*European Journal of Pharmacology*, 190 (1990) 337–345
Elsevier



EJP 51569

# Reactivity of guinea-pig isolated trachea to methacholine, histamine and isoproterenol applied serosally versus mucosally

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Received 28 May 1990, revised MS received 31 July 1990, accepted 7 August 1990

Guinea-pig tracheas were perfused with recirculating modified Krebs-Henseleit solution while monitoring changes in inflow-outflow pressure difference, which is an index of trachealis muscle tone. The reactivities of the trachealis muscle to methacholine, histamine and isoproterenol applied separately to the mucosal (intraluminal, IL) or serosal (extraluminal, EL) compartments were compared, and evidence for the agonist-induced release of epithelium-derived relaxing factor (EpDRF) was sought. All agents were more potent when added to the EL compartment, but the IL/EL $EC_{50}$ ratios were different: 100 for methacholine, 41 for histamine and 25 for isoproterenol. Methacholine or histamine added to the IL compartment, after the preparations were pre-contracted with the same concentration of the agonist or 30 mM KCl added EL, did not result in relaxation. Likewise, IL isoproterenol did not evoke contraction. IL KCl evoked relaxation. The results indicate that the epithelium reduces access of bronchoactive agents to the muscle, while an immediate relaxant effect of EpDRF released by agonists could not be demonstrated.

Trachea; Epithelium; EpDRF (epithelium-derived relaxing factor); Smooth muscle (airway); (Reactivity)

## 1. Introduction

The epithelium of the large airways of many mammals releases an as yet unidentified inhibitory substance, epithelium-derived relaxing factor (EpDRF), which diminishes the reactivity of the underlaying smooth muscle to a number of bronchoactive drugs (Flavahan et al., 1985; Fedan et al., 1988). Direct evidence for the release of EpDRF has been obtained using sandwich preparations (Tschirhart and Landry, 1986) or through bioassay with co-axial preparations in which recipient smooth muscles are placed within donor trachea (Ilhan and Sahin, 1986; Hay et al., 1987; Güc et al., 1988a,b,c).

A novel preparation, the isolated perfused trachea, has been described recently which allows examination of the degree to which the respiratory epithelium influences the activity of drugs affecting airway smooth muscle (Munakata et al., 1988; 1989). In this preparation reactivity to drugs applied to the mucosal surface (intraluminal compartment), where diffusion across the epithelium hinders access of drugs to the muscle, may be compared with that seen after the drugs are applied to the serosal surface (extraluminal compartment), where access to the muscle on the serosal surface is unobstructed.

In the present study the reactivity of the trachealis smooth muscle of the isolated, perfused guinea-pig trachea to methacholine, histamine and

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isoproterenol was compared after the drugs were applied to the mucosal or serosal surfaces. Evidence was then sought for the release of EpDRF in response to the luminal application of the drugs. A preliminary account of this work has been given (Fedan and Frazer, 1988).

## 2. Materials and methods

### *2.1. Tracheal perfusion*

Male English short hair guinea-pigs (500-1000 g; Camm Research Institute, Wayne, NJ, U.S.A.) were killed by stunning and bleeding. A segment of trachea 4 cm long was removed, placed in modified Krebs-Henseleit (MKH) solution (37 °C; equilibrated with 95% $O_2$-5% $CO_2$) and cleaned. The trachea was tied at each end to a perfusion apparatus (schematic in fig. 1) which was modified from Munakata et al. (1988; 1989) and fashioned from glass and plastic tubing. The apparatus was placed with the segment extended to its original length into a water-jacketed organ chamber (37 °C) containing 25 ml MKH; this is referred to as the serosal or extraluminal (EL) compartment. The lumen was perfused with recirculating MKH from a separate, 30 ml MKH bath; this is referred to as the mucosal or intraluminal (IL) compartment. Hydrostatic pressure was sampled at either end of the trachea with side hold catheters, centered in the lumen, that were connected to a differential pressure transducer. Inlet and outlet pressure difference ($\Delta P = P_{in} - P_{out}$) was plotted on a strip-chart recorder. The basal flow conditions established were essentially identical to those described by Munakata et al. (1988; 1989). The flow rate was set to give a $\Delta P$ of 0.5 to 1.5 cm $H_2O$. The transmural pressure at the middle of the trachea was set at ca. 0 cm $H_2O$ by adjusting the height of the tip of the tubing returning MKH to the IL bath. Flow rate was kept constant in each experiment, and ranged from 15 to 32 ml/min from experiment to experiment. Under constant flow conditions $\Delta P$ is an index of the tracheal resistance. Changes in tracheal resistance reflect changes in tracheal cross sectional area associated with muscle contraction or relaxation (Munakata et al., 1989). Thus, an increase or decrease in $\Delta P$ corresponds to muscle contraction or muscle relaxation.

