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Richard Alan Johnston
West Virginia University

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Characterization of the Airway Epithelial Bioelectric Mechanisms Associated with the Effects of Epithelium-Derived Relaxing Factor in Guinea-Pig Isolated Trachea

DISSERTATION

Submitted to the School of Medicine
at West Virginia University
In Partial Fulfillment of the Requirements
for the Degree of
Doctor of Philosophy
in Pharmacology and Toxicology

by

Richard A. Johnston

Jeffrey S. Fedan, Ph.D., Chairperson
Dale L. Birkle, Ph.D.
Charles R. Craig, Ph.D.
Michael I. Luster, Ph.D.
Mark J. Reasor, Ph.D.
Michael R. Van Scott, Ph.D.

Department of Pharmacology and Toxicology

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Abstract

Characterization of the Airway Epithelial Bioelectric Mechanisms Associated with the Effects of Epithelium-Derived Relaxing Factor in Guinea-Pig Isolated Trachea

Richard A. Johnston

In response to elevated serosal or mucosal tonicity, guinea-pig tracheal epithelium releases the non-nitric oxide, non-prostanoid, epithelium-derived relaxing factor (EpDRF), which subsequently diffuses to the underlying airway smooth muscle to initiate relaxation. Hypertonicity-induced smooth muscle relaxation *via* EpDRF involves epithelial Na^+ and Cl^- channels and is preceded by a depolarization of the transepithelial potential difference (V_{ms}). By utilizing two different experimental systems, this study sought to characterize further the airway epithelial bioelectric mechanisms associated with the effects of EpDRF in guinea-pig isolated trachea.

First, this study attempted to elucidate which epithelial ion channels and/or transporters are involved in the depolarization of the V_{ms} . This was accomplished by determining the effect of ion channel and transporter inhibitors on *in vitro* transepithelial short-circuit current responses to elevated mucosal tonicity in guinea-pig tracheal segments mounted in Ussing chambers. The data from these studies indicate that apical Na^+ channels and the basolateral $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter are intimately involved in the epithelial bioelectric response to elevated mucosal tonicity, and thus, may play an important role in the synthesis, release, and/or effects of EpDRF.

Secondly, this study sought to determine the consequences of altered epithelial ion transport, following LPS-treatment (4 mg/kg, i.p.; 18 hr post-treatment), on EpDRF-induced smooth muscle relaxation and its associated bioelectric events as well as airway reactivity to methacholine (MCh). *In vitro* tracheal smooth muscle contractile and relaxant responses as well as V_{ms} responses were measured utilizing the isolated and modified isolated, perfused trachea apparatuses while the two-chambered, whole-body plethysmograph was used to measure *in vivo* airway reactivity. LPS-treatment caused numerous and complex changes in EpDRF-induced smooth muscle relaxation and its associated bioelectric events. In addition, LPS-treatment causes *in vivo* airway hyporeactivity to MCh. These results suggest that the effects of EpDRF on smooth muscle reactivity are modulated by changes in the electrical activity of the epithelium which can ultimately impact on airway reactivity to MCh.

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List of Abbreviations

ACh	acetylcholine
ARDS	acute respiratory distress syndrome
ASL	airway surface liquid
ASM	airway smooth muscle
BF	breathing frequency
C.I.	confidence interval
DMSO	dimethyl sulfoxide
EC ₅₀	effective concentration producing 50% of the maximum response
EIA	exercise-induced asthma
EL	extraluminal
EpDRF	epithelium-derived relaxing factor
IL	intraluminal
I_{sc}	short-circuit current
KCl	potassium chloride
L-NAME	N ^T -nitro-L-arginine methyl ester
LPS	lipopolysaccharide
MCh	methacholine chloride
MCh PC ₂₀₀	provocative methacholine chloride concentration producing a two-fold increase in basal specific airway resistance
MKH	modified Krebs-Henseleit
NaCl	sodium chloride
NO	nitric oxide
RVI	regulatory volume increase
SRaw	specific airway resistance
V _{ms}	transepithelial potential difference
ΔP	differential pressure (inlet minus outlet pressure difference)

Introduction

The Mammalian Respiratory System

The mammalian respiratory system is comprised of the lungs, the extra- and intrapulmonary airways, the chest wall, and the respiratory control centers of the central nervous system (Levitzky, 1995). The primary function of the respiratory system is to exchange gases between the atmosphere and the blood. This ensures that mammals are supplied with oxygen while carbon dioxide is removed (West, 1995; Johnson, 1997). Oxygen, in conjunction with other organic molecules, is utilized by the cell to produce energy in the form of adenosine triphosphate during the process of oxidative phosphorylation (Lehninger et al., 1993). Aside from gas exchange, the respiratory system has non-respiratory functions which include playing a significant role in (1) acid-base balance, (2) body defense, (3) metabolism, (4) olfaction, (5) phonation, and (6) water loss (Levitzky, 1995).

In order for cells to be supplied with oxygen, the oxygen within the inspired air must first gain access to the alveoli, or gas exchanging regions of the lung, before being distributed throughout the body by means of the circulatory system. Air is primarily inspired through the nose and to a lesser extent, the mouth, and once inspired, it passes through the pharynx, larynx, trachea, bronchi, and bronchioles until it eventually reaches the alveoli. The most distal portion of the trachea branches into right and left primary bronchi which enter the right and left lungs, respectively. The right and left primary bronchi continue to branch even further into at least two smaller daughter branches. The airways continue to branch until terminating into alveolar sacs (Weibel, 1963; Nagaishi et al., 1972; Curtis and Barnes, 1989; Sherwood, 1997). Figure 1 illustrates the branching of the tracheobronchial tree, and because of anatomical and functional differences along its length, it is divided into a conducting zone and a respiratory or transitional zone. The conducting zone consists of airway generations zero to sixteen while generations seventeen to twenty-three make up the respiratory or transitional zone (Weibel, 1963).

Gas exchange is restricted to the respiratory or transitional zone while the conducting zone constitutes an anatomic dead space (Levitzky, 1995). When air moves through the conducting airways, it is humidified and warmed to body temperature (Levitzky, 1995; Sherwood, 1997). The respiratory or transitional zone occupies the last 5 mm of each airway branch and consists of small airways, namely bronchioles and alveoli, which are capable of gas exchange (Bouhuys, 1977). In addition to the functional differences between the conducting and respiratory or transitional zones, there is also a great deal of structural heterogeneity in the airways within and between these two regions.

The airways of the upper conducting zone are relatively nonmuscular, rigid tubes encircled by cartilaginous rings. Their walls are rich in connective tissue and cartilage but sparsely populated with smooth muscle (Johnson, 1997). The luminal surface of these airways are predominately lined with ciliated, pseudostratified, columnar epithelial cells, among which are dispersed submucosal glands, basal, brush, goblet, Kulchitsky, and serous cells as well as a small number of neuroendocrine cells (Divertie and Brass, 1980; Fawcett and Jensh, 1997; Johnson, 1997).

Figure 1. Divisions of the tracheobronchial tree. Z, generation; BR, bronchus; BL, bronchiole; TBL, terminal bronchiole; RBL, respiratory bronchiole; AD, alveolar duct; AS, alveolar sac. Reprinted with permission from Weibel ER (1963) *Morphometry of the Human Lung*, Academic Press Ltd, London UK.

The airways located at the junction between the conducting and respiratory or transitional zones differ from those either proximal or distal to them. Their walls are devoid of cartilage and contain little connective tissue but are rich in smooth muscle (Weibel, 1963; Nagaishi et al., 1972; Johnson, 1997). Ciliated, cuboidal epithelial cells are the predominant epithelial cell type of this region; however, they are smaller than their more proximal counterparts. There are fewer goblet cells in this region while submucosal glands are virtually absent. Clara cells, secretory cells of ambiguous function, also populate this region (Divertie and Brass, 1980; Fawcett and Jensh, 1997; Johnson, 1997). The differences in epithelial cell morphology within the conducting zone are shown in Figure 2.

The airway walls of the respiratory or transitional zone lack smooth muscle and contain very little connective tissue (Johnson, 1997). Type I alveolar epithelial cells are the predominant cell type covering the luminal surface of the alveoli. They are long, thin cells lacking cilia. Type II alveolar epithelial cells, which synthesize and secrete surfactant, are also found, to a lesser extent, along the luminal surface of the alveoli (Sherwood, 1997). In order for gas exchange to occur between the alveoli and the blood in the pulmonary capillaries, the gas must diffuse through the air-blood barrier which is formed by the pulmonary capillary endothelium, the type I alveolar epithelium, and the interstitial space between the two (Sherwood, 1997). An illustration of the air-blood barrier is shown in Figure 3. There are an estimated 300 million alveoli and nearly 1000 pulmonary capillaries surrounding each alveolus, providing a 50-100 m² surface area for gas exchange (Divertie and Brass, 1980; West, 1995). When this is taken with the fact that the air-blood barrier is only 0.5 µm thick, the mammalian respiratory system is indeed an exquisitely efficient gas exchanger (West, 1995; Sherwood, 1997).

Figure 2. Epithelial cell morphology of the (A) proximal and (B) distal airways of the conducting zone. Along the respiratory tract, there are distinct differences in epithelial cell morphology. Copyright 1980. Novartis. Reprinted with permission from Novartis Medical Education, illustrated by Frank H. Netter, MD. All rights reserved.

Figure 3. The air-blood barrier. This illustration depicts two of an estimated 300 million alveoli, the gas exchanging regions of the lung, and the other, affiliated structures of the air-blood barrier. Copyright 1980. Novartis. Reprinted with permission from Novartis Medical Education, illustrated by Frank H. Netter, MD. All rights reserved.

The Respiratory Smooth Muscle and Its Neural Innervation

The respiratory or airway smooth muscle is one of the six basic types of smooth muscle (Horowitz et al., 1996). It has many structural features in common with the other types of smooth muscle and is found from the trachea to the respiratory bronchioles, albeit in different quantities.

The parasympathetic nervous system has a profound impact on airway smooth muscle contraction. Postganglionic parasympathetic fibers directly innervate the smooth muscle. The release of acetylcholine (ACh) from these neurons initiates smooth muscle contraction via smooth muscle M_3 cholinergic receptors which are coupled to G_q -proteins on the muscle cells. Other spasmogens, by binding to their appropriate receptors, also initiate smooth muscle contraction through the activation of G_q -proteins. The α_q subunit of the G_q -protein activates the inner, plasma membrane-bound enzyme phospholipase C- β , leading to the synthesis of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol from a membrane-bound phospholipid, phosphatidylinositol 4,5 bisphosphate. IP_3 elevates cytosolic Ca^{2+} levels by stimulating, either directly or indirectly, the release of Ca^{2+} from intracellular stores (sarcoplasmic reticulum and mitochondria), the influx of extracellular Ca^{2+} through Ca^{2+} -selective ion channels and/or transporters, and Ca^{2+} -induced Ca^{2+} release. Elevations in cytosolic Ca^{2+} activate the Ca^{2+} /calmodulin-dependent myosin light chain kinase which enhances actin-myosin interactions resulting in smooth muscle contraction (Figure 4A) (Gross, 1988; Allen and Walsh, 1994; Horowitz et al., 1996; Janssen et al., 1998).

The sympathetic nervous system can also influence airway smooth muscle tone. There is evidence that postganglionic sympathetic fibers terminate on postganglionic parasympathetic nerves, thereby having the capability to modulate cholinergic neural mechanisms (Ind and Barnes, 1988). Activation of smooth muscle β_2 -adrenoceptors by adrenergic agonists initiates bronchodilation by activating adenylate cyclase which increases intracellular cyclic adenosine monophosphate (cAMP) levels. cAMP stimulates smooth muscle relaxation by down-

regulating myosin light chain kinase activity, stimulating the uptake of Ca^{2+} into the sarcoplasmic reticulum, and activating Ca^{2+} -dependent K^{+} channels (Figure 4B) (Kotlikoff and Kamm, 1996; Janssen et al., 1998). In humans, prostaglandin E_2 is capable of elevating intracellular smooth muscle cAMP concentrations and initiating relaxation. " - Adrenoceptors are also present on airway smooth muscle, but the consequences of their activation are more controversial (Ind and Barnes, 1988).

Besides the autonomic nervous system, there are other neural systems capable of modulating airway smooth muscle tone. The inhibitory nonadrenergic, noncholinergic (i-NANC) system is the only significant bronchodilator pathway in humans. Vasoactive intestinal peptide (VIP) and nitric oxide (NO) are the major neurotransmitters of this system (Barnes, 1988). VIP exerts its bronchodilating effects by elevating intracellular cAMP levels while NO initiates bronchodilation by activating soluble guanylate cyclase, resulting in the activation of smooth muscle K^{+} channels via cyclic guanosine monophosphate (cGMP) (Figure 4B) (Janssen et al., 1998). The excitatory nonadrenergic, noncholinergic (e-NANC) system is another neural system capable of influencing airway smooth muscle tone. e-NANC fibers release substance P and tachykinins from their terminals located beneath the airway epithelium. Both substance P and tachykinins initiate smooth muscle contraction by activation of their respective G_q -protein-coupled receptors (Barnes, 1988; Janssen et al., 1998).

Figure 4. Mechanisms of airway smooth muscle (A) contraction and (B) relaxation. See text for details. ACh, acetylcholine; G, G-protein-coupled receptor; PLC, phospholipase C- β ; IP₃, inositol 1,4,5-trisphosphate; ΔV , change in membrane potential: (A) depolarization and (B) hyperpolarization; NE, norepinephrine; PGE₂, prostaglandin E₂; NO, nitric oxide; AC, adenylyl cyclase; GC, soluble guanylate cyclase; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate. Reprinted with permission from Janssen LJ, Daniel EE and Rodger IW (1998) Airway smooth muscle cells: Structure and function, in *Asthma: Basic Mechanisms and Clinical Management* (Barnes PJ, Rodger IW and Thomson NC eds) pp 90-112, Academic Press Ltd, London UK.

The Respiratory Epithelium

The respiratory or airway epithelium constitutes a barrier to control the movement of substances between the external and internal environments of the body (Hulsmann and Jongste, 1996; Fawcett and Jensh, 1997); however, the epithelium functions as more than just a simple diffusion barrier (Widdicombe, 1991; Folkerts and Nijkamp, 1998). Under normal physiological conditions, the respiratory epithelium also functions as a mucus transporter (Widdicombe, 1991; Clarke and Boucher, 1993), a regulator of ion and fluid transport (Clarke and Boucher, 1993; Boucher, 1999), an osmotic sensor (Willumsen et al., 1994; Dortch-Carnes et al., 1999; Fedan et al., 1999), a site of metabolism for both endogenous and exogenous substances, and a source of spasmogenic and relaxant substances which are capable of modulating the reactivity of the underlying airway smooth muscle (Flavahan et al., 1985; Fedan et al., 1988; Goldie and Hay, 1997; Folkerts and Nijkamp, 1998).

Overlying the luminal or mucosal surface of the respiratory epithelium is the airway surface liquid (ASL) which consists of two layers: the sol and the gel. The sol layer is a thin, 5 to 7 μm in depth, watery layer which blankets the surface. Its thickness approximates the length of the cilia extending from the luminal surface of the epithelium. The gel layer rests upon the sol layer and is a discontinuous mucus blanket, consisting of 95% water (Figure 5). Since the mucus is both elastic and viscous, it can easily trap, and subsequently hold, inhaled, foreign matter. As the cilia beats, both the sol and gel layers move in tandem in a cephalad direction which allows for the effective clearance of inhaled, foreign substances and microorganisms from the respiratory tract. Epithelial ion and fluid transport play a major role in determining the depth of the ASL (Clarke and Boucher, 1993; Widdicombe and Widdicombe, 1995; Boucher, 1999).

Figure 5. Airway surface liquid (ASL). The ASL blankets the mucosal surface of the airway epithelium and consists of two layers: the sol and the gel. The depth of the ASL ranges between 5-100 μm (Widdicombe and Widdicombe, 1995). Reprinted from Clarke LL and Boucher RC (1993) Ion and water transport across airway epithelia, in *Pharmacology of the Respiratory Tract: Experimental and Clinical Research* (Chung KF and Barnes PJ eds) pp 505-550 by courtesy of Marcel Dekker Inc. For clarity, the author of this dissertation added the figure labels.

Under normal, physiological conditions, the epithelium of the conducting airways of most mammalian species, excluding the cow and the dog, is Na^+ -absorbing (Boucher et al., 1982; Langridge-Smith et al., 1984); however, all generate a lumen-negative potential with respect to the basolateral surface. A tentative localization of airway epithelial ion channels and transporters is shown in Figure 6. The activity of the $\text{Na}^+\text{-K}^+\text{-ATPase}$, localized to the basolateral surface of the epithelial cell, establishes an electrochemical gradient which draws Na^+ into the cell from the ASL through amiloride-sensitive Na^+ channels. The Na^+ is then extruded from the cell across the basolateral membrane by means of the $\text{Na}^+\text{-K}^+\text{-ATPase}$. K^+ channels and the electroneutral $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter also play a role in epithelial ion transport. Under normal, physiological conditions for most mammalian species, there is no net transcellular movement of Cl^- ; however, in order to achieve electrochemical equilibrium, Cl^- moves through the paracellular pathway as Na^+ is absorbed transcellularly (Figure 6) (Boucher et al., 1982; Clarke and Boucher, 1993; Knowles et al., 1995).

