/or a direct contractile response by the smooth muscle, the effect of papaverine on responses to intraluminal hypo-osmolarity and extraluminal MCh from intact and denuded preparations were compared (Fig. 10). Perfusion with intraluminal water elevated ΔP in intact as well as in epithelium-denuded preparations. In the presence of papaverine (10^{-4} M), responses were inhibited in intact and denuded tracheae, as were those to MCh.

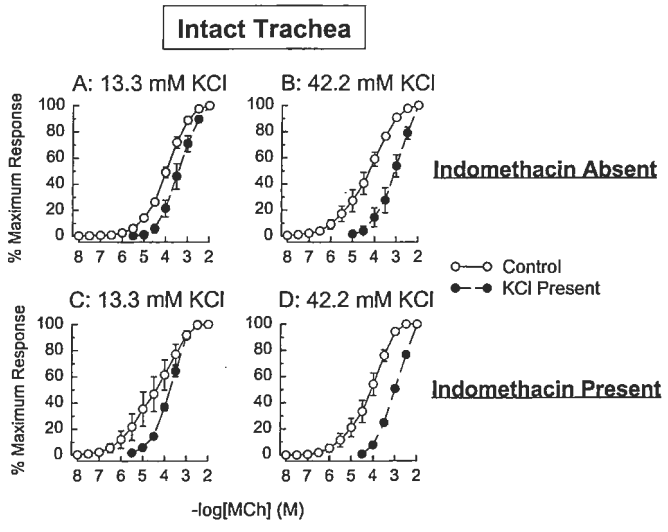

Fig. 7. Lack of effect of indomethacin (3×10^{-6} M) on inhibitory effect of hyperosmolar intraluminal KCl on intraluminal MCh concentration-response curves. In this experiment using intact tracheae, indomethacin was present during both concentration-response determinations (C and D). MCh concentration-response curves were obtained before (○) and after (●) elevating intraluminal KCl concentration with 13.3 mM KCl (A and C; $n = 4$ for both) or 42.2 mM KCl (B and D; $n = 4$ for both). Osmolar concentration-dependence of inhibitory effect of KCl on reactivity to MCh also is shown.

Effects of Serosally Applied Hyperosmolar Solution: Is EpDRF Released Only in Response to Elevated Mucosal Osmolarity? When added to preparations that had been contracted with MCh, the addition of NaCl or KCl to the extraluminal bath gave rise to concentration-dependent relaxation responses (Fig. 11). The relaxation due to NaCl was reasonably well maintained but rose gradually to the initial level of MCh-induced tone. The response to KCl was very transient and was followed by a contraction to a level well above the value caused by MCh alone (not shown). Intraluminally added NaCl elicited significantly larger responses at 5.62 and 13.3 mM than were seen after extraluminal NaCl addition. Because of its transient nature, the relaxation phase of the response to all concentrations of extraluminally applied KCl was significantly smaller than those after intraluminal addition. The contraction to extraluminally added KCl accounted for the greater "efficacy" of extraluminally added NaCl compared with extraluminally added KCl. A comparison of relaxation responses to extraluminally added NaCl in separate intact and epithelium-denuded tracheae revealed that epithelium removal decreased significantly the hyperosmolarity-induced relaxation response at 80 mM added extraluminal NaCl (Fig. 12).

Effects of Hyperosmolar Intraluminal MKH Solutions on Neurogenic Contractile Responses. Experi-

TABLE 3
Effect of intraluminal KCl on reactivity to intra- and extraluminal MCh in presence of indomethacin (3 times 10^{-6} M)

	Intraluminal MCh		Extraluminal MCh	
	EC_{50}	Maximum response	EC_{50}	Maximum response
	M		*M*	
13.3 mM KCl				
Control	0.37×10^{-5} (0.07–20.04)	14.4 ± 7.2	0.89×10^{-6} (0.40–2.00)	23.2 ± 5.7
KCl present	17.4×10^{-5} (13.20–22.90)	4.0 ± 1.2**	1.95×10^{-6}* (0.58–6.61)	21.1 ± 5.7
n	4		3	
42.2 mM KCl				
Control	7.08×10^{-5} (3.31–15.10)	17.3 ± 5.3	1.23×10^{-6} (0.81–1.86)	16.3 ± 4.2
KCl present	1.02×10^{-3}* (1.91–6.88)	4.6 ± 1.6**	2.51×10^{-6} (1.41–4.47)	8.2 ± 2.2**
n	4		4	

Maximum Response (ΔP) is given in centimeters H_2O; 95% C.I. in parentheses.
* Significantly larger than control.
** Significantly smaller than control.