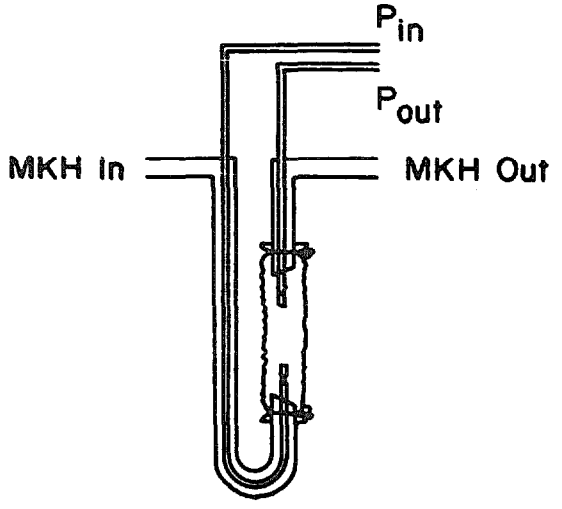


Fig. 1. Schematic of the perfusion apparatus used in these experiments (modified from Munakata et al. (1988; 1989)). The outside diameter of the inner catheters was 1.52 mm. The distance from the catheter side holes to the catheter tip was 5 mm; the side holes were 2 mm from the entrance of the catheter into the lumen. The tubing onto which the trachea was tied, and through which MKH was perfused, had an outside diameter of 2.42 mm and an inside diameter of 1.67 mm. Contractile and relaxant responses of the trachealis muscle caused, respectively, a decrease or an increase in tracheal diameter, an increase or a decrease in flow resistance and an increase or a decrease in pressure gradient ($\Delta P = P_{in} - P_{out}$) under constant flow rate conditions. The perfusion apparatus was placed into a 25 ml bath of MKH.

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), glucose (5.7); pH 7.4 (37 °C); aerated with 95% $O_2$-5%$CO_2$.

### *2.2. EL vs. IL concentration-response studies*

The preparations were equilibrated for 1 h, during which the EL and IL baths were replaced with fresh MKH ('washed') at 15 min intervals, before the actual experiment. Cumulative concentration-response curves for methacholine and histamine were obtained with the preparations at basal tone. Isoproterenol was added cumulatively after tone was elevated with 30 mM KCl added to the EL bath. Two concentration-response curves

for one agonist were obtained from each trachea. The EL curve was obtained first. Upon completion of the EL additions the preparations were washed EL and IL with fresh MKH. After 1.5 h with EL and IL washes every 15 min the IL concentration-response curve was obtained. At the conclusion of the IL concentration-response determinations the preparations were challenged with IL 120 mM KCl. Intraluminal KCl causes a relaxation of intact trachea but contraction of epithelium-denuded trachea (Munakata et al., 1988; 1989), and may be used to assess epithelial integrity. In no case was a contraction to IL KCl seen.

In preliminary experiments we observed no difference between two consecutive EL methacholine concentration-response curves. We also observed that the EL vs. IL differences were not dependent on the order in which the curves were obtained. From this it is inferred that differences between EL and IL concentration-response curves were not due to time-dependent changes in reactivity nor to the order in which the EL and IL curves were obtained.

### 2.3. *EL vs. IL single, equal concentration challenges*

Evidence for the agonist-induced release of EpDRF was sought using a protocol in which the trachea was first challenged with a single concentration of methacholine, histamine or isoproterenol added to one compartment. Without any intervening washes being performed, the same drug in the same concentration was then added to the other compartment. In these experiments methacholine and histamine were added to quiescent preparations or preparations contracted with EL 30 mM KCl, while isoproterenol was added to preparations contracted with EL $3 \times 10^{-7}$ M methacholine or EL 30 mM KCl.