Epithelium-Derived Relaxing Factor

The respiratory epithelium can synthesize and release substances which are capable of diffusing to the underlying airway smooth muscle and modulating its tone (Fedan et al., 1988; Morrison et al., 1990; Hulsman and Jongste, 1996). These autacoids can have either an excitatory or inhibitory effect upon airway smooth muscle tone. The inhibitory, non-nitric oxide, non-prostanoid epithelium-derived relaxing factor (EpDRF) is one such autacoid (Munakata et al., 1988, 1990; Fedan et al., 1999).

In 1985 Flavahan and colleagues provided the first evidence that the respiratory epithelium could modulate the tone of the underlying airway smooth muscle. After mechanical removal of the epithelium from canine bronchial rings, the bronchial smooth muscle became more sensitive to the effects of contractile agonists (ACh, histamine, and 5-hydroxytryptamine).

Figure 6. Distribution of airway epithelial ion channels and transporters and the major ion transport processes of the airway epithelium. This illustration holds true for most mammalian species (Boucher et al., 1982; Clarke and Boucher, 1993). The various ion channels and transporters of the airway epithelium are asymmetrically distributed between the apical and basolateral surfaces of these cells. The respective inhibitor of each ion channel and transporter is shown in parentheses. The arrows indicate the net, transcellular movement of Na^+ and the passive, paracellular movement of Cl^- (illustration produced by and courtesy of Jeffrey S. Fedan, Ph.D.).

In canine bronchial rings precontracted with ACh, isoproterenol-induced relaxations were attenuated in epithelium-denuded preparations. This same study also demonstrated that contractile responses to transmural nerve stimulation were potentiated by epithelium removal. In epithelium-intact preparations, the peak contraction elicited by a particular electrical frequency was followed by a decrease in tone as the stimulation continued; however, this decrease in tone was absent in epithelium-denuded preparations (Flavahan et al., 1985). Later that same year, a separate group reported that epithelium removal increased the sensitivity of bovine tracheal strips to the contractile effects of ACh, histamine, and 5-hydroxytryptamine (Barnes et al., 1985). In the months and years that followed, other studies with isolated, human (Raeburn et al., 1986a), guinea pig (Hay et al., 1986; Holroyde, 1986; Murlas, 1986), and rabbit (Raeburn et al., 1986b) airway preparations reported that epithelium removal increased the sensitivity of the airway smooth muscle to contractile agonists; however, the effect of epithelium removal upon airway smooth muscle reactivity to relaxant agonists was much more variable. Most of these studies, with the exception of Holroyde (1986) concluded that the increased sensitivity of the smooth muscle to contractile agonists following epithelium removal was due to the loss of an epithelium-derived inhibitory factor, EpDRF, which normally inhibits smooth muscle reactivity to contractile agonists. Some of these same studies also demonstrated that EpDRF was not a metabolite of arachidonic acid (Barnes et al., 1985; Flavahan et al., 1985), but nevertheless, a separate study concluded that the cytochrome P₄₅₀ enzyme system was involved in epithelial modulation of smooth muscle tone (Raeburn et al., 1988).

The first evidence supporting the hypothesis that the airway epithelium releases an inhibitory factor capable of modulating the reactivity of adjacent smooth muscles came in 1986 with the development of the coaxial bioassay preparation. This preparation consisted of an endothelium-denuded rabbit aortic strip (recipient tissue) positioned within the lumen of an epithelium-intact or -denuded guinea-pig trachea (donor tissue) (Figure 7A). By attaching the endothelium-denuded rabbit aortic strip to an isometric force transducer, contractile and relaxant responses of this tissue could be measured. Following the contraction of the aortic strip with

phenylephrine, an α_1 -adrenoceptor agonist, ACh was added to the preparation which subsequently relaxed the aortic strip. When the epithelium was removed from the guinea-pig trachea, and the same experiment repeated, the aortic strip failed to relax (Ilhan and Sahin, 1986) (Figure 7B). Coaxial bioassay experiments from other laboratories reported similar results when guinea-pig tracheal smooth muscle or rat anococcygeus muscle were used as the recipient tissue (Güc et al., 1988a; Güc et al., 1988b), or when human bronchus was used as a donor to relax phenylephrine-contracted rat aortic strips (Fernandes et al., 1990). In all of these coaxial bioassay experiments, as in the original, removal of the epithelium from the donor tissue abolished the relaxation response of the recipient tissue, supporting the notion that the relaxation of the recipient tissue was dependent upon an epithelium-derived relaxing factor, EpDRF. Further pharmacological characterization of EpDRF with the coaxial bioassay preparation demonstrated that this factor was not a product of nitric oxide synthase, cyclooxygenase, lipoxygenase, or the cytochrome P₄₅₀ enzyme systems. This same study showed that EpDRF was not related to free radical production, soluble guanylate cyclase, β -adrenoceptors, or platelet-activating factor (Fernandes and Goldie, 1990).

Until the late 1980s, EpDRF was studied using rings or strips of airway tissue, which had limited usefulness. In 1988 the guinea-pig isolated, perfused trachea apparatus was developed, allowing for much more elegant EpDRF studies (Munakata et al., 1988). This preparation allowed pharmacological agents to be added separately to either the extraluminal (serosal) or intraluminal (mucosal) surfaces of the trachea. In this preparation, contractile agonists added to the extraluminal surface were more potent since they had direct access to the smooth muscle. Agents added to the intraluminal surface must first diffuse through the epithelium before interacting with the smooth muscle. Early experiments with this preparation (Munakata et al., 1988) demonstrated that increasing the tonicity of the solution perfusing the intraluminal surface of the trachea was a major stimulus for the release of EpDRF.

Figure 7. Coaxial bioassay preparation. (A) A diagram of the coaxial bioassay preparation whose use was first reported by Ilhan and Sahin in 1986. (B) A schematic illustration of the experimental results obtained from this preparation. The addition of ACh to an endothelium-denuded, phenylephrine (PE)-contracted, rabbit aortic strip within an epithelium-intact (+Epi), guinea-pig trachea relaxed the aortic strip. When the same experiment was repeated using an epithelium-denuded (-Epi), guinea-pig trachea, the aortic strip failed to relax; thus, the ability of the epithelium to produce a transferrable EpDRF was concluded (illustration produced by and courtesy of Jeffrey S. Fedan, Ph.D.).

When hypertonic solutions of NaCl, KCl, urea, or mannitol were added to the intraluminal surface of a carbachol-contracted trachea, the smooth muscle relaxed. When the experiment was repeated in epithelium-denuded trachea, smooth muscle relaxation was significantly attenuated, demonstrating that the relaxation response evoked by hypertonic solution was epithelium-dependent (Munakata et al., 1988). A separate study later demonstrated that increasing the tonicity of the extraluminal surface of the trachea also stimulated EpDRF-induced smooth muscle relaxation (Fedan et al., 1999). It has also been shown that stimulating the release of EpDRF with hypertonic solution decreased smooth muscle reactivity to methacholine and transmural nerve stimulation (Fedan et al., 1999). Although it has been shown that elevated extraluminal and intraluminal tonicity elicit epithelium-dependent smooth muscle relaxation via EpDRF, EpDRF's mechanism of action as well as its identity remains largely obscure.

It has been demonstrated clearly that EpDRF-induced smooth muscle relaxation, evoked by hypertonic solution, is unaffected by nitric oxide synthase inhibitors, indomethacin, methylene blue, propranolol, and tetrodotoxin (Munakata et al., 1988, 1990; Fedan et al., 1999). Interestingly, gossypol, a dual cyclooxygenase-lipoxygenase inhibitor and oxygen radical scavenger, and hemoglobin attenuate relaxation responses to elevated intraluminal tonicity (Teeter et al., 1988; Munakata et al., 1990; Fedan and Johnston, unpublished). Because hemoglobin, and not nitric oxide synthase inhibitors, attenuates EpDRF-induced smooth muscle relaxation, this suggests that carbon monoxide may play a role in the relaxation response to elevated tonicity since carbon monoxide is capable of binding to hemoglobin (West, 1995).

Previous work has suggested that the synthesis, release, and/or effects of EpDRF may be functionally linked to the electrical activity of epithelial cells (Raeburn and Fedan, 1989). Fedan et al. (1999) reported that amiloride-sensitive Na^+ channels and 4-4'-diisothiocyanatostilbene-2-2'-disulfonic acid (DIDS)-sensitive Cl^- channels seem to be involved in EpDRF-induced smooth muscle relaxation. Both amiloride and DIDS attenuate EpDRF-induced smooth muscle relaxation responses elicited by elevated intraluminal tonicity. Fedan et al. (1999) also reported that the $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter and the $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ play virtually no role in EpDRF-

induced smooth muscle relaxation. A separate study reported that epithelial Ca^{2+} -activated K^+ channels may be involved in the synthesis and/or release of EpDRF (Tamaoki et al., 1997). Dortch-Carnes et al. (1999) recently modified the isolated, perfused trachea apparatus to measure transepithelial potential difference simultaneously with smooth muscle contraction and relaxation. This modified preparation has provided more evidence that the synthesis, release, and/or effects of EpDRF are functionally linked to epithelial electrical activity. Dortch-Carnes et al. (1999) have reported that depolarization of the transepithelial potential difference precedes EpDRF-induced smooth muscle relaxation elicited by elevating intraluminal tonicity with either ionic or non-ionic osmolytes (Dortch-Carnes et al., 1999). A summary of EpDRF's actions are illustrated in Figure 8.

Cell-Volume Regulation in Response to Hypertonic Challenge

When exposed to anisotonic solutions, cells activate volume regulatory mechanisms to either maintain or return to their normal volume (Parker, 1993; McManus et al., 1995; Häußinger, 1996). When the tonicity of the mucosal and serosal surfaces of the airway epithelium are elevated, this is a major stimulus for the synthesis, release, and/or effects of EpDRF (Munakata et al., 1988; Fedan et al., 1999), it is therefore essential to understand the principles of cell-volume regulation as they pertain to hypertonic challenge. A cell loses water when exposed to a hypertonic environment. In order to regain its normal volume, the cell activates regulatory volume increase (RVI) mechanisms. Increases or decreases in cell volume can only be achieved by the gain or loss, respectively, of osmotically active solutes. Osmotically active solutes include inorganic ions such as Na^+ , K^+ , and Cl^- and small, organic osmolytes. These small, organic osmolytes are divided into three major classes: (1) amino acids and their derivatives (i.e., alanine, proline, and taurine), (2) methylamines (i.e., betaine and glycylphosphorylcholine), and (3) polyols (i.e., sorbitol and *myo*-inositol). These molecules are sometimes referred to as non-perturbing osmolytes since the cell can accumulate

high concentrations of these substances without deleterious effects on cell structure and function (McManus et al., 1995; Lang et al., 1998). RVI mechanisms occur in two steps. First, within seconds after exposure to a hypertonic environment, a cell increases its net gain of Na^+ and Cl^- to maintain its volume through activation of the $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter and the $\text{Na}^+ - \text{H}^+$ exchanger, both of which are present on airway epithelial cells. Because the intracellular pH is altered by the activity of the $\text{Na}^+ - \text{H}^+$ exchanger, there is subsequent activation of the $\text{Cl}^- - \text{HCO}_3^-$ exchanger (Clarke and Boucher, 1993; McManus et al., 1995; Lang et al., 1998).

Figure 8. Actions of the airway epithelium-derived relaxing factor (EpDRF). EpDRF is known to be released when the tonicity of either the apical or basolateral surfaces of the airway epithelium are elevated, which in this illustration is denoted by NaCl. Once released, EpDRF can relax the airway smooth muscle. It is still questionable whether or not EpDRF can relax vascular smooth muscle or if it is capable of attenuating the effects of ACh released from postganglionic parasympathetic nerve terminals. Epi, airway epithelium; VSM, vascular smooth muscle; ASM, airway smooth muscle; ACh, acetylcholine (illustration produced by and courtesy of Jeffrey S. Fedan, Ph.D.).

The second step involves the accumulation of small organic osmolytes by activating the transcription of genes encoding osmolyte synthetic enzymes and transporters (Figure 9). These osmolytes are accumulated along with Na^+ by means of Na^+ -coupled osmolyte transporters, and this process can last for hours or days (McManus et al., 1995; Lang et al., 1998). In addition during RVI, cells breakdown cytoplasmic proteins while the anabolic pathways leading to the synthesis of organic osmolytes are increased (Haüssinger, 1996).

Exercise-Induced Asthma

In general, asthma can be defined as a chronic, inflammatory airway disease characterized by reversible airway obstruction. Asthma affects over 5% of the inhabitants of the world's industrialized countries (Shelhamer et al., 1995; Barnes et al., 1998). In the United States as many as twelve million persons may be afflicted with asthma (Shelhamer et al., 1995). The most common pathophysiological features of asthma include: (1) sloughing and desquamation of the respiratory epithelium, (2) thickening of the respiratory epithelial basement membrane, (3) goblet cell hyperplasia, (4) submucosal gland enlargement, (5) airway smooth muscle hypertrophy and hyperplasia, (6) dilation of the bronchial vasculature, (7) airway edema, (8) recruitment of inflammatory cells into the airways (Jeffrey, 1998), and (9) nonspecific airway hyperreactivity to nonsensitizing physical or chemical stimuli (Cockcroft and O'Byrne, 1993).

Approximately 70-80% of asthmatics experience exercise-induced asthma (EIA) which is a general term used to describe a transient increase in airway resistance following strenuous exercise. A decrease of 10% or more in the volume of air forcibly expired in one second (FEV_1) following exercise is used to diagnose EIA (Anderson, 1985; Anderson and Daviskas, 1999).

Figure 9. Mechanisms of regulatory volume increase (RVI). The RVI mechanisms occur in two steps. First, immediately after cells shrink due to exposure to a hypertonic environment, cells accumulate inorganic ions, namely Na^+ and Cl^- through the activation of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter, the $\text{Na}^+\text{-H}^+$ exchanger, and subsequently the $\text{Cl}^-\text{-HCO}_3^-$ exchanger. The second step occurs over many hours or days and involves the accumulation of organic osmolytes via the induction of genes encoding for osmolyte synthetic enzymes and ion-coupled transporters. 5'•AAA3' represents elevations in mRNA levels to convey an up-regulation of the transcription of genes encoding osmolyte synthetic enzymes and ion-coupled transporters. Figure adapted with permission from McManus ML, Churchwell KB and Strange K (1995) Regulation of cell volume in health and disease, *N Engl J Med* 333:1260-1266.

EIA is the most extreme five to ten minutes upon the cessation of exercise; however, a refractory period exists for up to three hours after the initial attack has ended (Anderson, 1985; McFadden, 1995). In contrast to asthmatics who may experience bronchoconstriction following exercise, normal individuals may actually experience bronchodilation during exercise (Kagawa and Kerr, 1970). Many hypotheses have tried to explain the phenomenon of EIA (Freed, 1995); however, two hypotheses in particular have gained the most attention: (1) the thermal hypothesis of EIA and (2) the osmotic theory of EIA. Most of the experimental evidence seems to support the osmotic theory of EIA (McFadden, 1995; Anderson and Daviskas, 1999) which is described in the following paragraphs.

Because of temperature and water vapor concentration gradients, heat and water are added to the inspired air as it passes over the respiratory mucosa of the conducting airways, a process referred to as conditioning. Under normal physiological conditions, most of the inspired air is conditioned by the time it reaches the oropharynx. During strenuous exercise, an individual's ventilation rate increases dramatically; therefore, the respiratory tract has to condition a greater volume of air over a much shorter period of time. As a consequence, more airways are actively recruited to condition the inspired air, and in the process, the respiratory mucosa loses more heat and water than normal to the inspired air. The ASL is the major source of water used to condition the inspired air during exercise. The water lost from the ASL during exercise is not readily replaced, and as a result, the osmolarity of the ASL increases (Anderson and Daviskas, 1999).

The osmotic theory of EIA suggests that the increased ASL osmolarity results in the release of bronchoactive mediators from the epithelium, bronchial mucosal mast cells, and sensory nerves which lead to EIA. Spasmogenic substances released from these cells include prostaglandin D₂, leukotrienes, histamine, and substance P, all of which can readily diffuse to the smooth muscle and initiate bronchoconstriction. Increasing the osmolarity of the ASL also leads to the release of bronchorelaxant substances from the airway epithelium, including NO,

prostaglandin E₂, and EpDRF

(Munakata et al., 1988; Anderson and Daviskas, 1999; Fedan et al., 1999). The effects of airway drying on the airway epithelium and surrounding cells of the airway wall are illustrated in Figure 10.

The osmotic theory of EIA explains why some asthmatics could experience bronchoconstriction following exercise whereas normal individuals do not. In asthmatics, the airway epithelium is sloughed and desquamated (Laitinen et al., 1985), and as a consequence, more airways would have to be recruited to condition the inspired air. This could lead to an increase in the ASL osmolarity throughout the respiratory tract, resulting in the release of bronchospastic mediators from various sources to initiate EIA (Anderson and Daviskas, 1999). In normal individuals, the intact epithelium may produce enough bronchorelaxant factors (NO, PGE₂, and EpDRF) to offset the effects of these bronchospastic mediators; therefore EIA is prevented.

Figure 10. The effects of airway drying on the airway epithelium and airway wall. (A) The net direction of ion and water movement across the airway epithelium under normal, physiological conditions is indicated by the arrows. (B) Evaporative water loss (airway drying) as a result of hyperpnea reduces the depth of the periciliary fluid or ASL and increases its concentration of inorganic ions; thus, its osmolarity is elevated. This results in the release of bronchoactive mediators from the epithelium and the vascular endothelium of bronchial vessels in the airway wall. These mediators are capable of diffusing to the airway smooth muscle and modulating its tone. In addition, water movement from the submucosal tissues increases during this time in order to replenish the water lost from the periciliary fluid during airway drying. PGs, prostaglandins; NO, nitric oxide. Reprinted from Anderson SD and Daviskas E (1999) Airway drying and exercise-induced asthma, in *Exercise-Induced Asthma* (McFadden ER ed) pp 77-113 by courtesy of Marcel Dekker Inc.