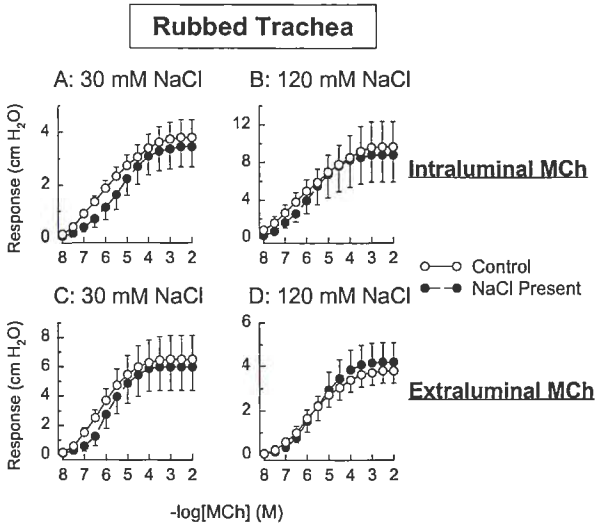

Fig. 8. Lack of effect of hyperosmolar intraluminal NaCl solution on reactivity of rubbed tracheae to intraluminal MCh (A and B) and extraluminal MCh (C and D). Note that higher NaCl concentrations were intentionally employed than in Fig. 6. $n = 4$ to 5 for each group.

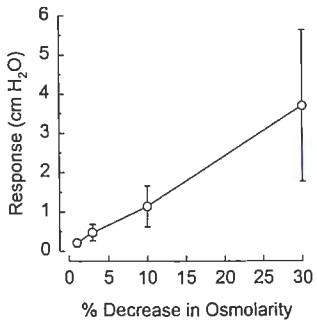

Fig. 9. Elevation of ΔP in response to intraluminal hypo-osmolarity. A response was seen with as little as a 1% reduction of the intraluminal MKH solution. This curve was generated with "cumulative" addition of water to the bath, each addition following the establishment of a stable, plateau response. $n = 7$.

ments were conducted to examine the effects of intraluminal hyperosmolarity on responses of the trachea elicited by endogenous transmitters released in response to electric field stimulation. Unlike responses to intraluminally applied exogenous agents, responses to endogenously released agonists would not be influenced by diffusion of the agent across the epithelium. Three patterns of response to electric field stimulation were observed in the tracheae of different animals: 1) rapid, transient, monophasic contraction only; 2) rapid, transient contraction followed by a slower developing and longer lasting contraction persisting beyond delivery of electrical impulses; and 3) rapid, transient contraction followed by relaxation below baseline. Often, but not always, transitions between the first response pattern to the second and/or third occurred with increasing stimulus frequency.

The rapid, initial neurogenic contractions of intact trachea were concentration-dependently inhibited by increasing the osmolarity of the perfusing solution with NaCl (Fig. 13). In the absence of epithelium, a significant inhibitory effect of NaCl did not occur at 13.3 mM; at 30 mM NaCl a small but significant inhibition occurred, and the inhibition became larger at 120 mM NaCl (Fig. 14). These effects of intraluminal NaCl on responses of intact and denuded tracheae to 30 Hz stimulation are compared in normalized fashion in Fig. 15, in which it can be seen that the concentration-response relationship was shifted to the right in the absence of the epithelium. Thus, inhibition of neurogenic contractions by intraluminal hyperosmolarity was mediated substantially by the epithelium.

All three concentrations of intraluminal NaCl inhibited the slower developing contractions when they were evident (pattern two, data not shown). Although of interest, the effects of intraluminal hyperosmolarity on neurogenic relaxation could not be determined because NaCl relaxed the MCh-induced tone that was required to visualize the neurogenic responses.