### 2.4. *Statistical analysis*

The results were quantified as $\Delta P$ in cm $H_2O$, as % maximum response and, in the case of isoproterenol, as percentage relaxation of the 30 mM KCl-induced contraction (% 30 mM KCl). The results shown in the figures and tables are presented as means $\pm$ S.E.; n is the number of separate experiments. Geometric mean $EC_{50}$ values (the concentration producing 50% of the maximum response) were determined from nonlinear curve fitting analysis (De Lean et al., 1978). The data were evaluated for differences with analysis of variance. The 0.05 level of probability was considered significant.

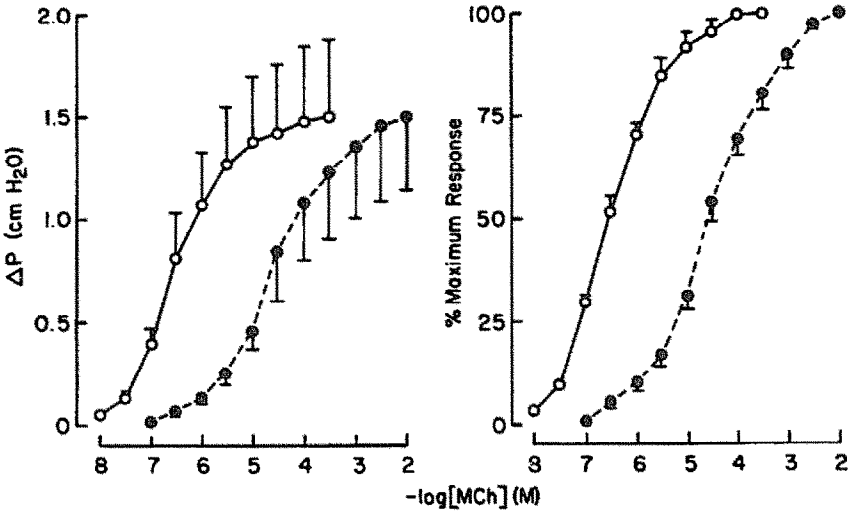


Fig. 2. Comparison of concentration-response curves following addition of methacholine (MCh) to the EL (serosal; open symbols) and IL (mucosal; filled symbols) compartments. Responses are expressed in figs. 2-4 as $\Delta P$ and % maximum response to show maximum response and sensitivity comparisons, respectively. n = 5.

TABLE 1

EL vs. IL reactivity of perfused guinea-pig trachea.

| Compartment | $EC_{50}$, M (95% C.I.) | IL $EC_{50}$/EL $EC_{50}$ | Maximum response | |
|---|---|---|---|---|
| | | | cm $H_2O$ | % 30 mM KCl |
| Methacholine (n = 5) | | | | |
| EL | $3.14\times10^{-7}$ (2.29-4.20) | 99.7 ± 17.7 [b] | 1.50 ± 0.38 | – |
| IL | $2.91\times10^{-5}$ [a] (1.74-4.88) | | 1.50 ± 0.36 | – |
| Histamine (n = 4) | | | | |
| EL | $4.80\times10^{-6}$ (3.38-6.81) | 40.7 ± 7.1 [b] | 0.56 ± 0.12 | – |
| IL | $1.85\times10^{-4}$ [a] (1.36-2.51) | | 0.56 ± 0.08 | – |
| Isoproterenol (n = 4) | | | | |
| EL | $3.41\times10^{-9}$ (2.43-4.77) | 24.6 ± 3.5 [b] | 1.46 ± 0.30 | 128.3 ± 16.1 |
| IL | $8.21\times10^{-8}$ [a] (6.02-11.2) | | 0.90 ± 0.16 [c] | 108.1 ± 14.4 |

[a] Significantly greater than EL. [b] These values are significantly different. [c] Significantly less than EL. $EC_{50}$ values are given as geometric means with 95% confidence interval (C.I.) in parentheses.

### 2.5. *Drugs*

Methacholine (acetyl-β-methylcholine) chloride, histamine diphosphate and (−)-isoproterenol (+)-bitartrate were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

## 3. Results

### 3.1. *EL vs. IL reactivity*

The IL methacholine, histamine and isoproterenol concentration-response curves were displaced significantly to the right of the EL curves.