Lipopolysaccharide, Sepsis, and Acute Respiratory Distress Syndrome

Lipopolysaccharide (LPS) is an amphiphilic macromolecule which is the major component of the outer membrane of gram-negative bacteria (Figure 11A). LPS consists of two major structural components: a polysaccharide portion and a lipid A portion. The lipid A component is covalently bound on one side to the outer membrane of the bacterium whereas the polysaccharide portion is bound to the other end of the lipid A component and extends into the extracellular space. As Figure 11B illustrates, the polysaccharide portion can be further subdivided into a core region and an O-specific chain. The lipid A portion is highly conserved among gram-negative bacteria and is the toxic component of the LPS macromolecule. As gram-negative bacteria die or begin to replicate, LPS is released and capable of initiating its pathological effects (Lüderitz et al., 1982; Rietschel et al., 1994, 1996; Raetz, 1996).

LPS, in itself, is not toxic, but it is the immunologic and inflammatory events that it initiates which are detrimental. Various serum proteins are capable of modulating the effects of LPS, but the most important is the lipopolysaccharide-binding protein (LBP). By binding to the LBP, the LPS macromolecule is delivered to a membrane-bound receptor, CD14, found on macrophages and monocytes, and once bound, LPS-induced signal transduction can commence (Rietschel et al., 1996). LPS stimulates a wide variety of cellular processes, some of which are summarized in Figure 12. An overwhelming gram-negative bacterial infection can result in sepsis and/or acute respiratory distress syndrome (ARDS).

Figure 11. Localization of lipopolysaccharide (LPS) and its chemical components. (A) LPS is the major component of the outer membrane of gram-negative bacteria. GluNAc, N-acetyl-D-glucosamine; KDO, 3-deoxy-D-manno-octulosonic acid; MDO, membrane-derived oligosaccharides. Reprinted with permission from Raetz CRH (1996) *Bacterial lipopolysaccharides: A remarkable family of bioactive macroamphiphiles*, in *Escherichia coli* and *Salmonella: Cellular and molecular biology* (Neidhardt FC ed) pp 1035-1063, ASM Press, Washington, D.C. (B) The LPS macromolecule is divided into two major regions: the lipid A portion and the polysaccharide portion. The polysaccharide region can be further subdivided. Reprinted with permission from, Academic Press Ltd, London UK.

Figure 12. Toxic effects of lipopolysaccharide (LPS). As gram-negative bacteria begin to replicate or die, LPS is released and capable of exerting its pathological effects. If a gram-negative bacterial infection is severe enough, it can result in sepsis. $\text{TNF}\alpha$, tumor necrosis factor α ; IL-1, interleukin-1; IL-6, interleukin-6; PAF, platelet-activating factor; IL-2, interleukin-2. Reprinted with permission from Bone RC (1991) The pathogenesis of sepsis, *Ann Int Med* 115(6):457-469.

As defined by the American College of Chest Physicians/Society of Critical Care Medicine, sepsis is the systemic response to infection (Bone, 1996), and as many as 500,000 new cases are reported annually in the United States (Sessler et al., 1996). Sepsis is a serious medical condition with a 40% mortality rate (Marsh and Wewers, 1996). A few of the clinical manifestations of sepsis include fever, hypothermia, tachycardia, tachypnea, oliguria, acidosis, and hypotension (Rietschel et al., 1996). Fifty percent of those individuals diagnosed with sepsis develop ARDS within the first twenty-four hours after the onset of sepsis. Acute lung injury is recognized clinically as ARDS (Sessler et al., 1996). The clinical manifestations of ARDS include arterial hypoxemia, dyspnea, decreased lung compliance, pulmonary edema, and atelectasis (McFeely and Hudson, 1994; Sessler et al., 1996).

This dissertation describes an investigation into two separate, but related questions pertaining to the role and mechanism(s) of action of EpDRF. In the first, the effects of epithelial ion transport inhibitors on the epithelial bioelectric response to elevated mucosal tonicity were examined in order to elucidate which ion channels and/or transporters are involved in the epithelial depolarization which precedes EpDRF-induced smooth muscle relaxation. In the second study, the effects of LPS-treatment on EpDRF-induced smooth muscle relaxation and related bioelectric events were examined, since LPS has been implicated in a number of pulmonary diseases.

The Relationship Between Epithelial Bioelectric Responses and Epithelium-Derived Relaxing Factor-Induced Airway Smooth Muscle Relaxation in Response to Hypertonic Solution

Objectives

When cells are exposed to a hypertonic environment, they activate RVI mechanisms which alter ion transport through perturbations in ion channel and/or transporter activity (McManus et al., 1995; Lang et al., 1998). In addition, when the extraluminal (serosal) and intraluminal (mucosal) surfaces of guinea-pig tracheal epithelium are challenged with hypertonicity, EpDRF is released (Munakata et al., 1988; Fedan et al., 1999), an event which seems to be functionally linked to epithelial ion transport (Dortch-Carnes et al., 1999; Fedan et al., 1999). With a consideration of these statements, the purpose of this study was to characterize further the epithelial bioelectric events associated with the synthesis, release, and/or effects of EpDRF in guinea-pig isolated trachea.

The hypothesis underlying this study was that hypertonicity-induced alterations in epithelial ion transport are functionally linked to the synthesis, release, and/or effects of EpDRF. The hypothesis was tested by measuring transepithelial short-circuit current (I_{sc}) responses to pharmacological agents added to either surface of guinea-pig isolated trachea mounted in Ussing chambers. Dortch-Carnes et al. (1999) have shown that EpDRF-induced smooth muscle relaxation is preceded by a depolarization of the transepithelial potential difference while Fedan et al. (1999) have shown that EpDRF-induced smooth muscle relaxation is significantly attenuated by amiloride (Na^+ channel blocker) and DIDS (Cl^- channel blocker). The experiments in this study will try to determine which epithelial ion channels and/or transporters are involved in the transepithelial depolarization preceding EpDRF-induced airway smooth muscle relaxation. This will be accomplished by examining the effect of various ion channel and transporter inhibitors on I_{sc} responses to hypertonic modified Krebs Henseleit (MKH) solution. In this study, the MKH solution was made hypertonic by adding either 120 mOsm NaCl or D-mannitol to the MKH solution.

Materials and Methods

Animals

Specific, pathogen-free, male Dunkin-Hartley guinea pigs (Harlan Sprague Dawley, Inc.; Indianapolis, IN; 370-714 g) were used during the course of this study. The guinea pigs were housed in separate, actively ventilated, micro-isolator cages (Lab Products, Inc.; Seaford, DE) within a single room. The room within which these animals were housed were part of an 8800 ft² modified barrier, animal care facility. Within this facility, the animals were acclimated to their new environment prior to experimentation, given food and water *ad libitum*, and breathed fresh air. All of the animals in the animal care facility, as well as the facility itself, were under the supervision of a full-time veterinarian.

Throughout this study, the guinea pigs were sacrificed according to a standard protocol. The animals were first anesthetized intraperitoneally (i.p.) with sodium pentobarbital (Veterinary Laboratories, Inc.; Lenexa, KS; 65 mg/kg), and once it was determined that the animals were unresponsive to any stimuli, they were exsanguinated by severing the abdominal aorta and cutting the heart.

All of the experimental protocols in this study were approved by the Institutional Animal Care and Use Committee (IACUC) while the facilities where the experimental protocols were conducted were fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. These facilities have been accredited by AAALAC International since February 18, 1987.

Ussing System

A classical Ussing system [World Precision Instruments (WPI); Sarasota, FL] was utilized to directly measure I_{sc} in freshly isolated, guinea-pig tracheal segments. I_{sc} can be defined as the current required to offset the spontaneous transepithelial potential difference

generated by the tissue. Under I_{sc} conditions there is no net ion transport; therefore, I_{sc} is a measurement of total active ion transport (Soybel, 1994).

The Ussing system consisted of two separate, water-jacketed, glass circulation reservoirs whose temperature was maintained at 37°C. The reservoirs were filled with recirculating, MKH solution, a physiological salt solution, which was maintained at 37°C and continuously gassed with 95% O₂-5% CO₂. The reservoirs were connected to opposing hemi-chambers, between which a guinea-pig tracheal segment was mounted. Because of the manner in which the tissue was mounted, pharmacological agents could be added selectively to either the mucosal or serosal surfaces of the trachea.

In order to prepare a guinea-pig tracheal segment for mounting, the animal was sacrificed as previously described. The trachea was exposed *in vivo*, the entire trachea excised, placed in MKH solution gassed with 95% O₂-5% CO₂, and cleaned of extraneous tissue. Once cleaned, the trachea was slit along its longitudinal axis by cutting through the airway smooth muscle. The trachea was then opened as a flat sheet and cut horizontally so that the proximal end of the trachea was mounted in the Ussing chamber. After mounting, only a 0.125 cm² surface area of the trachea was exposed to the gassed, MKH solution from the glass circulation reservoirs.

The fluid which bathed the mucosal and serosal surfaces of the trachea was in contact with silver/silver chloride electrode cartridges (WPI; Sarasota, FL) which were filled with 0.9 % sodium chloride irrigation, USP (saline; Baxter Healthcare Corporation; Deerfield, IL) in 4 % melted bacteriological agar. A two-channel DVC-1000 voltage/current clamp (WPI; Sarasota, FL) was connected to the Ussing chamber via four silver/silver chloride electrodes. Two electrodes, placed distal to the tissue, were connected to the current-passing circuit while two electrodes, placed proximal to the tissue, monitored transepithelial potential difference. I_{sc} was measured continuously, and the data recorded on a conventional strip chart recorder (Gould Instrument Systems; Valley View, OH). In addition, the I_{sc} was normalized to surface area. To accomplish this, the I_{sc} was multiplied by the inverse of the surface area of the trachea exposed to

the fluid bathing the mucosal and serosal surfaces. In this instance, the surface area was calculated from the formula describing the surface area of a circle:

$$\text{surface area of a circle} = \pi r^2$$

where r is the radius (0.2 cm). The following sample calculation demonstrates how the I_{sc} was eventually expressed in $\mu\text{A}/\text{cm}^2$:

$$I_{sc} \times (1/\pi r^2)$$

then, by substitution of arbitrary numerical values

$$5.0 \mu\text{A} \times 1/[\pi(0.2 \text{ cm})^2] = 39.8 \mu\text{A}/\text{cm}^2$$

The tissue was equilibrated for 3 hr prior to any experimental manipulations, with the MKH solution bathing the mucosal and serosal surfaces of the trachea being changed every 15 min.

Administration of Agents to the Ussing System

The Ussing system was used in this study to determine the effect of amiloride (Na^+ channel blocker), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB; Cl^- channel blocker), iberiotoxin (Ca^{2+} -activated K^+ channel blocker), bumetanide (Na^+ - K^+ - 2Cl^- cotransporter inhibitor), and ouabain (Na^+ - K^+ -ATPase inhibitor) on tracheal I_{sc} responses to hypertonic solution. During this portion of the study, the effect of a single concentration of hypertonic solution on I_{sc} was examined, and in this instance, the MKH solution was made hypertonic by adding either 120 mOsm of NaCl or D-mannitol to the MKH solution.

Following the 3 hr equilibration period, hypertonic NaCl or D-mannitol solution was added to the mucosal surface of the trachea in the presence of serosally-added methacholine chloride (MCh; 3×10^{-7} M) in order to mimic conditions in previous EpDRF studies (Dortch-Carnes et al., 1999; Fedan et al., 1999). Once the electrophysiological response to hypertonic solution had equilibrated, the preparation was then washed at 15 min intervals for 90 min with fresh MKH solution.

After the completion of the 90 min wash period, a respective ion channel and/or transporter inhibitor was added to either the mucosal or serosal surface of the trachea, depending upon the epithelial surface where its molecular target is believed to exist (Figure 6). Once the electrophysiological response of the inhibitor, if any, had equilibrated, hypertonic NaCl or D-mannitol solution was added again to the mucosal surface of the trachea in the presence of serosally-added MCh (3×10^{-7} M). There were a few exceptions to this standard protocol. Iberiotoxin was added to both the mucosal and serosal surfaces of the trachea for 30 min while ouabain was added prior to the addition of MCh to mimic conditions in a previous EpDRF study, which was done to prevent Na^+ loading of the tracheal smooth muscle (Fedan et al., 1999).

Solutions and Reagents

The MKH solution contained (mM): NaCl (113.0), KCl (4.8), CaCl_2 (2.5), KH_2PO_4 (1.2), MgSO_4 (1.2), NaHCO_3 (25.0), and glucose (5.7). The MKH solution was continuously gassed with 95% O_2 -5% CO_2 and maintained at 37°C and a pH of 7.4. In addition, the osmolarity of the MKH solution was 281.2 ± 0.6 mOsm ($n = 5$; Osmette A Automatic Osmometer, Precision Systems, Inc.; Sudbury, MA).

All chemical reagents used during the course of this study were purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in distilled water unless otherwise noted. Amiloride and ouabain were dissolved in warm, distilled water while bumetanide and NPPB were dissolved in dimethyl sulfoxide (DMSO).

Analysis of Results

When statistically comparing the ΔI_{sc} in response to hypertonic MKH solution in the presence and absence of ion channel or transporter inhibitors, the paired Student's *t* test or Mann-Whitney rank sum test was used, as appropriate.

All of the results were expressed as the mean $\pm$ S.E.M. (standard error of the mean) where n is the number of separate experiments. In every experiment, $p < 0.05$ was considered significant.

Results

Effect of ion transport inhibitors on the basal I_{sc} of isolated trachea

The basal I_{sc} of guinea-pig isolated, trachea mounted in Ussing chambers was 47.8 ± 1.6 $\mu\text{A}/\text{cm}^2$ ($n = 64$). Treatment of the tissue with mucosally-applied amiloride (3×10^{-5} M), mucosally-applied NPPB (10^{-4} M), or serosally-applied bumetanide (10^{-5} M) decreased the basal I_{sc} by $35.6 \pm 1.4\%$ ($n = 12$), $83.3 \pm 4.9\%$ ($n = 8$), and $8.9 \pm 1.6\%$ ($n = 12$), respectively. Mucosally- and serosally-applied iberiotoxin (10^{-7} M) had no apparent effect upon the basal I_{sc} . In order to mimic previous studies of EpDRF in the isolated, perfused trachea apparatus (Fedan et al., 1999), the effect of serosally-applied ouabain (10^{-5} M) on I_{sc} was measured in the presence of serosally-applied MCh (3×10^{-7} M). In this instance, ouabain decreased the I_{sc} by $94.3 \pm 3.3\%$ ($n = 8$).

Effect of ion transport inhibitors on the bioelectric responses of isolated trachea to hypertonic NaCl and D-mannitol solution

In order to characterize further the ion channels and/or transporters involved in EpDRF-induced smooth muscle relaxation (Dortch-Carnes et al., 1999; Fedan et al., 1999), this study examined the effect of ion channel and transporter inhibitors on the I_{sc} responses of isolated trachea to MKH solution made hypertonic with either added NaCl (120 mOsm) or D-mannitol (120 mOsm), both of which have been shown to elicit EpDRF-induced smooth muscle relaxation (Munakata et al., 1988; Fedan et al., 1999). Again, in order to mimic previous studies of EpDRF (Dortch-Carnes et al.,

1999; Fedan et al., 1999), MCh (3×10^{-7} M) was added serosally prior to the mucosal application of hypertonic NaCl or D-mannitol solution. MCh, itself, increased the I_{sc} which was significantly attenuated by bumetanide and NPPB (data not shown).

In the presence of serosally-added MCh (3×10^{-7} M), the bioelectric responses to hypertonic NaCl and D-mannitol solution were different (Figure 13). Mucosally-applied hypertonic NaCl solution produced a triphasic response which consisted of a rapid decrease, an increase, and finally, a very slow, sustained decrease in I_{sc} . The triphasic I_{sc} responses were difficult to quantify since the first two phases, the rapid decrease and then increase, were variable from tissue to tissue; however, the very slow, sustained decrease in I_{sc} was characteristic of all tissue responses to hypertonic NaCl solution. As a consequence, the very slow, sustained decrease in I_{sc} was the only phase which was quantified. The response to hypertonic D-mannitol solution was much less complex, consisting of only a very slow, sustained decrease in I_{sc} .

Pretreatment with either serosally-applied amiloride (3×10^{-5} M) or bumetanide (10^{-5} M) significantly inhibited the ΔI_{sc} induced by NaCl and D-mannitol (Figures 14 and 15) while mucosally- and serosally-applied iberitoxin (10^{-7} M) had no apparent effect on the I_{sc} responses to either NaCl or D-mannitol (Figure 16). In the presence of mucosally-applied NPPB (10^{-4} M), the I_{sc} increased rather than decreased following the addition of NaCl while the ΔI_{sc} induced by D-mannitol was almost completely abolished (Figure 17). Serosally-added ouabain (10^{-5} M) caused a progressive reduction in I_{sc} toward zero. This prevented assessment of the effect of ouabain on responses to NaCl and D-mannitol; however, in the presence of ouabain, the I_{sc} increased rather than decreased following the application of NaCl and D-mannitol (Figure 18).