Discussion

The perfused trachea responds to both increases and decreases in the osmolarity of the perfusing solution. Indeed, the trachea was sensitive to very small changes in osmolarity. The effects of mucosal hyperosmolarity involve EpDRF; an epithelial component may also exist in the response to intraluminal hypo-osmolarity. We also observed that the epithelium is involved in relaxation responses to extraluminally applied hyperosmolarity.

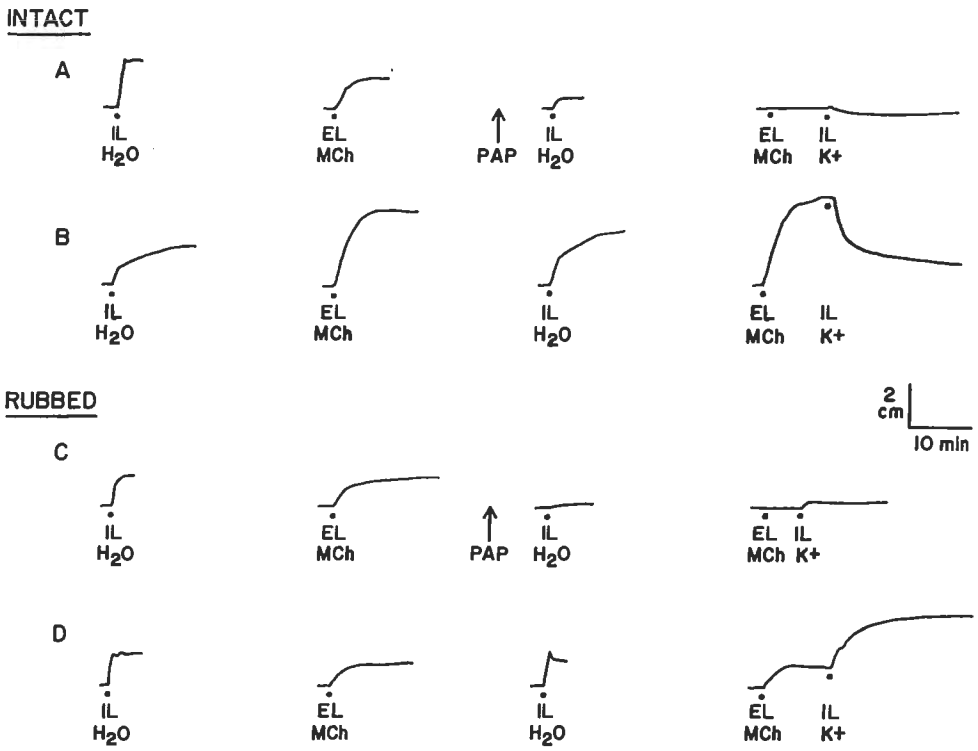

Fig. 10. Elevation in ΔP in response to intraluminal hypo-osmolarity: effects of papaverine (10^{-4} M; PAP) and epithelium removal. Epithelium-containing (intact, A and B) and epithelium-denuded (rubbed, C and D) preparations were perfused for 5 min with intraluminal water (IL H_2O) to provide an extreme hypo-osmolar challenge. After returning normal MKH solution to perfusate and perfusing for 1 h, tracheae were contracted with extraluminal (EL) MCh (3.5×10^{-7} M). After 1 h of washing, preparations were incubated with papaverine (A and C) or with normal MKH solution (B and D), and challenged a second time with water and MCh. Intraluminal KCl (120 mM) was added at end of experiment to verify that epithelium was removed when desired, as demonstrated by contraction to intraluminal KCl in denuded tracheae or relaxation of intact tracheae. (In some cases elevation in ΔP in response to water persisted beyond 5-min exposure period.) Tracings are representative of $n = 2$ to 4 separate experiments in each protocol.