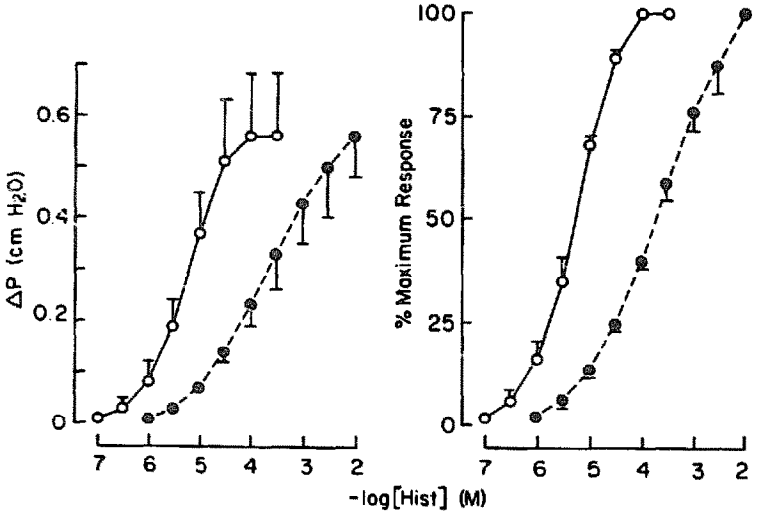


Fig. 3. Comparison of concentration-response curves following addition of histamine (Hist) to the EL (serosal; open symbols) and IL (mucosal; filled symbols) compartments. n = 4.

IL/EL $EC_{50}$ ratios were significantly different, and greatest for methacholine (99.7), followed by histamine (40.7) and isoproterenol (24.6) (figs. 2-4; table 1). The EL and IL maximum responses to methacholine were not different, nor were those for histamine. The maximum relaxation response to IL isoproterenol in terms of $\Delta P$ was significantly less than that for EL addition. This was a consequence of smaller contractions to KCl preceding the IL concentration-response determination, because the maximum responses to isoproterenol normalized in terms of % 30 mM KCl were not different.

### *3.2. EL vs. IL challenge with single, equivalent concentrations*

The reactivity of the trachealis muscle to IL agonists is, as just described, reduced greatly by the epithelium. Since the trachealis muscle is situated on the serosal surface of the preparation with no intervening barrier to drug diffusion, we reasoned that if an equilibrium response is elicited by the EL application of an agonist, IL addition of the same concentration of the agent would not change (i.e. elevate) the local concentration of the drug at the smooth muscle biophase per se. The agonist when added IL might, however, cause the release of factors from the epithelium which might cause a detectable change in trachealis muscle tone. A series of experiments was performed to evaluate these possibilities.

Concentrations of the agonists were used which approximate their EL $EC_{50}$ values. IL methacholine when added prior to or after EL methacholine did not cause a relaxation response (fig. 5). Likewise, IL histamine added in the absence or presence of EL histamine, did not evoke relaxation (fig. 6). In preparations in which tone had first been induced with 30 mM KCl, neither IL or EL methacholine (fig. 5) nor IL or EL histamine (fig. 6) induced relaxation. The IL application of isoproterenol evoked only relaxation of preparations with tone induced with either EL methacholine or EL KCl (fig. 7).