Figure 13. Representative tracing showing the effect of mucosally-applied MKH solution made hypertonic with added (A) NaCl (120 mOsm) or (B) D-mannitol (120 mOsm) on I_{sc} in the presence of serosally-applied MCh ($3 \times 10^{-7} M$) in isolated trachea. MCh increased the I_{sc} . NaCl elicited a triphasic response which consisted of (1) a rapid decrease, (2) an increase, and finally, (3) a very slow, sustained decrease in I_{sc} . D-mannitol elicited a very slow, sustained decrease in I_{sc} . As explained in the text, it was the very, slow sustained decrease in I_{sc} which was quantified.

Figure 14. Effect of mucosally-applied amiloride (3×10^{-5} M) on the I_{sc} responses of mucosally-applied MKH solution made hypertonic with added (A) NaCl (120 mOsm) or (B) D-mannitol (120 mOsm) in the presence of serosally-applied MCh (3×10^{-7} M) in isolated trachea. Amiloride significantly inhibited the ΔI_{sc} induced by both NaCl and D-mannitol. $n = 6$ for NaCl and D-mannitol. * $p < 0.05$ compared to -Amiloride as determined by a paired Student's t test.

Figure 15. Effect of serosally-applied bumetanide (10^{-5} M) on the I_{sc} responses of mucosally-applied MKH solution made hypertonic with added (A) NaCl (120 mOsm) or (B) D-mannitol (120 mOsm) in the presence of serosally-applied MCh (3×10^{-7} M) in isolated trachea. Bumetanide significantly inhibited the ΔI_{sc} induced by both NaCl and D-mannitol. $n = 6$ for NaCl and D-mannitol. * $p < 0.05$ compared to -Bumetanide as determined by a paired Student's t test.

Figure 16. Lack of effect of mucosally- and serosally-applied iberiotoxin (10^{-7} M) on the I_{sc} responses of mucosally-applied MKH solution made hypertonic with added (A) NaCl (120 mOsm) or (B) D-mannitol (120 mOsm) in the presence of serosally-applied MCh (3×10^{-7} M) in isolated trachea. $n = 4$ for NaCl and D-mannitol.

Figure 17. Effect of mucosally-applied NPPB (10^{-4} M) on the I_{sc} responses of mucosally-applied MKH solution made hypertonic with added (A) NaCl (120 mOsm) or (B) D-mannitol (120 mOsm) in the presence of serosally-applied MCh (3×10^{-7} M) in isolated trachea. In the presence of NPPB, the I_{sc} increased rather than decreased following the addition of NaCl while the ΔI_{sc} induced by D-mannitol was almost completely abolished. $n = 4$ for NaCl and D-mannitol. * $p < 0.05$ compared to ! NPPB as determined by a paired Student's t test.

Figure 18. Effect of serosally-applied ouabain (10^{-5} M) on the I_{sc} responses of mucosally-applied MKH solution made hypertonic with added (A) NaCl (120 mOsm) or (B) D-mannitol (120 mOsm) in the presence of serosally-applied MCh (3×10^{-7} M) in isolated trachea. Ouabain (10^{-5} M) caused a progressive reduction in I_{sc} toward zero. This prevented assessment of the effect of ouabain on responses to NaCl and D-mannitol; however, in the presence of ouabain, the I_{sc} increased rather than decreased following the application of NaCl and D-mannitol. $n = 4$ for NaCl and D-mannitol. * $p < 0.05$ compared to -Ouabain as determined by a paired Student's t test.

In order to make certain that the responses seen in the presence of the inhibitors were not due to the effects of the vehicle, DMSO, the effect of DMSO on the responses was tested. DMSO had no effect upon the responses to hypertonic NaCl or D-mannitol solution (Figure 19). Finally, to ensure that tissue desensitization did not contribute to the apparent effect of the inhibitors, the effect of hypertonic NaCl and D-mannitol solution on I_{sc} responses was measured twice in the same preparation (Figure 20). The second response was measured 90 min after the first, with washes every 15 min. There was no difference between the first and second response to D-mannitol, but there was a significant decrease in the second NaCl response when compared to the first.

Figure 19. Lack of effect of mucosally- and serosally-applied DMSO on the I_{sc} responses of mucosally-applied MKH solution made hypertonic with added (A) NaCl (120 mOsm) or (B) D-mannitol (120 mOsm) in the presence of serosally-applied MCh (3×10^{-7} M) in isolated trachea. The final concentration of DMSO bathing the mucosal surface of the trachea was 0.08% while the final concentration bathing the serosal surface of the trachea was 0.01%. $n = 4$ for NaCl and D-mannitol.

Figure 20. Comparison of the first and second I_{sc} responses to mucosally-applied MKH solution made hypertonic with added (A) NaCl (120 mOsm) or (B) D-mannitol (120 mOsm) in the presence of serosally-applied MCh (3×10^{-7} M) in isolated trachea. The second ΔI_{sc} induced by NaCl was slightly, but significantly less than the first while there was no difference between the first and second ΔI_{sc} induced by D-mannitol. $n = 4$ for NaCl and D-mannitol. * $p < 0.05$ compared to the first response as determined by a paired Student's t test.

Discussion

The results of this study suggest that apical Na^+ channels and basolateral $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporters are intimately involved in the airway epithelial bioelectric response to elevated mucosal tonicity while the role of apical Cl^- channels and basolateral $\text{Na}^+-\text{K}^+-\text{ATPases}$ is unclear. Since apical Na^+ channels are involved in both the epithelial bioelectric response to elevated mucosal tonicity and hypertonicity-induced smooth muscle relaxation *via* EpDRF, this further strengthens the hypothesis that the synthesis, release, and/or effects of EpDRF are functionally linked to the electrical activity of the epithelium (Raeburn and Fedan, 1989; Dortch-Carnes et al., 1999; Fedan et al., 1999).

Although the major objective of this study was to elucidate which epithelial ion channels and/or transporters were involved in the depolarization of the transepithelial potential difference (V_{ms}) preceding EpDRF-induced smooth muscle relaxation, this study has made other observations regarding the basic electrophysiological properties of guinea-pig tracheal epithelium. The basal I_{sc} value obtained in this study was not consistent with two previous studies measuring I_{sc} in guinea-pig tracheal epithelium (Croxtan, 1993; Robison and Kim, 1994). The reasons for these discrepancies are not clear; however, both of these studies used animals in a lower weight range which may be important if epithelial ion transport is size- and/or age-dependent. More importantly, the difference may lie in the arrangement of the tissue. Croxtan (1993) measured I_{sc} in intact trachea while Robison and Kim (1994) measured I_{sc} in cultured, tracheobronchial epithelial cells. This is most significant when comparing our results with those from cultured cells since submucosal tissues have been shown to modulate the bioelectric properties of the airway epithelium (Freed and Croxtan, 1993). The lack of an airway wall and its modulatory effects in cultured cells may explain these differences. Our basal I_{sc} value ($47.8 \pm 1.6 \mu\text{A}/\text{cm}^2$), however, was consistent with a separate study ($37.3 \pm 2.9 \mu\text{A}/\text{cm}^2$) which also measured I_{sc} in guinea-pig tracheal segments mounted in Ussing chambers (Stutts and Bromberg, 1987).

The effects of several ion transport inhibitors on the basal I_{sc} produced results which contradicted the current, presumed model of ion transport in guinea-pig tracheal epithelium. The current model proposes that under resting I_{sc} conditions Na^+ absorption is the major ion transport process which accounts for the majority of the I_{sc} (Boucher et al., 1982). In this study, amiloride decreased the basal I_{sc} by $35.6 \pm 1.4\%$ which is consistent with a Na^+ absorptive epithelium; however, other studies have shown the effect of amiloride on baseline I_{sc} to be much greater. Croxton (1993) reported that amiloride (3×10^{-5} M) decreased the baseline I_{sc} by approximately 50% while Robison and Kim (1994) reported that amiloride (10^{-5} M) decreased the baseline I_{sc} by approximately 75%. Again, these discrepancies may be explained by the same reasons given for the differences seen in the basal I_{sc} among the different preparations.

Our results became more contradictory when the effect of NPPB, a Cl^- channel blocker, on baseline I_{sc} was examined. NPPB abolished $83.3 \pm 4.9\%$ of the basal I_{sc} . Despite the fact that the current model of ion transport in guinea-pig tracheal epithelium proposes that there is no net Cl^- secretion (Boucher et al., 1982), our results with NPPB suggest that Cl^- secretion may be the predominant ion transport process of this epithelium. Robison and Kim (1994) used another Cl^- channel blocker, N-phenylanthranilic acid, which decreased the basal I_{sc} of cultured, tracheobronchial epithelial cells by approximately 20 %. On the surface, the effect of NPPB suggests that the majority of the I_{sc} is due to Cl^- secretion; however, NPPB is a nonselective Cl^- channel blocker. In fact, NPPB has been shown to inhibit the Ca^{2+} release-activated Ca^{2+} channel as well as the activity of the $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ which may account for its large inhibition of baseline I_{sc} (Ito et al., 2000; Li et al., 2000).

With respect to the effect of hypertonic solution on I_{sc} responses in the presence of serosally-added MCh (3×10^{-7} M), the results of this study demonstrate that these responses are dependent upon the osmolyte used to elevate mucosal tonicity. The triphasic change in I_{sc} following the addition of hypertonic NaCl solution is very intriguing; however, the electrophysiological basis of the first two phases, the rapid decrease followed by an increase, could not be elucidated because these particular responses were inconsistent between and within

animals. In response to MKH solution made hypertonic with either NaCl or D-mannitol, there was always a slow, sustained decrease in I_{sc} , indicating that this electrophysiological response is the characteristic bioelectric response of the airway epithelium to elevated mucosal tonicity.

Since MKH solution made hypertonic with either added NaCl or D-mannitol elicited a slow, sustained decrease in I_{sc} , the effect of ion transport inhibitors on this particular response was evaluated. This decrease in I_{sc} following an elevation in mucosal tonicity may be explained by the same mechanisms believed to account for hyperosmotic stress of the airways during dry air hyperpnea (Figure 10; Anderson and Daviskas, 1999). Because elevated mucosal tonicity would decrease the osmotic pressure of the ASL, water would exit the cell; thus, intracellular Na^+ and Cl^- concentrations would increase. Consequently, this would decrease Na^+ absorption and increase Cl^- secretion. A decrease in Na^+ absorption and an increase in Cl^- secretion would decrease and increase the I_{sc} respectively, and the net effect on I_{sc} would be dependent upon which ion transport process dominates. In addition, the effect on I_{sc} elicited by elevated mucosal tonicity is also dependent upon the paracellular pathway conductance. A previous study reported that the decrease in I_{sc} following elevated mucosal tonicity was due to a decrease in Na^+ absorption. In fact, this same study also reported that Cl^- secretion was not induced by elevated mucosal tonicity (Willumsen et al., 1994). This explanation describing the decrease in I_{sc} fits with the effects of D-mannitol since it is membrane-impermeable; however, the effects of NaCl are more complicated because it is membrane-semipermeable. In order to fully elucidate the particular ion fluxes which are altered following exposure of the tracheal epithelium to elevated mucosal tonicity, isotope flux studies are necessary.

The significant attenuation of the hypertonicity-induced ΔI_{sc} by amiloride, regardless of the osmolyte used to elevated tonicity, suggests that apical Na^+ channels are involved in the epithelial bioelectric response to elevated mucosal tonicity. In the presence of amiloride, the I_{sc} response to elevated mucosal tonicity is most likely reduced for two reasons. First, Na^+ is unable to leave the cell across the apical membrane in the presence of amiloride. Secondly, amiloride

stimulates Cl^- secretion (Boucher et al., 1982), which would offset any decrease in I_{sc} by hypertonic solution.

The significant, inhibitory effect of bumetanide on hypertonicity-induced ΔI_{sc} , regardless of the osmolyte used to elevate tonicity, suggests that basolateral $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporters along with apical Na^+ channels are involved in the epithelial bioelectric response to elevated mucosal tonicity. The reason bumetanide attenuates the ΔI_{sc} elicited by elevated mucosal tonicity is dependent upon the existence and activation of another basolateral Cl^- -coupled transporter when the $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter is inhibited. Two candidates include the $\text{Cl}^- - \text{HCO}_3^-$ exchanger and the Na^+ -coupled Cl^- transporter. In fact, the Na^+ -coupled Cl^- transporter is postulated to exist in the basolateral membranes of Cl^- secretory epithelia (Frizzell et al., 1979). How would the action of the Na^+ -coupled Cl^- transporter, if it exists in guinea-pig tracheal epithelium, offset the decrease in I_{sc} elicited by elevated mucosal tonicity? According to the model of hyperosmotic stress of the airways during dry air hyperpnea, not only do the intracellular concentrations of Na^+ and Cl^- increase, but also the extracellular concentrations of these ions in the submucosa (Figure 10) (Anderson and Daviskas, 1999) which would tend to drive these ions into the overlying epithelial cell by means of the Na^+ -coupled Cl^- transporter if the $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter is inhibited by bumetanide. Na^+ and Cl^- would begin to accumulate in the cell, and in order to obtain electrochemical equilibrium, they must be extruded across either the apical or basolateral membranes. According to the model proposed by Frizzell et al. (1979), Na^+ is extruded from the cell by the action of the basolateral $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ while Cl^- is driven from the cell across the apical membrane. In the presence of hypertonicity, the intracellular Cl^- concentration is increased so any further increase would accentuate its secretion across the apical membrane. This stimulation of Cl^- secretion would offset the decrease in I_{sc} elicited by hypertonic solution. This scenario holds true only if another Cl^- -coupled transporter is activated following inhibition of the $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter.

Unlike amiloride and bumetanide, the effects of NPPB on hypertonicity-induced ΔI_{sc} was osmolyte dependent. In the presence of NPPB, hypertonic NaCl solution increased rather

than decreased the I_{sc} . Under basal conditions, NPPB will hyperpolarize the apical membrane; therefore, it will consequently stimulate Na^+ absorption and increase I_{sc} . The addition of hypertonic NaCl solution will increase the inward Na^+ gradient, which, in all likelihood, will potentiate Na^+ influx when Cl^- is elevated inside the cell by NPPB. This enormous inward Na^+ flux will increase the I_{sc} response in such a way that it overwhelms any I_{sc} decrease evoked by elevated mucosal tonicity. NPPB almost completely abolished the ΔI_{sc} induced by hypertonic D-mannitol. Since NPPB did not have the same effect on ΔI_{sc} elicited by hypertonic D-mannitol and NaCl, this suggests that role of apical Cl^- channels in the epithelial bioelectric response to elevated mucosal tonicity is osmolyte dependent, and at this moment, unclear. The application of hypertonic D-mannitol to the mucosal surface will drive Na^+ across the apical membrane, decreasing I_{sc} ; however, this decrease is almost completely abolished, meaning it must be opposed by an increase in I_{sc} by some other ion, namely Cl^- . The only manner in which this can occur is by movement of Cl^- out of the cell across the basolateral membrane or through apical Cl^- channels which are insensitive to NPPB. Exit across the basolateral membrane could occur through either the $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter or the $\text{Cl}^-/\text{HCO}_3^-$ exchanger and/or possibly the Na^+ -coupled Cl^- transporter if they exist in guinea-pig tracheal epithelium.

The lack of effect of iberitoxin on ΔI_{sc} induced by hypertonic solution suggests that Ca^{2+} -activated K^+ channels have no apparent role in the epithelial bioelectric response to elevated mucosal tonicity; however, this does not rule out the possibility of other K^+ channels being involved. Although the effects of ouabain, a Na^+-K^+ -ATPase inhibitor, on ΔI_{sc} changes induced by hypertonic solution were examined in this study, the results were inconclusive since ouabain completely abolished the I_{sc} . A further characterization of the possible involvement of the Na^+-K^+ -ATPase in the epithelial bioelectric response to elevated mucosal tonicity is impossible since its pharmacological inhibitor, ouabain, completely abolishes the baseline I_{sc} .

When comparing the first and second epithelial bioelectric responses to hypertonic NaCl or D-mannitol solution in the same tissue, the second response to NaCl, not D-mannitol, was significantly less than the first; however, this effect was not as great as the effect of the tested

inhibitors on reducing I_{sc} . This indicates that the tissue desensitizes in response to hypertonic NaCl solution and that some of the reduction in I_{sc} seen with the inhibitors is partially due to desensitization.

The overall goal of this study was to demonstrate that those ion channels involved in hypertonicity-induced smooth muscle relaxation *via* EpDRF were also involved in the epithelial bioelectric response to elevated tonicity, and if this was found to be the case, this would further support the hypothesis that the synthesis, release, and/or effects of EpDRF are functionally linked to epithelial electrical activity. Fedan et al. (1999) reported that apical Na^+ and Cl^- channels were involved in EpDRF-induced smooth muscle relaxation; however, this study has shown that apical Na^+ channels and basolateral $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporters are involved in the epithelial bioelectric response to elevated mucosal tonicity while the role of apical Cl^- channels and basolateral $\text{Na}^+ - \text{K}^+ - \text{ATPases}$ is unclear.