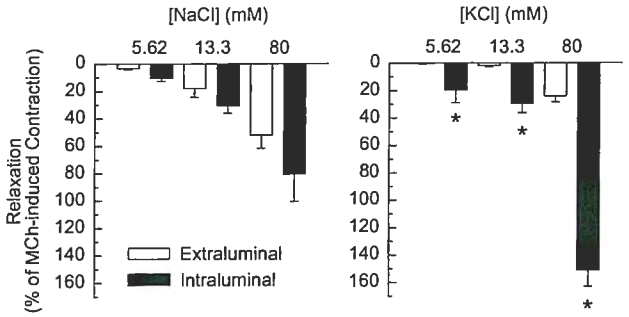

Fig. 11. Comparison of relaxation responses of extraluminal MCh (3.5×10^{-7} M)-contracted perfused tracheae to elevated tonicity in the extraluminal (EL) and intraluminal (IL) baths. Left, tonicity elevated with NaCl; right, tonicity increased with KCl. *Significantly larger than value for extraluminal bath addition. $n = 8$ for 5.62 mM; $n = 8$ for 13.3 mM; $n = 6$ for 80 mM.

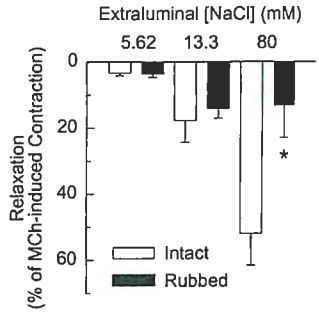

Fig. 12. Comparison of relaxation responses of MCh (3.5×10^{-7} M)-contracted intact and epithelium-denuded (rubbed) tracheae to extraluminally applied NaCl. *Significantly smaller than value for intact tracheae. $n = 8, 6$ for 5.62 mM; $n = 8, 9$ for 13.3 mM; and $n = 6, 6$ for intact, rubbed, respectively.

Relaxation Induced by Intra- and Extraluminal Hyperosmolarity. Our results indicate that the epithelium is an osmotic sensor which, upon elevation in osmolarity, brought about three effects that can affect airway diameter. The first effect was relaxation of the airway smooth muscle. Relaxation was produced equipotently by intraluminal KCl and NaCl, despite the fact that KCl is a powerful contractile agent when added to the extraluminal bath of intact tracheae or to the extra- or intraluminal baths of epithelium-denuded tracheae. This reiterates the conclusion by Munakata et al. (1988) that relaxation was stimulated by hyperosmolarity per se rather than by agent-specific mechanisms.

In the absence of the epithelium, relaxation occurred to the higher intraluminal NaCl concentrations. Jongejan et al. (1990, 1991) observed that hyperosmolar NaCl elicited relaxation followed by contraction in human isolated bronchial rings; in that preparation added agents have access to both sides of the airway wall. In the present study, although luminal hyperosmolarity relaxed the trachea in the absence of the epithelium, these results do not indicate that the muscle was affected to this degree in the presence of the epithelium. In intact trachea the solute concentrations in the lumen of the trachea would not be attained in the smooth muscle milieu in amounts achieved in the denuded trachea. It is reasonable to suggest that there are only slight eleva-

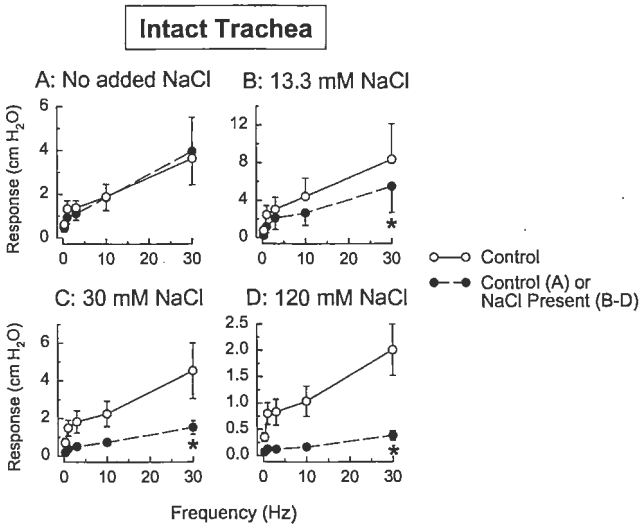

Fig. 13. Effect of intraluminal hyperosmolar NaCl on electric field stimulation-induced contractile responses of intact tracheae. Two frequency-response curves were obtained from each trachea, first in the absence of NaCl (○), and second (●) without any treatment (A, $n = 4$) or 30 min after addition of NaCl to perfusing solution as follows: B, 13.3 mM ($n = 5$); C, 30 mM ($n = 8$); and D, 120 mM ($n = 8$).