Shown in figs. 5 and 6 is the relaxation re-

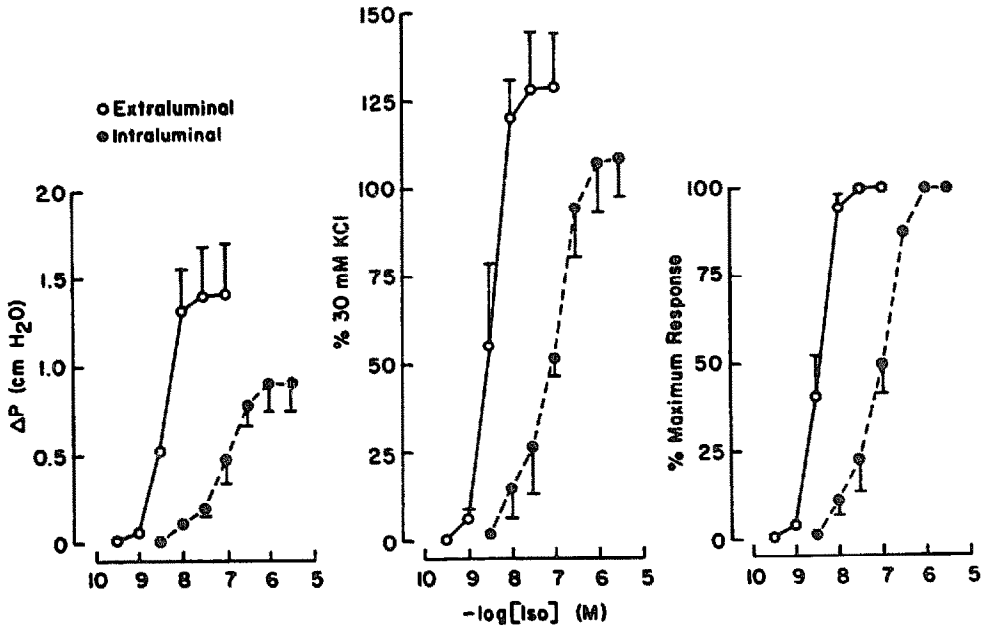


Fig. 4. Comparison of concentration-response curves following addition of isoproterenol (Iso) to the EL (serosal; open symbols) and IL (mucosal; filled symbols) compartments. In this figure the results are also given as % 30 mM KCl to normalize the magnitude of the relaxation response with respect to the tone induced by KCl. n = 4.

sponse to IL KCl, which confirms the phenomenon reported by Munakata et al. (1988; 1989). This is in contrast to contraction elicited with EL KCl (fig. 7).

## 4. Discussion

The degree to which the respiratory epithelium reduces the reactivity of trachealis smooth muscle to drugs is evident in this study, which confirms the findings of Munakata et al. (1988; 1989). Munakata et al. (1989) observed IL/EL $EC_{50}$ ratios of 115-151 for acetylcholine and 35-59 for histamine. Our values were quite comparable: 100 for methacholine and 41 for histamine. The smaller value for methacholine vis à vis acetylcholine could reflect laboratory variability or, more likely, an action of epithelial cholinesterase degrading acetylcholine more than methacholine as the drugs diffuse across the mucosa. Munakata et al. (1989) did not examine isoproterenol, but we observed isoproterenol to be 25 times more potent EL compared with IL. An extraneuronal uptake mechanism for isoproterenol in epithelium has been described (Farmer et al., 1986), which, presumably, could reduce the potency of IL isoproterenol. In functional terms, as opposed to the actual permea-