Because amiloride significantly attenuated hypertonicity-induced smooth muscle relaxation *via* EpDRF and the epithelial bioelectric response to elevated mucosal tonicity, this provides further evidence that apical Na^+ channels are intimately involved in the synthesis, release, and/or effects of EpDRF. The role of these channels in mediating EpDRF-induced relaxation responses is unclear. Because the mechanisms by which eukaryotic cells sense increases or decreases in extracellular tonicity are poorly understood (Cohen, 1997), it is possible that these Na^+ channels are in fact osmotic sensors which initiate EpDRF synthesis and/or signaling by relaying extracellular changes in tonicity to the intracellular environment. If the normal activity of these channels are interfered with by amiloride, it may alter their ability to sense changes in tonicity and/or communicate these changes to the intracellular environment. It is very unlikely that amiloride-sensitive Na^+ channels are the only, possible osmotic sensors of the airway epithelial cells given the fact that elevations in serosal tonicity also lead to the synthesis, release, and/or effects of EpDRF (Fedan et al., 1999). It is possible that each epithelial surface may have its only osmotic sensor. Because the $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter is also

involved in the epithelial bioelectric response to elevated mucosal tonicity, the possibility exists that this transporter is the osmotic sensor of the basolateral surface.

Fedan et al. (1999) reported that bumetanide produced a modest inhibition ($p < 0.07$) of EpDRF-induced smooth muscle relaxation at added NaCl concentrations equal or greater than 150 mOsm; however, this study has shown that bumetanide attenuated ΔI_{sc} changes evoked by only 120 mOsm of added NaCl or D-mannitol. The inconsistency between the effects of bumetanide on EpDRF-induced smooth muscle relaxation and hypertonicity-induced ΔI_{sc} suggests that the $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter is involved in the epithelial bioelectric response to elevated hypertonicity but not appreciably involved in EpDRF-induced smooth muscle relaxation. From this study, the relationship between the mechanical and bioelectric effects of Cl^- channel blockers on hypertonicity-induced smooth muscle relaxation and epithelial bioelectric responses, respectively, cannot be determined. Fedan et al. (1999) used DIDS, a Cl^- channel blocker and $\text{Cl}^- - \text{HCO}_3^-$ exchanger inhibitor (Lamb et al., 2000), rather than NPPB, a nonselective Cl^- channel blocker, to come to the conclusion that apical Cl^- channels are involved in mediating EpDRF-induced smooth muscle relaxation. The effect seen with DIDS may have nothing to do with its effect upon apical Cl^- channels but everything to do with its effect upon the $\text{Cl}^- - \text{HCO}_3^-$ exchanger. In order for further clarification of the role of Cl^- channels and/or transporters, the effect DIDS on ΔI_{sc} and NPPB on EpDRF-induced smooth muscle relaxation, respectively, will have to be examined. Finally, there is a discrepancy between the lack of effect of iberiotoxin on the epithelial bioelectric response to elevated mucosal tonicity and its inhibitory effect on the synthesis and/or release of EpDRF. Tamaoki et al. (1997) used iberiotoxin to examine the basal synthesis and/or release of EpDRF, that is, they did not evoke the effects of EpDRF by elevating mucosal or serosal tonicity. For further clarification of the possible involvement of Ca^{2+} -activated K^+ channels in this response, the effect of iberiotoxin on EpDRF-induced smooth muscle relaxation should be examined by stimulating the effects of EpDRF with hypertonic solution. If iberiotoxin fails to have any effect on EpDRF-induced smooth muscle

relaxation elicited with hypertonic solution, this could suggest that the basal and stimulated release of EpDRF are controlled by different mechanisms.

The mechanisms by which hypertonicity-induced changes in epithelial bioelectric activity lead to the synthesis, release, and/or effects of EpDRF are unclear; however, the phenomenon of electrical signals releasing autacoids is not unheard of in physiological systems. For example, the release of EpDRF from the airway epithelium, due to altered epithelial electrical activity, may be analogous to the release of ACh from nerve terminals following the propagation of an action potential along the length of a neuron. It is possible that EpDRF, like ACh, is prepackaged in vesicles and released from its cell of origin following a change in the potential difference across the cell membrane.

There were two major points brought about following the conclusion of this study. First, the basic physiological ion transport properties of guinea-pig tracheal epithelium need to be further investigated since this study has shown, contrary to the accepted model, that guinea-pig tracheal epithelium may be a Cl^- secretory epithelium rather than a Na^+ absorbing epithelium. Secondly, these results demonstrate that the epithelial bioelectric response to elevated mucosal tonicity is mediated by the actions of amiloride-sensitive Na^+ channels and the bumetanide-sensitive $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter while the role of apical Cl^- channels and basolateral $\text{Na}^+ - \text{K}^+ - \text{ATPases}$ is unclear. Because amiloride attenuated hypertonicity-induced smooth muscle relaxation *via* EpDRF (Fedan et al., 1999) and the epithelial bioelectric response to elevated mucosal tonicity, apical Na^+ channels appear to be heavily involved in the synthesis, release, and/or effects of EpDRF.

**Lipopolysaccharide-Induced Alterations in Epithelial
Electrophysiology in Relation to Epithelium-Derived
Relaxing Factor and Airway Reactivity**

Objectives

Previous work has suggested that the synthesis, release, and/or effects of EpDRF are functionally linked to the electrical activity of epithelial cells (Raeburn and Fedan, 1989; Dortch-Carnes, et al., 1999; Fedan et al., 1999); therefore, this study sought to determine what effects alterations in epithelial ion transport had upon EpDRF-induced airway smooth muscle relaxation. The hypothesis of this study was that alterations in epithelial ion transport were associated with changes in EpDRF-induced smooth muscle relaxation since the two appear to be functionally linked; therefore, any change in the bioelectric properties of the airway epithelium should subsequently effect the synthesis, release, and/or effects of EpDRF. Because LPS-treatment (4 mg/kg, i.p.; 18 hr post-treatment) has been shown to alter the basal bioelectric properties of guinea-pig tracheal epithelium (Johnston and Fedan, 1999), this model system was utilized to examine what effect altered epithelial ion transport had upon EpDRF-induced smooth muscle relaxation.

The hypothesis was tested through a variety of different experiments. First, tracheal smooth muscle mechanical responses (contraction and relaxation) and epithelial bioelectric responses (V_{ms}) were measured in isolated, perfused trachea from saline (control)- and LPS-treated guinea pigs. If it is found that LPS-treatment alters EpDRF-induced smooth muscle relaxation, it will then be hypothesized that airway reactivity to contractile agonists *in vitro* and *in vivo* will also be altered.

Materials and Methods

Animals

Specific, pathogen-free, male Dunkin-Hartley guinea pigs (Harlan Sprague Dawley, Inc.; Indianapolis, IN; 405-700 g) were used during the course of this study. The guinea pigs were housed in separate, actively ventilated, micro-isolator cages (Lab Products, Inc.; Seaford, DE)

within a single room. The room within which these animals were housed were part of an 8800 ft² modified barrier, animal care facility. Within this facility, the animals were acclimated to their new environment prior to experimentation, given food and water *ad libitum*, and breathed fresh air. All of the animals in the animal care facility, as well as the facility itself, were under the supervision of a full-time veterinarian.

Throughout this study, the guinea pigs were sacrificed according to a standard protocol. The animals were first anesthetized intraperitoneally (i.p.) with sodium pentobarbital (Veterinary Laboratories, Inc.; Lenexa, KS; 65 mg/kg), and once it was determined that the animals were unresponsive to any stimuli, they were exsanguinated by severing the abdominal aorta and cutting the heart.

All of the experimental protocols in this study were approved by the Institutional Animal Care and Use Committee (IACUC) while the facilities where the experimental protocols were conducted were fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. These facilities have been accredited by AAALAC International since February 18, 1987.

***In Vivo* Saline- and Lipopolysaccharide-Treatments**

Guinea pigs were injected i.p. with 4 mg/kg lipopolysaccharide (LPS) from *Salmonella typhimurium* or a volume equivalent of 0.9 % sodium chloride irrigation, USP (saline, control; Baxter Healthcare Corporation; Deerfield, IL). Eighteen hr after treatment, *in vitro* or *in vivo* experiments were conducted.

Isolated, Perfused Trachea Apparatus

The isolated, perfused trachea apparatus (Figure 21) is a unique *in vitro* preparation which is used to measure tracheal smooth muscle contractile and relaxant responses in response to various pharmacological agents (Munakata et al., 1988; Fedan and Frazer, 1992).

This preparation consisted of two separate, water-jacketed organ baths whose temperature was maintained at 37°C. The 30 ml extraluminal (EL) or serosal bath and the 30 ml intraluminal (IL) or mucosal bath were filled with MKH solution that was continuously gassed with 95% O₂-5% CO₂. In order to remove the trachea from the guinea pig, the animal was sacrificed as previously described. The trachea was exposed *in vivo*, a 4.2 cm segment excised, placed in MKH solution gassed with 95% O₂-5% CO₂, and cleaned by removing extraneous tissue. The trachea was then mounted on a stainless steel perfusion holder, stretched to its *in situ* length, and placed into the EL bath containing gassed MKH solution. After attaching the necessary tubing, the trachea was perfused, at a constant rate of 34 ml/min, with gassed, recirculating MKH solution (37°C) from the separate IL bath. Since the tracheal smooth muscle and epithelium were in direct contact with the gassed MKH solution in the EL and IL baths, respectively, pharmacological agents added to the EL bath have direct access to the smooth muscle while those agents added to the IL bath must first diffuse through the epithelium before reaching the smooth muscle. This preparation, therefore, allowed one to study the degree to which the epithelium can modulate airway reactivity.

Figure 21. Isolated, perfused trachea apparatus. A 4.2 cm length of guinea-pig trachea, shown in **blue**, is attached to a perfusion holder and placed in the extraluminal (EL) or serosal bath containing modified Krebs-Henseleit (MKH) solution maintained at 37°C. Once all of the necessary tubing is attached, the intraluminal (IL) or mucosal surface of the trachea is perfused at a constant rate with recirculating MKH solution also maintained at 37°C. The **red arrows** indicate the direction of fluid flow through the preparation. As explained in the text, this preparation can measure contractile and relaxant responses of the tracheal smooth muscle (illustration produced by and courtesy of Jeffrey S. Fedan, Ph.D.).

Contractile and relaxant responses of the tracheal smooth muscle were measured as changes in the inlet minus outlet pressure difference (ΔP ; cm H₂O) between the side holes of two indwelling catheters which became inserted into the lumen upon mounting the trachea to the holder (Figure 22). The proximal and distal ends of the trachea were attached with suture (Deknatel, Inc.; Fall River, MA) around the inlet and outlet catheters, respectively. The inlet and outlet catheters were connected by pressure lines to the positive and negative sides, respectively, of a differential pressure transducer (Validyne Engineering Corp.; Northridge, CA). Because the rate of fluid movement through the trachea was kept constant (34 ml/min), the ΔP responses were representative of changes in tracheal cross-sectional area associated with contraction and relaxation; therefore, an increase or decrease in ΔP was associated with smooth muscle contraction and relaxation, respectively (Munakata et al., 1989). These responses were recorded on a conventional strip chart recorder (Gould Instrument Systems; Valley View, OH). In all instances, the transmural pressure was zeroed, and the preparations were equilibrated for 3 hr before any experimental manipulations were initiated. During the equilibration period, however, the MKH solution in both baths was changed every 15 min.

Figure 22. Differential pressure measurement in the isolated, perfused trachea apparatus. Contractile and relaxant responses of the tracheal smooth muscle are measured as changes in the inlet minus outlet pressure difference (ΔP) between the side holes of the indwelling perfusion holder catheters. The trachea is shown in **blue**, and the **red arrows** indicate the direction of fluid flow through the trachea (illustration produced by and courtesy of Jeffrey S. Fedan, Ph.D.).

Modified Isolated, Perfused Trachea Apparatus

Recent modifications to the isolated, perfused trachea apparatus now permit transepithelial potential difference (V_{ms} ; Dortch-Carnes et al., 1999) to be measured simultaneously with smooth muscle contractile and relaxant responses at the proximal and distal ends of the trachea. The experimental setup of this preparation was unchanged from before (Figure 21); however, modifications of the perfusion holder were made to measure epithelial bioelectric responses. Because epithelial bioelectric responses were measured with this preparation, a plastic perfusion holder, rather than a stainless steel one, was used. For the most part, the plastic perfusion holder did not affect the manner in which smooth muscle mechanical responses (ΔP) were measured; however, the flow rate in this preparation was kept constant at 22 ml/min instead of the 34 ml/min used with the stainless steel holder, which permitted transmural pressure to be zeroed in the face of a greater fluid resistance of the plastic holder. A schematic illustration of the modified perfusion holder is shown in Figure 23.

During the course of this study, V_{ms} measurements were made at the proximal end of the trachea, and the epithelium surrounding the opening of the inlet catheter was the point source for the measurement of the V_{ms} . Two silver/silver chloride calomel half cells, one designated as IL or mucosal (V_m) and the other as EL or serosal (V_s), were used to measure the V_{ms} . Each calomel half cell was in contact with the fluid bathing either the EL or IL surfaces of the trachea by means of Ussing voltage electrode cartridges [World Precision Instruments (WPI); Sarasota, FL] filled with 3 M KCl in 4 % melted bacteriological agar. The V_{ms} was equal to the sum of V_m , V_s , and an offset potential. Before the trachea was mounted on the perfusion holder, any potential difference between the electrodes, not exceeding ± 2 mV, was dialed to zero (Dortch-Carnes et al., 1999).

Figure 23. V_{ms} measurement in the modified isolated, perfused trachea apparatus. The V_{ms} can be measured at either the proximal or distal end of the trachea through the side hole of the indwelling perfusion holder catheter; however, this study measured the V_{ms} only at the proximal end of the trachea. Again, the trachea is shown in **blue** and the **red arrows** indicate the direction of fluid flow through the trachea. The negative sign (!) refers to the fact that the airway epithelium generates a lumen-negative potential with respect to the positive (+), basolateral surface (illustration produced by and courtesy of Jeffrey S. Fedan, Ph.D.).

A two-channel DVC-1000 voltage/current clamp (WPI; Sarasota, FL) was used in the measurement of tracheal V_{ms} responses. The V_{ms} was recorded continuously under open-circuit conditions on a conventional strip chart recorder (Gould Instrument Systems; Valley View, OH).

***In Vitro* Elevated Tonicity Concentration-Response Curves**

EpDRF release was stimulated by increasing the tonicity of the MKH solution perfusing the IL surface of the trachea with either NaCl, KCl, D-mannitol, or urea. After obtaining a stable contraction with extraluminally-added MCh (3×10^{-7} M; $\sim EC_{50}$ for contraction), concentration-response curves for relaxation were generated by adding the osmolytes in stepwise-increasing, cumulative concentrations to the IL bath. Equiosmolar, rather than equimolar, concentrations of the osmolytes were added to the IL bath. The relaxation responses were normalized as a percentage of the MCh-induced contraction.

In addition, concentration-response curves for ΔV_{ms} were generated by adding NaCl in stepwise-increasing, cumulative concentrations to the EL and/or IL baths in the presence and absence of MCh. The EL and IL curves were obtained from each trachea, the IL curve first, followed 90 min later by the EL curve.

***In Vitro* Effects of Agents on EpDRF-Induced Smooth Muscle Relaxation**

During the course of this study, inhibitors of enzymes important to the synthesis of nitric oxide and prostanoids were utilized to determine whether or not they affected EpDRF-induced smooth muscle relaxation. Each inhibitor was added to the EL and IL baths 30 min prior to inducing muscle tone with extraluminally-added MCh (3×10^{-7} M; $\sim EC_{50}$). When relaxation concentration-response curves were generated, the effect of an inhibitor upon responses was compared to the responses in control preparations where the inhibitor was absent.

***In Vitro* Effects of Agents on the Basal V_{ms}**

In some experiments, the effects of indomethacin, N^T -nitro-L-arginine methyl ester (L-NAME), atropine, sodium nitroprusside, and amiloride on the basal V_{ms} were determined. Indomethacin, L-NAME, atropine, and sodium nitroprusside were added to the EL and IL baths simultaneously while amiloride was only added to the IL bath.

The effect of LPS on the basal V_{ms} was determined in tracheas from naive animals by adding 10 $\mu\text{g/ml}$ of LPS to the EL and IL baths simultaneously. An untreated, control preparation was run in parallel as a time control.

***In Vitro* MCh Concentration-Response Curves**

MCh concentration-response curves were generated by adding MCh in stepwise-increasing, cumulative concentrations to the EL and IL baths. EL and IL MCh concentration-response curves were obtained from each trachea, the EL curve first, followed 90min later by the IL curve, with washes every 15 min. The contractile responses to MCh were normalized as a percentage of the maximum MCh-induced contraction.

Tracheal Epithelium Removal

In isolated, perfused trachea experiments requiring removal of the tracheal epithelium, a trimmed, 6-cm segment of pipe cleaner (Fisher Scientific; Pittsburgh, PA) was slowly and carefully inserted into the tracheal lumen and then withdrawn while rotating slowly (Fedan and Frazer, 1992).

Pulmonary Function Measurements

A plethysmograph is used to measure lung volumes and pulmonary function parameters *in vivo*. In this study, a two-chambered, whole-body plethysmograph (Figure 24) was used (Buxco Electronics, Inc.; Sharon, CT).

A conscious guinea pig was placed in the plethysmograph so that its head and thorax were in the head and body chamber, respectively. In addition, two latex collars were placed around the animal's neck in order to restrain it. Besides restraining the animal, the collars form an airtight seal between the two chambers. The guinea pig breathed fresh air through a port in the head chamber, and administered agents entered the head chamber through a separate, aerosol port. The head and thoracic chambers were equipped with identical wire screen pneumotachographs and SEN1 differential pressure transducers. The differential pressure transducers measured pressure changes within each chamber and were connected to an LS-20 noninvasive airway mechanics analyzer. The results were then logged and displayed on a computer screen.