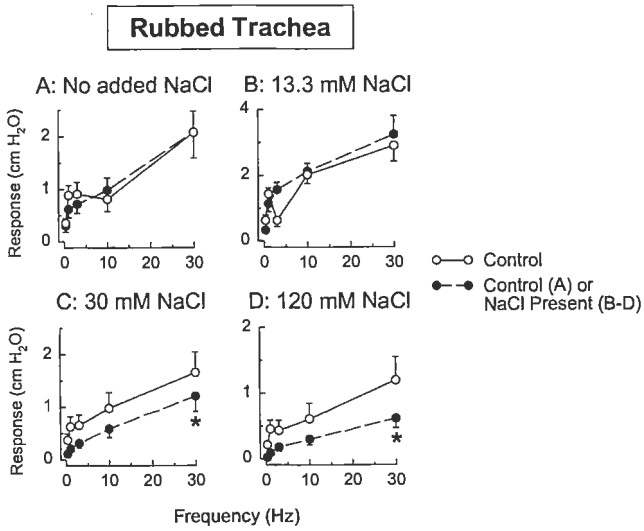

Fig. 14. Effect of intraluminal hyperosmolar NaCl on electric field stimulation-induced contractile responses of epithelium-denuded (rubbed) tracheae. Protocol was similar to that in Fig. 13, except that denuded tracheae were used. A, no treatment ($n = 6$) or 30 min after the addition of NaCl to the perfusing solution, as follows: B, 13.3 mM ($n = 6$); C, 30 mM ($n = 8$); and D, 120 mM ($n = 7$). *Significantly less than control.

tions in osmolarity at the level of the smooth muscle when NaCl is added to the intraluminal compartment of intact trachea. Therefore, we conclude that hyperosmolarity-induced relaxation of guinea pig intact perfused tracheae initiated by elevated intraluminal osmolarity is primarily, if not exclusively, mediated by the epithelium.

Relaxant responses of intact tracheae to intraluminal hyperosmolarity were not affected by indomethacin. The small but significant inhibitory effect of L-NAME was comparable with between-curve changes in control tracheae in the absence of the inhibitor. We interpret these findings to suggest that the relaxation response to elevated osmolarity in intact trachea is not mediated by prostaglandins or appreciably by nitric oxide.

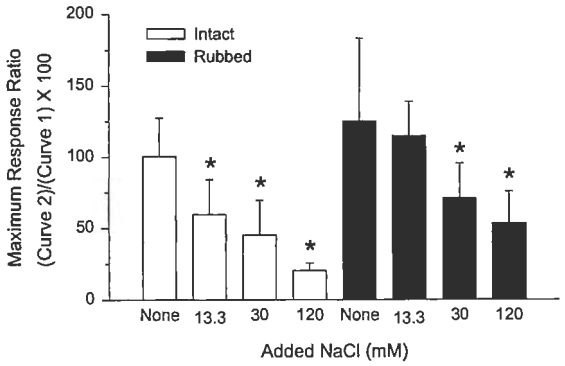

Fig. 15. Comparison of effects of intraluminal hyperosmolar NaCl solution on electric field stimulation-induced contractile responses of intact and epithelium-free (rubbed) tracheae to maximal, 30 Hz stimulation. This figure depicts ratios of contractile responses to 30-Hz stimulation obtained in the absence of intraluminal hyperosmolar NaCl (Fig. 13) divided by responses obtained in the presence (Fig. 14) of intraluminal hyperosmolar NaCl, i.e., (curve 2)/(curve 1). This ratio provides a normalized index of relative magnitudes of inhibitory effect of NaCl on responses obtained from intact versus epithelium-free tracheae, irrespective of magnitudes of responses. n values are given in legends to Figs. 13 and 14. *Significantly less than ratio obtained in the absence of NaCl (None).