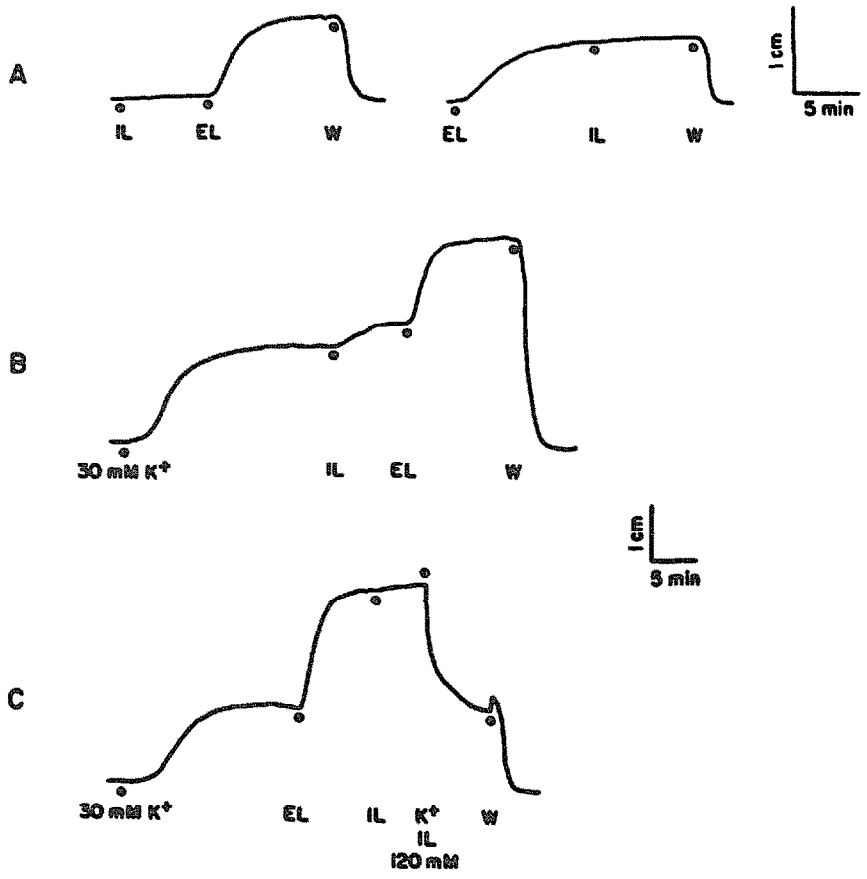


Fig. 5. Responses to methacholine ($10^{-6}$ M) added sequentially to the IL and EL compartments. The additions of methacholine are indicated by the dots labeled IL and EL. (A) Methacholine added to resting trachea. In the left tracing IL methacholine was applied and remained during EL exposure. In the right tracing the order of application was reversed. (B,C) Methacholine added to 30 mM KCl-contracted trachea. (B) IL addition preceded EL addition; the order of addition was reversed in (C). Note (C) that the IL application of 120 mM KCl (at $K^+$) caused an immediate and rapid relaxation response. W indicates washout from EL and IL compartments. The lower calibration lines apply to tracings (B, C); cm = cm $H_2O$. Tracings (A) were from the same trachea, tracings (B,C) were from a different trachea, and all are representative of results obtained from three separate experiments of each type.

bility of the drugs, these results taken together indicate that the mucosa is not an equivalent barrier to the actions of the three agonists on the smooth muscle.

The difference in EL vs. IL reactivity to acetylcholine and histamine (Munakata et al., 1989) and methacholine (Smith et al., 1990) is eliminated in preparations from which the epithelium has been removed mechanically. A possibility suggested by Munakata et al. (1989) to explain the serosal vs. mucosal reactivity differences is that EpDRF is released upon IL challenge with methacholine and histamine, and that the inhibitory effects of released EpDRF in concert with hindrance of diffusion of the agonists may explain the lesser potency of IL-administered agents. The present results do not support the view that IL application of the contractile agonists causes a release of EpDRF. IL methacholine or IL histamine, added to preparations which were precontracted with the same concentration of the agonist added EL or with EL KCl, did not cause the relaxation response expected from rapid EpDRF release. Likewise, no evidence was obtained that isoproterenol causes a release of an excitatory factor (see Flavahan et al., 1985). IL