Figure 24. Two-chambered, whole-body plethysmograph. The two-chambered, whole-body plethysmograph is used along with its associated equipment to measure *in vivo* pulmonary function parameters and reactivity to inhaled, MCh aerosol. Reprinted from *J Pharmacol Toxicol Methods*, Chong BTY, Agrawal DK, Romero FA and Townley RG, Measurement of bronchoconstriction using whole-body plethysmograph: Comparison of freely moving versus restrained guinea pigs, pp 163-168, 1998, with permission from Elsevier Science.

The plethysmograph was capable of measuring ten pulmonary function parameters from a single breath, but this study was only concerned with two: breathing frequency (BF) and specific airway resistance (SRaw). The first parameter of interest, BF, was expressed as the number of breaths per minute (BPM). The computer analyzes each breath with regard to four exclusion criteria: (1) the minimum inspiratory time, (2) the maximum inspiratory time, (3) the peak expiratory flow, and (4) the percent maximum deviation between the inspiratory and expiratory volume. A breath must fall in the normal range of all four criteria in order to be recorded (Lomask, 1996).

SRaw is the product of airway resistance and thoracic gas volume and is expressed in cm H₂O•s. SRaw is an indicator of airway resistance, and in mathematical terms, it can be described by the following equation:

$$\text{SRaw} = \tan 2(P_{\text{atm}} - 47)(1.36)(T_i/B)$$

Figure 25 illustrates the derivation of theta (2) which can also be expressed mathematically by the following equation:

$$2 = B(\Delta T)/T_i$$

ΔT is the delay time between the zero crossings of nasal and thoracic flows at the end of inspiration. ΔT is computed in seconds and used to calculate 2, which is the phase shift between the nasal and thoracic flows. The additional components of the SRaw equation are as follows: (1) 1.36 is the conversion factor from mm Hg to cm H₂O, (2) P_{atm} is the barometric pressure in mm Hg, and (3) T_i is the inspiratory time (Lomask, 1996).

Both SRaw and BF data were logged at 6 sec intervals with the mean of ten consecutive averages taken as the measurement at each time point.

***In Vivo* MCh Dose-Response Curves**

The guinea pig was placed in the two-chambered, whole-body plethysmograph 24 hr prior to generating an *in vivo* MCh concentration-response curve, and the animal was acclimated to the chamber for 2 hr in air. This reduced the stress that the animal experienced when placed in the plethysmograph a second time. On the second day, the animal was again placed in the plethysmograph and acclimated in air for 20 min. During this 20 min period, SRaw and BF were continuously recorded to get basal SRaw and BF values. Next, a saline aerosol vehicle was delivered to the head chamber for 3 min, and at the end of this period, SRaw and BF data were taken at 6 sec intervals for 3 min as the animal breathed air. After a 30 min resting period, the lowest concentration of MCh aerosol was delivered to the head chamber for 3 min. Once the aerosol delivery was stopped, SRaw and BF were again measured for 3 min as the animal breathed air. After 30 min, SRaw and BF returned to their basal values, and the next higher concentration of MCh was delivered to the chamber and the procedure was repeated with increasing MCh concentrations. Unlike *in vitro* experiments, a full *in vivo* dose-response curve was impossible to generate because the animals cannot tolerate dyspnea accompanying a maximum response; therefore, ED₅₀'s and maximum responses could not be determined. MCh concentrations ranging between 0.0316 and 3.16 mg/ml were delivered.

Figure 25. Definition of theta. A schematic illustration of guinea-pig head (nasal) and body (thoracic) airflow during inspiration and expiration as recorded by the data collection system associated with the two-chambered, whole-body plethysmograph. Since thoracic airflow precedes nasal airflow at the zero crossing following inspiration, ΔT (delay time) can be calculated, which is subsequently used to calculate theta (2). Reprinted from Lomask K (1996) *BioSystem XA User's Manual, Version 0.9.3*, Buxco Electronics, Inc., Sharon, CT with permission from Buxco Electronics, Inc.

The MCh PC₂₀₀, the provocative MCh concentration producing a two-fold increase in basal SRaw, was calculated by linear interpolation of graphed data. All aerosols were generated using an Ultra Neb 99 DeVilbiss nebulizer (DeVilbiss Co.; Somerset, PA) containing 5 ml of solution.

When examining the effects of saline- and LPS-treatments on airway reactivity to inhaled MCh aerosol, a standard protocol was followed. Naive animals were acclimated on day 1 and the same animals exposed to MCh aerosols on day 2. These animals were then treated with saline or LPS, and 18 hr post-treatment, airway reactivity to inhaled MCh was determined. In all instances, each animal served as its own control since reactivity to MCh was determined before and after saline- and LPS-treatments.

***In Vivo* Administration of L-NAME**

To test the possibility that inhaled L-NAME aerosol may affect airway reactivity to inhaled MCh in saline- and LPS-treated animals, guinea pigs were exposed to L-NAME aerosol (1.2 mM) for 5 min prior to the delivery of each MCh concentration. Two MCh concentration-response curves were generated in both saline- and LPS-treated animals, the first in the absence and the second after treatment with L-NAME.

Histological Examination of Guinea-Pig Airways

Following anesthesia and exsanguination, trachea and lungs from saline- and LPS-treated animals were inflated with 15-20 ml of 10% buffered formalin phosphate (Fisher Scientific; Pittsburgh, PA) and removed *en bloc*. Sections of the trachea as well as the azygous, left, right apical, right cardiac, and right diaphragmatic lobes of the lung were then embedded with paraplast-plus paraffin before being cut into 5 µm slices with a microtome. Individual sections were then placed on glass microscope slides where they were stained according to Harris' hematoxylin and eosin procedure. Following the conclusion of section staining, section mounting

was completed by placing a cover slip over the tissue section. Finally, the slides were examined in a “blind” fashion under the light microscope by a veterinary pathologist.

Solutions and Reagents

The MKH solution contained (mM): NaCl (113.0), KCl (4.8), CaCl₂ (2.5), KH₂PO₄ (1.2), MgSO₄ (1.2), NaHCO₃ (25.0), and glucose (5.7). The MKH solution was continuously gassed with 95% O₂-5% CO₂ and maintained at 37°C and a pH of 7.4. In addition, the osmolality of the MKH solution was 281.2±0.6 mOsm ($n = 5$; Osmette A Automatic Osmometer, Precision Systems, Inc.; Sudbury, MA).

All chemical reagents used during the course of this study were purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in saline unless otherwise noted. Indomethacin was prepared in 100 mM sodium carbonate.

Analysis of Results

The data obtained from *in vitro* concentration-response curves examining EpDRF-induced smooth muscle relaxation were normalized as a percent of the MCh-induced contraction while *in vitro* ΔP MCh concentration-response curves were normalized as a percent of the maximum response. Data normalization was necessary in these instances to avoid large variability among ΔP values (Fedan and Frazer, 1992). Since ΔP is dependent upon the fifth power of the radius of the trachea, small differences in the internal diameter of the trachea can lead to large variability among ΔP values (Munakata et al., 1989). Geometric EC₅₀ values were derived from the least squares analysis of a sigmoidal curve fit (Sigma Plot[®]). When comparing EC₅₀ values statistically, normally-distributed EC₅₀ values were used. The EC₅₀'s and maximum responses were then analyzed using either the paired or nonpaired Student's *t* test, the Mann-Whitney rank sum test, or one-way repeated measures analysis of variance (ANOVA), as appropriate. For concentration-response curves examining ΔV_{ms} , the EC₅₀'s and maximum

responses were derived and statistically compared in the same manner as described for the ΔP responses.

When examining the effect of agents on the V_{ms} , the data were statistically analyzed using either the paired or nonpaired Student's t test or the Mann-Whitney rank sum test, as appropriate.

For *in vivo* airway reactivity studies, pre-treatment and post-treatment BF, SRaw, and MCh PC₂₀₀ values among saline- and LPS-treated animals were statistically compared using the paired or nonpaired Student's t test or Mann-Whitney rank sum test, as appropriate. The effect of L-NAME on MCh PC₂₀₀ values among saline- and LPS-treated animals was compared using the paired or nonpaired Student's t test, as appropriate.

All of the results, except where noted, were expressed as the mean $\pm$ S.E.M. (standard error of the mean) where n is the number of separate experiments. In every experiment, $p < 0.05$ was considered significant.

Results

Effect of LPS-treatment on basal V_{ms} in isolated, perfused trachea

LPS-treatment increased the basal V_{ms} in isolated, perfused trachea. The basal V_{ms} was -14.6 ± 1.2 mV and -28.3 ± 1.9 mV for the saline- and LPS-treated groups, respectively (Figure 26).

Effect of LPS-treatment on EpDRF-induced smooth muscle relaxation in isolated, perfused trachea

In order to test the hypothesis that LPS-induced alterations in epithelial ion transport were associated with perturbations in EpDRF-induced smooth muscle relaxation, smooth muscle reactivity to EpDRF was measured in isolated, perfused tracheas from saline- and LPS-treated guinea pigs. EpDRF-induced smooth muscle relaxation was stimulated by increasing the tonicity of the IL MKH solution with four different osmolytes (NaCl, D-mannitol, urea, and KCl) following contraction of the smooth muscle with extraluminally-applied MCh (3×10^{-7} M; $\sim EC_{50}$). Figures 27-29 and Table 1 summarize the results of this study. When NaCl and D-mannitol were used to increase the tonicity of the IL bath, EpDRF-induced smooth muscle relaxation was potentiated in tracheas from LPS-treated animals (Figure 27). When urea was used, there was no difference in the maximum relaxation responses between the two treatment groups; however, there was a small, but significant decrease in smooth muscle reactivity to EpDRF in the LPS-treated group, as demonstrated by a rightward shift of the concentration-response curve (Figure 28).

Figure 26. Effect of LPS-treatment on the basal V_{ms} in isolated, perfused trachea. Basal V_{ms} values were obtained from tracheas removed from saline- and LPS-treated animals 18 hr post-treatment. Saline- and LPS-treated, $n = 20$. * $p < 0.05$ compared to saline as determined by a nonpaired Student's t test.

Figure 27. Effect of LPS-treatment on EpDRF-induced smooth muscle relaxation elicited with IL MKH solution made hypertonic with added (A) NaCl or (B) D-mannitol. The curves were obtained from tracheas removed from saline- and LPS-treated animals 18 hr post-treatment. The preparations were contracted with extraluminally-applied MCh (3×10^{-7} M; $\sim EC_{50}$) before NaCl or D-mannitol were added in stepwise-increasing, cumulative concentrations to the IL bath. EpDRF-induced smooth muscle relaxation was potentiated in tracheas from LPS-treated animals when NaCl or D-mannitol were used to evoke EpDRF release. The osmolyte concentrations shown in the abscissa of the figures refer to the osmolar concentrations of osmolyte added to the IL MKH solution. Equiosmolar, rather than equimolar, concentrations of NaCl and D-mannitol were added to the IL bath. For NaCl the osmolarity is twice the molarity and for D-mannitol the osmolarity is the same as the molarity. Saline- and LPS-treated, $n = 8$ for NaCl and $n = 6$ for D-mannitol. * $p < 0.05$ compared to saline as determined by a nonpaired Student's t test.

Figure 28. Effect of LPS-treatment on EpDRF-induced smooth muscle relaxation elicited with IL MKH solution made hypertonic with added urea. The curves were obtained from tracheas removed from saline- and LPS-treated animals 18 hr post-treatment. The preparations were contracted with extraluminally-applied MCh (3×10^{-7} M; $\sim \text{EC}_{50}$) before urea was added in stepwise-increasing, cumulative concentrations to the IL bath. LPS-treatment did not affect the maximum relaxation response; however, it caused a small, but significant decrease in smooth muscle reactivity to EpDRF, as demonstrated by an increase in the EC_{50} for relaxation. (A) and (B) are the same data but expressed in a different manner to illustrate the significant difference in EC_{50} values between the treatment groups which is clearly seen in (B). The data in (B) is expressed as a percentage of the maximum relaxation response. The urea concentrations shown in the abscissa of the figures refer to the osmolar concentrations of urea added to the IL MKH solution. For urea the osmolarity is the same as the molarity. Saline- and LPS-treated, $n = 6$.

Figure 29. Lack of effect of LPS-treatment on EpDRF-induced smooth muscle relaxation elicited with IL MKH solution made hypertonic with added KCl. The curves were obtained from tracheas removed from saline- and LPS-treated animals 18 hr post-treatment. The preparations were contracted with extraluminally-applied MCh (3×10^{-7} M; $\sim EC_{50}$) before KCl was added in stepwise-increasing, cumulative concentrations to the IL bath. Neither the maximum relaxation response nor the EC_{50} were affected by LPS-treatment when KCl was used to evoke EpDRF release and subsequent smooth muscle relaxation. The KCl concentrations shown in the abscissa of the figure refer to the osmolar concentrations of KCl added to the IL MKH solution. For KCl the osmolarity is twice the molarity. Saline- and LPS-treated, $n = 4$.

TABLE 1

**Effect of LPS-treatment on *in vitro* airway reactivity to
EpDRF in isolated, perfused trachea**

Treatment (<i>n</i>)	EC ₅₀ (mOsm) ^a (95% C.I.)	Maximum Response (% of MCh) ^b
NaCl		
Saline (8)	11.4 (8.2-15.8)	78.2±6.2
LPS (8)	10.4 (8.4-12.8)	113.3±9.5 ^c
D-Mannitol		
Saline (6)	15.2 (8.2-28.2)	86.1±4.9
LPS (6)	15.9 (11.1-22.7)	109.7±8.5 ^c
Urea		
Saline (6)	10.7 (9.0-12.9)	110.3±7.0
LPS (6)	23.3 ^c (20.2-26.9)	122.3±7.5
KCl		
Saline (4)	16.8 (9.4-30.2)	110.2±15.0
LPS (4)	14.0 (10.6-18.2)	99.0±7.2

The tracheas were removed from the animals 18 hr post-treatment. ^aValues refer to mOsm of osmolyte added to the intraluminal MKH solution. ^bRelaxation responses in cm H₂O were normalized as a percentage of the extraluminal MCh (3×10^{-7} M; ~EC₅₀)-induced contraction. ^cSignificantly greater than saline-treated controls.

Finally, when KCl was used to evoke EpDRF release and subsequent smooth muscle relaxation, there was no difference in the concentration-response curves among the saline- and LPS-treated groups (Figure 29).

Effect of indomethacin and L-NAME on EpDRF-induced smooth muscle relaxation in isolated, perfused trachea from saline- and LPS-treated guinea pigs

Because LPS is known to up-regulate both inducible cyclooxygenase and nitric oxide synthase (Okamoto et al., 1998), the effect of indomethacin, a cyclooxygenase inhibitor, and L-NAME, a nitric oxide synthase inhibitor, on EpDRF-induced smooth muscle relaxation was tested. This was done to rule out the possible contribution of prostaglandins and NO to the potentiation of EpDRF-induced smooth muscle relaxation following LPS-treatment. Neither indomethacin nor L-NAME affected EpDRF-induced smooth muscle relaxation when NaCl was used to elevate tonicity in isolated, perfused trachea from saline- and LPS-treated guinea pigs (Figures 30 and 31).

Effect of LPS-treatment on the epithelial bioelectric events associated with EpDRF-induced smooth muscle relaxation

A previous study has shown that EpDRF-induced smooth muscle relaxation, elicited by hypertonicity, was associated with epithelial bioelectric events, namely a depolarization of the V_{ms} (Dortch-Carnes et al., 1999). The current study has shown that LPS-treatment potentiated EpDRF-induced smooth muscle relaxation when NaCl and D-mannitol were used to elicit EpDRF release; therefore, according to the hypothesis, the epithelial bioelectric events associated with increased EpDRF-induced smooth muscle relaxation should also be potentiated following LPS-treatment.

Figure 30. Lack of effect of extraluminally- and intraluminally-applied indomethacin (3×10^{-6} M) on EpDRF-mediated relaxation responses elicited with IL MKH solution made hypertonic with added NaCl. The curves were obtained from tracheas removed from (A) saline- and (B) LPS-treated animals 18 hr post-treatment. Indomethacin was present for 30 min prior to contracting the tracheal smooth muscle with extraluminally-applied MCh (3×10^{-7} M; $\sim EC_{50}$) and initiating EpDRF-induced smooth muscle relaxation by adding NaCl in stepwise-increasing, cumulative concentrations to the IL bath. Saline- and LPS-treated, $n = 4$.

Figure 31. Lack of effect of extraluminally- and intraluminally-applied L-NAME (10^{-4} M) on EpDRF-mediated relaxation responses elicited with IL MKH solution made hypertonic with added NaCl. The curves were obtained from tracheas removed from (A) saline- and (B) LPS-treated animals 18 hr post-treatment. L-NAME was present for 30 min prior to contracting the tracheal smooth muscle with extraluminally-applied MCh (3×10^{-7} M; $\sim EC_{50}$) and initiating EpDRF-induced smooth muscle relaxation by adding NaCl in stepwise-increasing, cumulative concentrations to the IL bath. Saline- and LPS-treated, $n = 4$.