The relaxation response to intraluminal hyperosmolarity appears to involve Na^+ and Cl^- channels. Individually, both amiloride and DIDS inhibited relaxations to intraluminal NaCl; together the two blockers gave an additive effect. When amiloride was administered only in the extraluminal bath, the blocker did not inhibit the responses as it had when it was present in both baths. Thus, the relevant site of amiloride's action would appear to be the apical membrane of the epithelium. Future experiments will be needed to clarify whether the effects of DIDS resulted from an apical site of action.

Earlier studies on tracheal muscle strips involving relaxation responses to KCl when added to K^+-free MKH solution led to the conclusion that either the production of EpDRF by the epithelium and/or its inhibitory effect on the tracheal smooth muscle were linked to Na^+,K^+-pumping (Raeburn and Fedan, 1989). At the time of those experiments the possibility was not considered that relaxation to K^+ involved an osmotic component, even though such responses were not blocked completely by ouabain. In the present study ouabain did not inhibit the relaxations of the perfused trachea to intraluminally added NaCl but produced a nearly significant potentiation (Fig. 5), suggesting that the Na^+,K^+-pump is not involved in the release of effects of EpDRF.

The Na^+-K^+-$2Cl^-$ transporter blocker, bumetanide, inhibited relaxant responses only to the highest concentrations of intraluminal NaCl and KCl, and in the case of NaCl the effect neared but did not achieve statistical significance. These findings suggest that EpDRF release over the full range of added salt concentrations is not associated intimately with Na^+-K^+-$2Cl^-$ cotransport.

A surprising finding in this study were the epithelium-dependent relaxation responses elicited by extraluminal elevated NaCl and KCl. These findings indicate that the epithelium has a bipolar function as an osmotic sensor capable of transmitting inhibitory signals to the smooth muscle. In human nasal epithelium, Willumsen et al. (1994) observed that apical, not basolateral, application of hyperosmotic solution elicited bioelectric responses. A species or upper versus lower

airway difference may account for the differing results. Munakata et al. (1988) did not report that relaxation of guinea pig perfused trachea was obtained with extraluminal hyperosmolarity. However, they added extraluminal KCl to unstimulated preparations, whereas we observed the effect in tracheae that were contracted with MCh.

Inhibition of Reactivity to MCh by Stimulated Release of EpDRF. The second way in which the epithelium, acting as osmotic sensor, can regulate airway diameter is by affecting reactivity to contractile agents. Heretofore, the inhibitory effect of the epithelium on reactivity has been demonstrated by removing the epithelium. In the present study we used intraluminal hyperosmolarity to provoke EpDRF release from intact trachea and observed an osmolar concentration-dependent decrease in reactivity to intra- and extraluminally applied MCh, only in the presence of the epithelium. The fact that intraluminally added NaCl had no effect on reactivity of denuded tracheae to MCh is additional support for the conclusion that the smooth muscle is not the primary site of the relaxant effects of elevated mucosal tonicity. The inhibition was independent of the means used to increase osmolarity, because added KCl and NaCl were equieffective, and it occurred both in the absence and in the presence of indomethacin.

One difference in the effects of elevated intraluminal tonicity on extra- and intraluminal concentration-response curves was noted, namely, reactivity was decreased to a greater degree when MCh was administered to the intraluminal bath. There are several possible explanations for the difference. First, MCh added to the intraluminal bath may itself have caused the release of EpDRF and/or other inhibitory substances, which enhanced the effect of hyperosmolarity released EpDRF. This possibility has specifically been investigated (Fedan et al., 1990), and the result showed that intraluminally applied MCh does not relax an extraluminal MCh-contracted trachea. A second possible explanation is that intraluminal reactivity to MCh was reduced because of an alteration in the permeation of the drug through the epithelium, perhaps through tight junctions. For example, hypo-osmolar solutions uncouple gap junction electrical connectivity in pancreatic acinar cells (Ngezahayo and Kolb, 1990); hyperosmolar solutions might have caused an opposite effect and heightened the diffusion barrier. Direct evidence against this possibility was provided by the finding that hyperosmolarity in the intraluminal bath resulted in a decrease in reactivity to extraluminally applied MCh. This is among the strongest evidence obtained to date that EpDRF is released from the epithelium and diffuses through the submucosa to the smooth muscle to inhibit contractility. The third and most likely possibility is that EpDRF is more efficacious against intraluminal MCh because the potency and efficacy of intraluminal MCh is already substantially reduced by the epithelial diffusion barrier (and other mechanisms; Fedan and Frazer, 1992). That is, the weaker the efficacy of an agonist, the greater will be the effect of a physiological antagonist such as EpDRF.