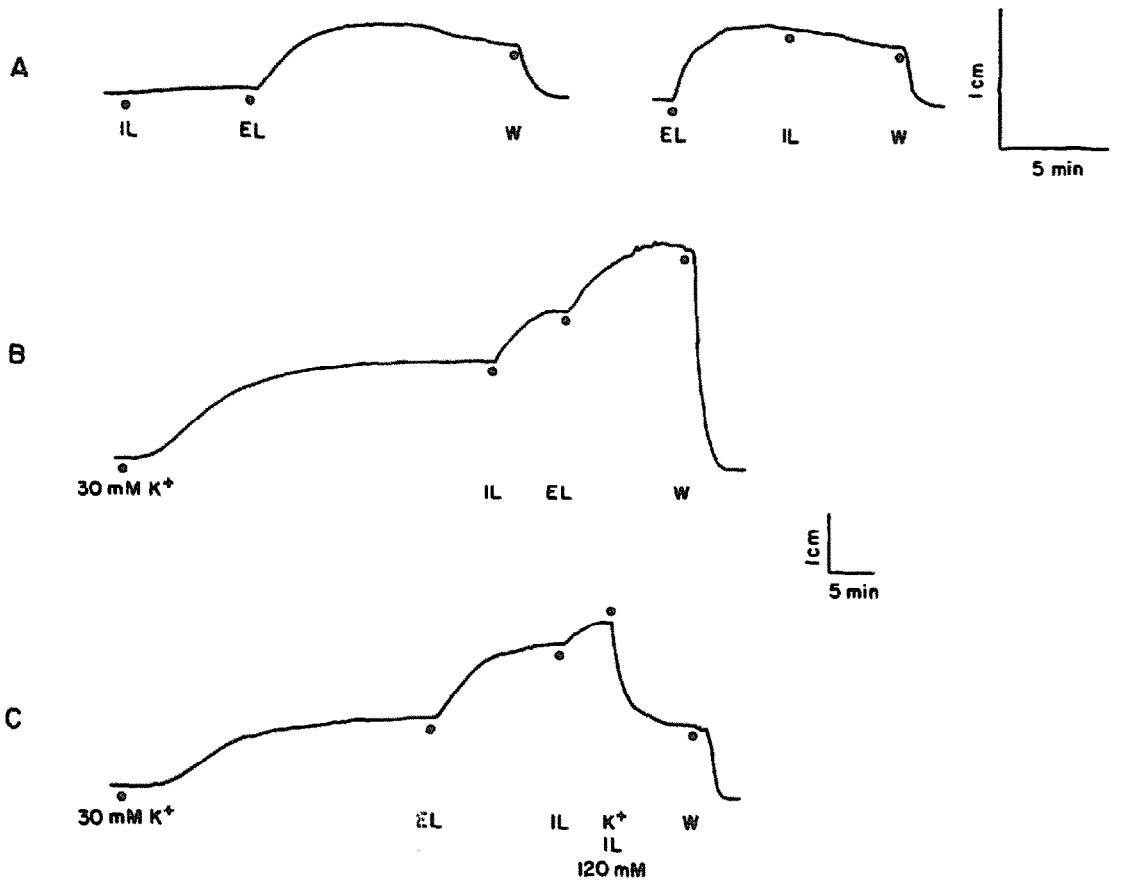


Fig. 6. Responses to histamine ($3\times10^{-6}$ M) added sequentially to the IL and EL compartments. The additions of histamine are indicated by the dots labeled IL and EL. (A) Histamine added to resting trachea. In the left tracing IL addition preceded EL addition; in the right tracing the order of application was reversed. (B,C) Histamine added to 30 mM KCl-contracted trachea. (B) IL addition preceded EL addition; the order of addition was reversed in (C). Note (C) that the IL application of 120 mM KCl (at $K^+$) caused an immediate and rapid relaxation response. W indicates washout from EL and IL compartments. The lower calibration lines apply to tracings (B,C); cm = cm $H_2O$. Tracings (A) were from the same trachea, tracings (B,C) were from a different trachea, and all are representative of results obtained from three separate experiments of each type.