NaCl concentration-response curves with respect to ΔV_{ms} were generated from isolated, perfused tracheas from saline- and LPS-treated animals in the presence of extraluminally-applied 3×10^{-7} M MCh (Figure 32). For an easy comparison of the relationship between hypertonicity-induced epithelial depolarization and smooth muscle relaxation, depolarization and relaxation concentration-response curves were plotted alongside each other as a percentage of the maximum depolarization or relaxation, respectively (Figure 33). In tracheas from LPS-treated animals, the IL addition of NaCl significantly increased epithelial depolarization and EpDRF-induced smooth muscle relaxation compared to the saline-treated controls; however, the V_{ms} of each treatment group eventually depolarized to the same level (Figure 32 and Table 2). In both treatment groups, the EC_{50} for depolarization was significantly greater than the EC_{50} for EpDRF-induced smooth muscle relaxation (Figure 33 and Table 2).

Besides examining epithelial bioelectric reactivity to NaCl in the presence of extraluminally-applied MCh (3×10^{-7} M), this study also examined epithelial bioelectric reactivity to NaCl in the absence of MCh (Figures 34 and 35 and Table 3). The addition of hypertonic NaCl to the EL and IL baths depolarized the V_{ms} in a concentration-dependent manner. In both treatment groups, extraluminally-applied NaCl was more potent than intraluminally-applied NaCl. When comparing responses between the two treatment groups, the IL EC_{50} for depolarization was significantly greater in the LPS-treated group compared to the saline-treated group. Finally, extraluminally- and intraluminally-applied NaCl depolarized the V_{ms} to a greater extent in the LPS-treated group; however, it did not reach statistical significance, except for one concentration added intraluminally (Figure 35).

Figure 32. Effect of LPS-treatment on epithelial bioelectric reactivity to hypertonic NaCl in the presence of extraluminally-added MCh (3×10^{-7} M). The curves were obtained from tracheas removed from saline- and LPS-treated animals 18 hr post-treatment. MCh (3×10^{-7} M) was added to the EL bath before NaCl was added in stepwise-increasing, cumulative concentrations to the IL bath. (A) The mV change in the V_{ms} evoked by the addition of IL NaCl. LPS-treatment significantly increased the degree of depolarization while the EC_{50} 's were unaffected. (B) These are data from the same experiments in (A); however, they are expressed in a different manner. These plots show the actual V_{ms} following the addition of IL NaCl. In both treatment groups, intraluminally-applied NaCl eventually depolarized the V_{ms} to the same level. Saline- and LPS-treated, $n = 4$. * $p < 0.05$ compared to saline-treated as determined by a nonpaired Student's t test.

Figure 33. Effect of LPS-treatment on the relationship between EpDRF-induced smooth muscle relaxation and epithelial depolarization elicited by hypertonic NaCl. The curves were obtained from tracheas removed from saline- and LPS-treated animals 18 hr post-treatment. The preparations were contracted with extraluminally-applied MCh (3×10^{-7} M; $\sim EC_{50}$) before NaCl was added in stepwise-increasing, cumulative concentrations to the IL bath. This figure illustrates that the EC_{50} 's for depolarization are significantly greater than the EC_{50} 's for relaxation in both treatment groups. Saline- and LPS-treated for relaxation, $n = 8$ and saline- and LPS-treated for depolarization, $n = 4$.

TABLE 2

**Relationship between *in vitro* smooth muscle relaxation and epithelial depolarization
in isolated, perfused trachea from saline- and LPS-treated guinea pigs**

Treatment	EC ₅₀ (mOsm) ^a (95% C.I.)		Maximum Response	
	Relaxation (% of MCh) ^b	Depolarization ΔV_{ms} (mV) ^c	Relaxation (% of MCh) ^b	Depolarization ΔV_{ms} (mV) ^c
Saline	11.4 (8.2-15.8)	121.6 ^d (75.0-197.2)	78.2±6.2	15.3±0.5
LPS	10.4 (8.4-12.8)	183.6 ^e (120.2-280.4)	113.3±9.5 ^f	38.0±4.1 ^f

The tracheas were removed from the animals 18 hr post-treatment. ^aValues refer to mOsm of NaCl added to the intraluminal MKH solution. ^bRelaxation responses in cm H₂O were normalized as a percentage of the extraluminal MCh (3×10^{-7} M; $\sim$ EC₅₀)-induced contraction; saline- and LPS- treated, $n = 8$. ^cValues refer to the change of the V_{ms} after the administration of extraluminal MCh (3×10^{-7} M); saline- and LPS-treated, $n = 4$. ^dSignificantly greater than saline (relaxation). ^eSignificantly greater than LPS (relaxation). ^fSignificantly greater than saline-treated controls.

Figure 34. Effect of LPS-treatment on epithelial bioelectric reactivity to extraluminally- and intraluminally-applied NaCl in the absence of MCh (a within-treatment group comparison). EL and IL NaCl concentration-response curves were obtained from tracheas removed from (A) saline- and (B) LPS-treated animals 18 hr post-treatment. NaCl was added in stepwise-increasing, cumulative concentrations to the EL and IL baths. In both treatment groups, extraluminally-applied NaCl was more potent. The arrows refer to the fact that the next highest concentration of intraluminally-applied NaCl reversed the depolarization, or in other words, hyperpolarized the V_{ms} . In addition, because of the size of the EL bath and the limits of solubility of NaCl, only 843.4 mOsm of NaCl could be added. Saline-treated, $n = 7$ and LPS-treated, $n = 6$.

Figure 35. Effect of LPS-treatment on epithelial bioelectric reactivity to (A) extraluminally- and (B) intraluminally-applied NaCl in the absence of MCh (a between-treatment group comparison). This is the same data shown in Figure 34 but in a different manner. EL and IL NaCl concentration-response curves were obtained from tracheas removed from saline- and LPS-treated animals 18 hr post-treatment. NaCl was added in stepwise-increasing, cumulative concentrations to the EL and IL baths. NaCl depolarized the V_{ms} to a greater extent in the LPS-treated group; however, it did not reach statistical significance, except for one concentration added intraluminally. The arrows refer to the fact that the next highest concentration of intraluminally-applied NaCl reversed the depolarization, or in other words, hyperpolarized the V_{ms} . In addition, because of the size of the EL bath and the limits of solubility of NaCl, only 843.4 mOsm of NaCl could be added. Saline-treated, $n = 7$ and LPS-treated, $n = 6$. * $p < 0.05$ compared to saline-treated as determined by a nonpaired Student's t test.

TABLE 3

Effect of LPS-treatment on *in vitro* epithelial bioelectric reactivity to hypertonic NaCl in isolated, perfused trachea

Treatment (n)	EC ₅₀ (mOsm) ^a (95% C.I.)	
	EL	IL
Saline (7)	68.2 (57.6-81.0)	157.4 ^b (95.0-261.0)
LPS (6)	88.6 (73.0-107.2)	313.4 ^{c,d} (283.0-347.0)

EL, extraluminal; IL, intraluminal. The tracheas were removed from the animals 18 hr post-treatment. ^aValues refer to mOsm of NaCl added to the extraluminal or intraluminal MKH solution. ^bSignificantly greater than saline (EL). ^cSignificantly greater than LPS (EL). ^dSignificantly greater than saline (IL).

Effects of indomethacin and L-NAME on LPS-induced alterations of the basal V_{ms} in isolated, perfused trachea

In order to elucidate the mechanism underlying the increase in the basal V_{ms} following LPS-treatment, the effect of indomethacin (3×10^{-6} M) and L-NAME (10^{-4} M) on the basal V_{ms} was examined. Both indomethacin and L-NAME were chosen because LPS up-regulates their targets, cyclooxygenase and nitric oxide synthase (Okamoto et al., 1998) whose products, prostaglandins and NO, respectively, have been shown to stimulate Cl^{-} secretion (Tamaoki et al., 1992; Tamai and Gaginella, 1993).

Indomethacin depolarized the V_{ms} in both treatment groups (Figure 36), but significantly more so in the LPS-treated group: $+3.1 \pm 0.5$ mV and $+5.5 \pm 0.7$ mV for the saline- and LPS-treated groups, respectively ($p < 0.05$).

L-NAME slightly hyperpolarized the V_{ms} in both treatment groups (Figure 37); however, there was no significant difference between the magnitude of the hyperpolarization among the saline- and LPS-treated groups.

Because L-NAME has been shown to possess muscarinic receptor antagonist properties (Buxton et al., 1993), the effect of atropine (10^{-6} M), a muscarinic receptor antagonist, on the basal V_{ms} was characterized to determine if the small effect of L-NAME was due to the inhibition of nitric oxide synthesis or muscarinic receptor blockade.

Figure 36. Effect of indomethacin on the basal V_{ms} of isolated, perfused trachea from saline- and LPS-treated animals. The tracheas were removed from saline- and LPS-treated animals 18 hr post-treatment. Following a 3 hr equilibration period, indomethacin (3×10^{-6} M) was simultaneously added to the EL and IL baths. (A) The actual mV change in the basal V_{ms} evoked by the addition of indomethacin. The depolarizing effect of indomethacin was significantly greater in the LPS-treated group. (B) In the presence of indomethacin, the V_{ms} was significantly decreased in both treatment groups. Saline- and LPS-treated, $n = 5$. * $p < 0.05$ compared to saline-treated as determined by a nonpaired Student's t test. † $p < 0.05$ compared to saline-treated (! Indomethacin) as determined by a paired Student's t test. †† $p < 0.05$ compared to saline-treated (! Indomethacin) as determined by a nonpaired Student's t test. ††† $p < 0.05$ compared to LPS-treated (! Indomethacin) as determined by a paired Student's t test.

L-NAME

Figure 37. Effect of L-NAME on the basal V_{ms} of isolated, perfused trachea from saline- and LPS-treated animals. The tracheas were removed from saline- and LPS-treated animals 18 hr post-treatment. Following a 3 hr equilibration period, L-NAME (10^{-4} M) was added simultaneously to the EL and IL baths. (A) The actual mV change in the V_{ms} evoked by the addition of L-NAME. There was no difference in the hyperpolarizing effect of L-NAME between the treatment groups. (B) There was no difference in the V_{ms} before and after the addition of L-NAME within each treatment group. Saline- and LPS-treated, $n = 4$. * $p < 0.05$ compared to saline-treated (! L-NAME) as determined by a nonpaired Student's t test. † $p < 0.05$ compared to saline-treated (+L-NAME) as determined by a nonpaired Student's t test.

The effect of atropine on the basal V_{ms} in isolated, perfused trachea from saline- and LPS-treated animals is shown in Figure 38. Atropine's effect was variable in both treatment groups. Atropine initiated either a depolarization or a hyperpolarization of the V_{ms} which did not exceed ± 2 mV.

Since atropine did not clarify the effects of nitric oxide and its possible contribution to the basal V_{ms} , the effect of sodium nitroprusside (SNP), a nitric oxide donor, on the basal V_{ms} of isolated, perfused trachea from saline- and LPS-treated animals was determined (Figure 39). SNP (10^{-6} M) caused a very small depolarization of the basal V_{ms} ; however, there was no difference in the magnitude of the depolarization among the treatment groups.

Effect of amiloride on LPS-induced alterations of the basal V_{ms} in isolated, perfused trachea

Since the effects of indomethacin and L-NAME gave little insight into the mechanism responsible for the increased basal V_{ms} in tracheas from LPS-treated animals, the Na^+ channel blocker, amiloride, was used (Figure 40). Amiloride was chosen because its target plays an important role in epithelial ion transport (Figure 6). The application of amiloride (3×10^{-5} M) to the IL surface of isolated, perfused tracheas from saline- and LPS-treated animals depolarized the V_{ms} , but significantly more so in the LPS-treated group. After the addition of amiloride, there was no longer any difference between the V_{ms} 's among the saline- and LPS-treated groups.

Atropine

Figure 38. Effect of atropine on the basal V_{ms} of isolated, perfused trachea from (A) saline- and (B) LPS-treated animals. The tracheas were removed from saline- and LPS-treated animals 18 hr post-treatment. Following a 3 hr equilibration period, atropine (10^{-6} M) was added simultaneously to the EL and IL baths. In both groups, atropine had variable effects, sometimes depolarizing and other times hyperpolarizing. Since there was no consistent effect, the raw data from four individual experiments was plotted and an average value shown underneath each graph. Saline- and LPS-treated, $n = 4$.

Sodium Nitroprusside (SNP)

Figure 39. Effect of SNP on the basal V_{ms} of isolated, perfused trachea from saline- and LPS-treated animals. The tracheas were removed from saline- and LPS-treated animals 18 hr post-treatment. Following a 3 hr equilibration period, SNP (10^{-6} M) was added to the EL and IL baths. (A) The actual mV change in the basal V_{ms} evoked by the addition of SNP. There was no significant difference between the degree to which SNP depolarized the V_{ms} among the treatment groups. (B) There was no difference in the V_{ms} before and after the addition of SNP within each treatment group. Saline- and LPS-treated, $n = 3$. * $p < 0.05$ compared to saline-treated (! SNP) as determined by a nonpaired Student's t test. † $p < 0.05$ compared to saline-treated (+SNP) as determined by a nonpaired Student's t test.

Amiloride

Figure 40. Effect of amiloride on the basal V_{ms} of isolated, perfused trachea from saline- and LPS-treated animals. The tracheas were removed from saline- and LPS-treated animals 18 hr post-treatment. Following a 3 hr equilibration period, amiloride (3×10^{-5} M) was added to the IL bath. (A) The actual mV change in the basal V_{ms} evoked by the addition of amiloride. The depolarizing effect of amiloride was significantly greater in the LPS-treated group. (B) Amiloride significantly altered the V_{ms} in both treatment groups. After the IL application of amiloride, there was no longer a difference between the V_{ms} 's among the two groups. Saline- and LPS-treated, $n = 5$. * $p < 0.05$ compared to saline-treated as determined by a nonpaired Student's t test. † $p < 0.05$ compared to saline-treated (! Amiloride) as determined by a paired Student's t test. †† $p < 0.05$ compared to saline-treated (! Amiloride) as determined by a nonpaired Student's t test. ††† $p < 0.05$ compared to LPS-treated (! Amiloride) as determined by a paired Student's t test.

Effect of the *in vitro* addition of LPS on the basal V_{ms} of isolated, perfused trachea from naive animals

Because this study has shown that *in vivo* LPS-treatment (4 mg/kg, i.p; 18 hr post-treatment) significantly hyperpolarized the basal V_{ms} (Figure 26), it was of interest to investigate whether or not the *in vitro* application of LPS to the organ bath did the same. The addition of LPS (10 μ g/ml) to the EL and IL surfaces of isolated, perfused trachea from naive guinea pigs caused a nearly significant ($p = 0.055$) hyperpolarization of the basal V_{ms} when compared to the control preparation to which nothing was added (Figure 41). In the untreated, control preparation, the reported ΔV_{ms} was due to a slight hyperpolarization of the V_{ms} over time which is inherent to the tissue. In addition, LPS caused a significant change in the basal V_{ms} when compared to the ΔV_{ms} in the absence of LPS.

Effect of LPS-treatment on *in vitro* airway reactivity to MCh in epithelium-intact and -denuded isolated, perfused trachea

Previous studies have shown that increases and decreases in EpDRF-induced smooth muscle relaxation were associated with alterations in *in vitro* and *in vivo* airway reactivity to MCh (Lawrence et al., 1996; Fedan et al., 2000). In this study LPS-treatment was shown to alter EpDRF-induced smooth muscle relaxation; therefore, it is hypothesized that *in vitro* and/or *in vivo* airway reactivity to MCh should be altered in accordance with the aforementioned studies.

LPS

Figure 41. Effect of the *in vitro* addition of LPS on the basal V_{ms} of isolated, perfused trachea from naive animals. Following a 3 hr equilibration period, LPS (10 $\mu\text{g/ml}$) was simultaneously added to the EL and IL baths. (A) The hyperpolarizing effect of LPS on the basal V_{ms} reached a nearly significant level when compared to the untreated, control preparation. The reported ΔV_{ms} for the untreated, control preparation was due to a slight hyperpolarization of the V_{ms} over time which was inherent to the tissue. (B) There was a significant difference between the V_{ms} 's before and after the addition of LPS. $t = 0$ hr refers to the time immediately before the start of the control- or LPS-treatment period. $t = x$ hr refers to the length of the control- or LPS-treatment period which was dependent upon the time required for the response to LPS to equilibrate, and the length of this period was variable between tracheas. Control- and LPS-treated, $n = 5$. $\dagger p = 0.055$ compared to control-treated as determined by a nonpaired Student's t test. $*p < 0.05$ compared to ! LPS as determined by a paired Student's t test.

In epithelium-intact tracheas, there was no difference in reactivity to extraluminally- and intraluminally-applied MCh between the two treatment groups; however, the tracheas from both treatment groups were more sensitive to extraluminally-applied MCh (Figure 42A, Table 4). In addition, there was no difference in the IL/EL maximum response ratio between the saline- and LPS-treated groups.

In epithelium-denuded tracheas, the differences between EL and IL reactivity to MCh were abolished, and the IL/EL maximum response ratio approached unity (Figure 42B, Table 5). In addition, there was no difference in reactivity to extraluminally- and intraluminally-applied MCh in epithelium-denuded tracheas from saline- and LPS-treated animals.

Effect of LPS-treatment on tracheal epithelial bioelectric reactivity to MCh in isolated, perfused trachea

Although LPS-treatment did not affect *in vitro* smooth muscle reactivity to MCh in epithelium-intact and -denuded isolated, perfused tracheas, it had a profound effect upon tracheal epithelial bioelectric reactivity to MCh.