It is well to consider whether epithelial cell shrinkage and an increase in tracheal diameter in response to intraluminal hypertonicity (Willumsen et al., 1994) could have contributed to decreased reactivity to MCh. The resistance of the perfusion holder containing the indwelling cannulas varies with $1/(\text{diameter})^5$ (Munakata et al., 1989). Two lines of evidence argue against cell shrinkage as the mechanism of reduction in ΔP. First, added intraluminal NaCl or KCl did not affect baseline ΔP (except in preparations containing spontaneous tone). Second, Hay et al. (1986a) observed in guinea pig tracheal strips that isometric contractile responses to low but not high concentrations of KCl were potentiated after epithelium removal. This effect, no doubt, reflected the loss of the effect of released EpDRF where diameter is not relevant.

Inhibition of Neurotransmission by EpDRF. The third way that the epithelium, acting as an osmotic sensor, can affect airway diameter is by inhibiting neurotransmission. Raised intraluminal osmolarity produced a concentration-dependent inhibition of neurogenic contractile responses; the inhibition was substantially greater in the presence of the epithelium. In tracheae demonstrating two phases in the contractile response, both phases were inhibited. Because in intact trachea the effect of a given concentration of intraluminal NaCl would not reflect the direct effect of the salt seen in the absence of the epithelium, and because NaCl had no effect on concentration-response curves for either extra- or intraluminally administered MCh in the absence of the epithelium, these findings indicate that released EpDRF inhibited cholinergic postganglionic and excitatory nonadrenergic, noncholinergic neurotransmission in the trachea. A decrease in acetylcholine release after incubation with cultured epithelial cell supernatant has been observed in canine tracheal smooth muscle; reactivity to exogenous acetylcholine was not affected (Matsumoto et al., 1996). On the other hand, we found that responses to both exogenous and endogenous cholinergic agonists were inhibited by intraluminal hyperosmotic solution, which suggests that pre- and postjunctional mechanisms may operate in the guinea pig trachea. For technical reasons we could not determine whether EpDRF affected the neurogenic inhibitory phase of the responses. Nevertheless, our results agree with those of Flavahan et al. (1985), who observed that epithelium removal potentiated contractile responses of dog airways to electric field stimulation. Several approaches, therefore, have indicated that neural efferent function in the airways is modulated by EpDRF.

Hypotonic Intraluminal Solutions. Very small decrements in intraluminal osmolarity elevated ΔP. Whether these responses involved epithelial swelling, a contractile factor from epithelium, and/or a direct effect on the airway smooth muscle, must be considered. Our findings suggest that the elevation of ΔP in intact tracheae did not result primarily from swelling of the epithelium. First, responses were elicited by very small decreases in luminal osmolarity, i.e., ca. 1% reduction. Second, the pressor response to luminal hypo-osmolarity occurred both in the absence and presence of the epithelium. Third, responses to both hypo-osmolarity and MCh were inhibited by papaverine.

It is difficult to gauge precisely the involvement of an epithelial, contractile mediator in these responses. Whether or not the epithelium mediated the responses to luminal hypo-osmolarity is dependent upon whether or not the MKH solution became diluted at the level of the smooth muscle. Due to the epithelial barrier it is unlikely that an appreciable reduction in osmolarity occurred in the smooth muscle with small decreases in intraluminal osmolarity. The notion that the epithelium mediates hypo-osmolarity-induced contractile responses through the release of a contractile factor will require further examination.

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