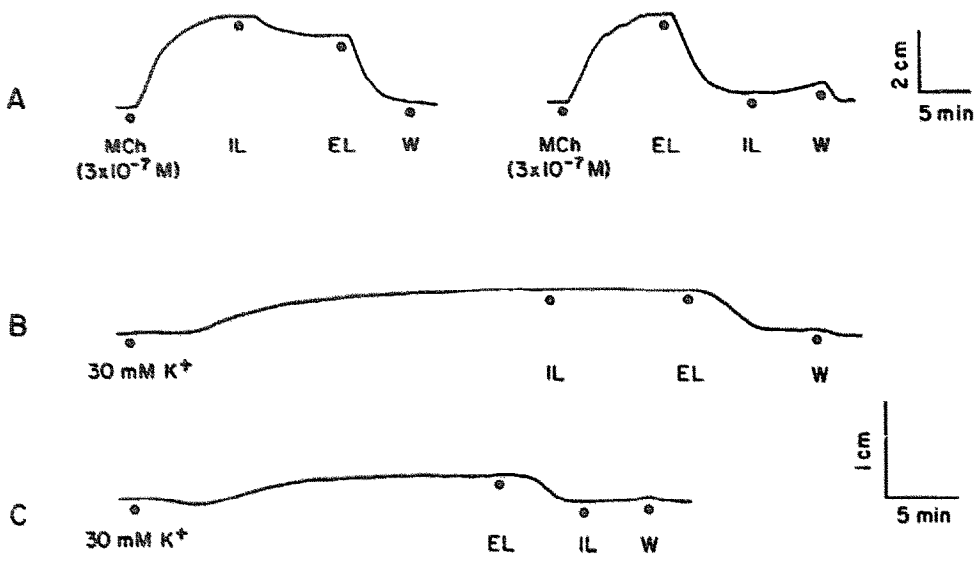


Fig. 7. Responses to isoproterenol added sequentially to the IL and EL compartments. The additions of isoproterenol are indicated by the dots labeled IL and EL. (A) Isoproterenol ($10^{-8}$ M) added to methacholine ($3\times10^{-7}$ M)-contracted trachea. In the left tracing IL addition preceded EL addition; in the right tracing the order of application was reversed. (B,C) isoproterenol ($3\times10^{-9}$ M) added to 30 mM KCl-contracted trachea. (B) IL addition preceded EL addition; the order of addition was reversed in (C). W indicates washout from EL and IL compartments. The lower calibration lines apply to tracings (B,C); cm = cm $H_2O$. Tracings (A) were from the same trachea, tracings (B,C) were from a different trachea, and all are representative of results obtained from three separate experiments of each type.

isoproterenol, in the absence or presence of EL isoproterenol, did not cause contraction.

There are a number of explanations why such evidence was not seen. The receptors which stimulate EpDRF release may be localized on the basolateral rather than on the apical surface of the epithelial cells. If such is the case, access of agonists to these receptors would be limited by diffusion, and the small amount of additional stimulation would be masked by that already achieved by serosally applied drugs.

It is possible that EpDRF is released continually and not in response to the agonists studied. This situation would be compatible with the results of some studies utilizing co-axial preparations. For example, the reactivity of recipient guinea-pig tracheal strips to ovalbumin (Hay et al., 1987) and the time course of force maintenance in response to carbachol (Güc et al., 1988a) and histamine (Güc et al., 1988c), were affected by the availability of a diffusable substance provided by donor guinea-pig tracheal epithelium.

A number of studies employing co-axial preparations have, however, demonstrated the agonist-evoked release of EpDRF. The rate of EpDRF release and the rapidity of its action on smooth muscle may be variable. For example, acetylcholine but not histamine caused EpDRF release from donor guinea-pig tracheal epithelium and relaxation of recipient rabbit aorta; this relaxation was slow in development (Ilhan and Sahin, 1986). The rat anococcygeus muscle within guinea-pig trachea relaxed in response to acetylcholine (Güc et al., 1988b). The relaxation had a slow time course. In contrast, Fernandes et al. (1989) observed that both histamine and methacholine caused a rapidly developing and large relaxation of rat aorta placed within guinea-pig trachea. It would thus appear that EpDRF is released from guinea-pig tracheal epithelium by both methacholine and histamine, and that the release is rapid. Such release can be anticipated to be occurring in the present experiments. A substantial variability in the rapidity of the relaxant effect of EpDRF in various smooth muscles also seems apparent. The effect of EpDRF on guinea-pig trachealis tone may be slow in onset, like rabbit aorta and rat anococcygeus, and not rapid, as occurs in rat aorta. This might explain

why no relaxation to methacholine and histamine was seen during the exposure periods used in our experiments.

While the rate of EpDRF effect on different smooth muscles may be variable when receptor agonists are involved, the epithelium-dependent, $K^+$-induced relaxation of guinea-pig perfused trachea (Munakata et al., 1988; fig. 5 and 6) and tracheal strips (Raeburn and Fedan, 1989) is, nevertheless, abrupt in onset and large in magnitude. Likewise, rapid temperature increase evokes a rapid, epithelium-dependent relaxation (Lamport and Fedan, 1990). It would appear that many unknown factors affect the release and actions of EpDRF.

Whether EpDRF is released from the apical or basolateral surface of epithelial cells is not known. The fact that the tone of a smooth muscle placed within the lumen of a trachea can so readily be decreased by agonists which release EpDRF suggests that the factor is released from the apical surface of epithelial cells, regardless of the location of the receptors involved. Unless there are other target cells for EpDRF in the airway lumen, the release of EpDRF into the lumen would seem to be an inefficient means of providing EpDRF to the submucosal muscle. The rapidity of relaxation of perfused trachea to IL KCl also must be reconciled, and it is convenient to suggest that EpDRF is liberated from the basolateral surface.

## Acknowledgements

Early studies with perfused trachea were performed with support of M.E.N. through a West Virginia University School of Medicine Summer Research Fellowship. We thank Terry Stewart for expert secretarial assistance and manuscript preparation. Mention of brand name does not constitute product endorsement.

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