In tracheas from both saline- and LPS-treated animals, extraluminally-applied MCh produced a biphasic concentration-response curve (Figure 43 and Table 6). At MCh concentrations less than 10^{-6} M, the V_{ms} hyperpolarized while at MCh concentrations greater than 10^{-6} M, the V_{ms} depolarized. The EC_{50} 's and maximum responses for hyperpolarization were smaller and larger, respectively, in the LPS-treated group; however, the MCh EC_{50} 's and maximum responses for depolarization were not different. The IL application of MCh also generated biphasic electrical concentration-response curves in both treatment groups (hyperpolarizing at MCh concentrations less than 10^{-3} M and depolarizing at MCh concentrations greater than 10^{-3} M; Figure 43 and Table 6).

Figure 42. Lack of effect of LPS-treatment on tracheal smooth muscle reactivity to extraluminally- and intraluminally-applied MCh in (A) epithelium-intact and (B) -denuded isolated, perfused trachea. EL and IL MCh concentration-response curves were obtained from tracheas removed from saline- and LPS-treated animals 18 hr post-treatment. In epithelium-intact tracheas from saline- and LPS-treated animals, extraluminally-applied MCh was more potent. Removal of the epithelium abolished the difference between reactivity to extraluminally- and intraluminally-applied MCh. Saline- and LPS-treated, $n = 6$ for epithelium-intact and $n = 4$ for epithelium-denuded.

TABLE 4

**Effect of LPS-treatment on *in vitro* airway reactivity to
MCh in epithelium-intact, isolated, perfused trachea**

Treatment (n)	EC ₅₀ (M) (95% C.I.)		Maximum Response ΔP (cm H ₂ O)		IL/EL Maximum Response Ratio ^a
	EL	IL	EL	IL	
Saline (6)	1.9×10 ⁻⁷ (1.4-2.5)	3.7×10 ^{-4b} (1.5-9.1)	10.7±1.5	8.4±1.1 ^c	0.78±0.03
LPS (6)	2.1×10 ⁻⁷ (1.7-2.4)	3.2×10 ^{-4d} (1.8-5.8)	6.6±0.9	4.7±0.9 ^e	0.70±0.12

EL, extraluminal; IL, intraluminal. The tracheas were removed from the animals 18 hr post-treatment. ^aThe IL/EL maximum response ratio is used to gauge the modulatory effect of the epithelium on the EL and IL maximum contractile responses. In the absence of the epithelium, the ratio is unity while it becomes less than unity in the presence of the epithelium. As the modulatory effect of the epithelium becomes more inhibitory, the ratio becomes smaller (Fedan and Frazer, 1992; Fedan et al., 2000). ^bSignificantly greater than saline (EL). ^cSignificantly less than saline (EL). ^dSignificantly greater than LPS (EL). ^eSignificantly less than LPS (EL).

TABLE 5

**Effect of LPS-treatment on *in vitro* airway reactivity to
MCh in epithelium-denuded, isolated, perfused trachea**

Treatment (n)	EC ₅₀ (M) (95% C.I.)		Maximum Response ΔP (cm H ₂ O)		IL/EL Maximum Response Ratio ^a
	EL	IL	EL	IL	
Saline (4)	2.1×10 ⁻⁶ (0.8-5.2)	7.7×10 ⁻⁶ (2.0-30.0)	3.8±0.7	3.5±0.6	0.97±0.12
LPS (4)	1.1×10 ⁻⁶ (0.5-2.2)	1.5×10 ⁻⁶ (0.8-2.8)	3.9±0.4	3.5±0.3	1.00±0.06

EL, extraluminal; IL, intraluminal. The tracheas were removed from the animals 18 hr post-treatment. ^aThe IL/EL maximum response ratio is used to gauge the modulatory effect of the epithelium on the EL and IL maximum contractile responses. In the absence of the epithelium, the ratio is unity while it becomes less than unity in the presence of the epithelium. As the modulatory effect of the epithelium becomes more inhibitory, the ratio becomes smaller (Fedan and Frazer, 1992; Fedan et al., 2000).

In the case of intraluminally-applied MCh, the maximum hyperpolarization response was greater in the LPS-treated group with respect to the saline-treated controls while there was no difference in the depolarization responses among the two treatment groups.

Effect of LPS-treatment on *in vivo* pulmonary function and airway reactivity to MCh

Besides investigating whether or not LPS-treatment altered *in vitro* airway reactivity to MCh in the isolated, perfused trachea, this study also investigated whether or not LPS-treatment altered *in vivo* airway reactivity to inhaled, MCh aerosol.

The post-treatment saline BF was slightly, but significantly, less than the pre-treatment value; however, there were no other differences between the pre- or post-treatment SRaw or BF values within each treatment group (Figure 44).

Eighteen hr following saline-treatment, the animals' reactivity to inhaled, MCh aerosol increased which was demonstrated by a decrease in the MCh PC₂₀₀ (Figure 45 and Table 7). On the other hand, LPS-treatment failed to produce any changes in airway reactivity to MCh when the LPS pre-and post-treatment groups were compared; however, when comparing the saline and LPS post-treatment groups to allow for the increase in reactivity in the saline-treated group, the LPS-treated animals were 3-fold *hyporeactive* to the effects of inhaled MCh as determined by a comparison of their MCh PC₂₀₀ values (Table 7).

Figure 43. Effect of LPS-treatment on epithelial bioelectric reactivity to (A) extraluminally- and (B) intraluminally-applied MCh in isolated, perfused trachea. EL and IL MCh concentration-response curves were obtained from tracheas removed from saline- and LPS-treated animals 18 hr post-treatment. The results were expressed as changes in the transepithelial potential difference (ΔV_{ms}). Positive values refer to depolarization of the V_{ms} while negative values refer to hyperpolarization of the V_{ms} . In both groups, extraluminally-applied MCh was more potent. Saline-treated, $n = 7$ and LPS-treated, $n = 6$. * $p < 0.05$ compared to saline-treated controls as determined by a nonpaired Student's t test.

TABLE 6

Effect of LPS-treatment on *in vitro* epithelial bioelectric reactivity to**MCh in isolated, perfused trachea**

Treatment (n)	EC ₅₀ (M) (95% C.I.)		Maximum Response ΔV_{ms} (mV) ^a	
	EL	IL	EL	IL
Hyperpolarization				
Saline (7)	6.2×10 ⁻⁸ (2.9-13.0)	9.8×10 ^{-6b} (2.1-45.0)	! 0.9±0.3	! 1.6±0.3
LPS (6)	4.2×10 ^{-9c} (1.6-11.0)	2.7×10 ^{-6d} (1.8-5.9)	! 3.0±0.7 ^b	! 5.3±1.6 ^e
Depolarization				
Saline (7)	2.6×10 ⁻⁶ (1.5-4.3)	7.6×10 ^{-2b} (2.3-23.0)	2.3±0.6	4.0±1.1
LPS (6)	1.9×10 ⁻⁶ (1.0-3.7)	2.8×10 ^{-2d} (0.7-10.0)	4.8±1.6	5.0±1.1

EL, extraluminal; IL, intraluminal. The tracheas were removed from the animals 18 hr post-treatment. ^aValues refer to the change in the V_{ms} . ^bSignificantly greater than saline (EL).

^cSignificantly less than saline (EL). ^dSignificantly greater than LPS (EL). ^eSignificantly greater than saline (IL).

Figure 44. Effect of saline- and LPS-treatments on post-treatment basal (A) specific airway resistance (SRaw) and (B) breathing frequency (BF). Eighteen hr following saline- and LPS-treatments, basal SRaw and BF were measured. There was a slight, but significant, decrease in the saline post-treatment BF when compared to the pre-treatment value. There were no other differences between the pre- and post-treatment values. Saline- and LPS-treated, $n = 6$. * $p < 0.05$ compared to saline pre-treatment as determined by a paired Student's t test.

Figure 45. Effect of (A) saline- and (B) LPS-treatments on *in vivo* airway reactivity to inhaled MCh aerosol. Eighteen hr post-treatment, (A) the reactivity of the saline-treated animals to inhaled, MCh aerosol increased while (B) LPS-treatment had no significant effect on airway reactivity. When comparing the saline- and LPS-treated groups to allow for the increase in reactivity of the saline-treated group, the LPS-treated animals were *hyporeactive* to the effects of inhaled MCh. Saline- and LPS-treated, $n = 6$.

TABLE 7

**Effect of saline- and LPS-treatments on *in vivo* airway
reactivity to inhaled MCh**

Treatment (<i>n</i>)	MCh PC ₂₀₀ (mg/ml) (95% C.I.)	
	Pre-treatment	Post-treatment
Saline (6)	0.17 (0.11-0.27)	0.08 ^a (0.05-0.14)
LPS (6)	0.18 (0.13-0.25)	0.26 ^b (0.08-0.79)

^aSignificantly less than saline (pre-treatment). ^bSignificantly greater than saline (post-treatment).

Effect of L-NAME on *in vivo* airway reactivity to MCh in saline- and LPS-treated animals

In order to determine if an acquired NO component contributed to the *in vivo* airway *hyporeactivity* of the LPS-treated animals, the effect of an L-NAME aerosol on *in vivo* airway reactivity to MCh was determined in saline- and LPS-treated animals. Prior to the generation of *in vivo* MCh concentration-response curves in the presence of L-NAME, the LPS-treated animals were again found to be *hyporeactive* to the effects of inhaled MCh as demonstrated by a comparison of MCh PC₂₀₀ values from saline- and LPS-treated animals (Figure 46 and Table 8). The administration of L-NAME prior to MCh had no effect upon airway reactivity in either the saline- or LPS-treated animals.

Histological Examination of Guinea-Pig Airways Following LPS-Treatment

Since L-NAME failed to provide any clues to the mechanism underlying LPS-induced *in vivo* airway *hyporeactivity* to MCh, this study then examined the effects of LPS-treatment on the microscopic structure of various regions of the guinea-pig respiratory tract. This was done to determine if LPS-treatment altered the physical structure of the airways in such a way as to limit access of MCh to the airway smooth muscle (i.e., excessive mucus secretion, epithelial and basement membrane hyperplasia and/or hypertrophy, etc.). Histological sections of the distal trachea as well as the azygous, left, right apical, right cardiac, and right diaphragmatic lobes of the lung from saline- and LPS-treated animals were examined. The sections from both treatment groups were indistinguishable from one another, at least at the level of the light microscope.

Figure 46. Lack of effect of L-NAME on *in vivo* airway reactivity to inhaled MCh aerosol in (A) saline- and (B) LPS-treated guinea pigs. Eighteen hr post-treatment, L-NAME did not alter airway reactivity to MCh in either treatment group. Saline- and LPS-treated, $n = 4$.

TABLE 8

**Effect of L-NAME on *in vivo* airway reactivity to
inhaled MCh in saline- and LPS-treated guinea pigs**

Treatment (<i>n</i>)	MCh PC ₂₀₀ (mg/ml) (95% C.I.)	
	! L-NAME	+L-NAME
Saline (4)	0.07 (0.06-0.10)	0.01 (0.00-0.94)
LPS (4)	0.78 ^a (0.31-1.97)	0.40 (0.11-1.45)

^aSignificantly greater than saline (! L-NAME).

Representative sections of the distal trachea and left lobe of the lung from saline- and LPS-treated animals are shown in Figure 47A-F. The bronchial and tracheal epithelium as well as the alveolar sacs remained structurally intact within each group. Finally, in both treatment groups, a few inflammatory cells, namely eosinophils and neutrophils, could be seen infiltrating the airways in some regions of the respiratory tract which were examined (data not shown).

Figure 47. Histological sections of guinea-pig airways following saline- and LPS-treatments. Representative Harris' hematoxylin-eosin stained histological sections from saline (A, C, and E)- and LPS (B, D, and F)-treated animals made 18 hr post-treatment. A thorough examination of histological sections from saline- and LPS-treated animals showed that LPS-treatment did not alter airway appearance or structure, at least at the level of the light microscope. A and B are 40 $\times$ sections of distal trachea, C and D are 10 $\times$ sections of bronchi, and E and F are 20 $\times$ sections of the alveolar regions from saline- and LPS-treated animals, respectively. Sections of the bronchi and alveolar regions were taken from the left lobe of the lung. The measurement bars (50, 200, and 100 μ m) found in B, D, and F also pertain to their respective counterpart in A, C, and E. These results are representative of eight different experiments from each treatment group.

Discussion

There are three general conclusions which can be drawn from the results of this study. First, the effects of EpDRF on the airway smooth muscle are associated with changes in the electrical activity of the airway epithelium following LPS-treatment; however, these changes in the effects of EpDRF are dependent upon the osmolyte used to elicit EpDRF release. Secondly, epithelial depolarization does not always precede EpDRF-induced smooth muscle relaxation, suggesting that EpDRF release is not always dependent on epithelial depolarization. Finally, LPS-induced *in vivo* airway hyporeactivity to inhaled, MCh aerosol may be due, at least in part, to an increase in the actions of EpDRF.

Both *in vivo* and *in vitro* LPS-treatment significantly hyperpolarized the basal V_{ms} of isolated, perfused trachea. To our knowledge, there have been very few studies examining the effect of LPS on ion channel and/or transporter function; however, those studies which have examined this phenomenon aid in the explanation of our results. In rat tail artery smooth muscle cells, LPS increased the inward flux of Ca^{2+} ions through voltage-sensitive Ca^{2+} channels (Wilkinson et al., 1996) while two other, separate studies reported that LPS increased the conductance of Ca^{2+} -activated K^{+} channels in vascular smooth muscle cells (Miyoshi and Nakaya, 1994; Hoang et al., 1997). In our system, LPS or one of its mediators may be increasing epithelial cell Ca^{2+} influx which could activate Ca^{2+} -activated K^{+} channels found on the basolateral surface of the airway epithelium (Clarke and Boucher, 1993). It is intriguing to note that Ca^{2+} -activated K^{+} channels have been shown to be involved in the synthesis and/or release of EpDRF (Tamaoki et al., 1997), and their activation by LPS or one of its many mediators may account for the alterations in EpDRF-induced smooth muscle relaxation following LPS-treatment.

The alteration of EpDRF-induced smooth muscle relaxation following LPS-treatment was dependent upon the osmolyte used to elevate IL tonicity, suggesting that the synthesis, release, and/or effects of EpDRF are osmolyte-dependent, at least after LPS-treatment. It is

difficult to reconcile these results with the fact that EpDRF-induced smooth muscle relaxation is initiated regardless of the osmolyte used to elevate EL or IL tonicity (Munakata et al., 1988; Fedan et al., 1999). One study has examined the potency of different osmolytes to elicit EpDRF-induced smooth muscle relaxation. This study reported that NaCl and KCl were equipotent in their ability to evoke EpDRF-induced smooth muscle relaxation; however, no other agents were examined (Fedan et al., 1999). The differences among the osmolytes used in the present study could be due to their ability or inability to interact with specific epithelial ion channels and/or transporters, namely apical Na^+ and Cl^- channels, which seem to be involved in EpDRF-induced smooth muscle relaxation (Fedan et al., 1999). D-mannitol is membrane-impermeable so it would be unable to pass through any ion channel and/or transporter while urea is freely, membrane-permeable. On the other hand, both NaCl and KCl can enter the cell through their respective ion channels and/or transporters. In addition, this study showed that LPS-treatment altered the basal bioelectric properties of the epithelium which could further modify EpDRF-induced smooth muscle relaxation in response to these osmolytes if a specific ion channel and/or transporter involved in these relaxation responses were altered following LPS-treatment. It is of interest to note that a previous study examined the effects of NaCl, mannitol, and urea on isolated, human bronchial strips, and they reported that hypertonic NaCl and mannitol initiated bronchoconstriction while urea did not (Jongejan et al., 1990). As a side note, the contractile responses elicited by these osmolytes may be attributed to species differences or more importantly to the fact that one cannot selectively add pharmacological agents to either the serosal or mucosal surfaces of strip preparations. Although our study looked at EpDRF-induced smooth muscle relaxation elicited by these agents, our study and that of Jongejan et al. (1990) demonstrated that hypertonic NaCl and D-mannitol behave in a similar manner regardless of the endpoint, contraction or relaxation, examined. In our study, NaCl and D-mannitol seem to be working by a similar mechanism while urea and KCl act by a different mechanism from each other as well as from NaCl and D-mannitol following LPS-treatment.

We investigated whether the potentiation of EpDRF-induced smooth muscle relaxation, elicited by NaCl, following LPS-treatment was due to an acquired NO and/or prostanoid component because both NO and certain prostanoids are known smooth muscle relaxants (Folkerts and Nijkamp, 1998). In addition, LPS can up-regulate inducible nitric oxide synthase as well as potentiate the smooth muscle relaxant effects of some prostanoids in guinea pigs (Stein et al., 1996; Myers et al., 1999). Both L-NAME and indomethacin, inhibitors of nitric oxide synthase and cyclooxygenase, respectively, failed to have any effect upon EpDRF-induced smooth muscle relaxation in tracheas from saline- and LPS-treated animals. This further supports the conclusion that EpDRF is neither NO nor a prostanoid and that the potentiation of EpDRF-induced smooth muscle relaxation following LPS-treatment was not due to an acquired NO or prostanoid component.

The potentiation of EpDRF-induced smooth muscle relaxation following LPS-treatment was also associated with a potentiation of the epithelial bioelectric events, depolarization of the V_{ms} , preceding EpDRF-induced smooth muscle relaxation when NaCl was used to elevate IL tonicity. These results further strengthen the hypothesis that the two events are functionally linked. Both hypertonicity-induced smooth muscle relaxation and epithelial depolarization were potentiated following LPS-treatment; however, in both treatment groups, the epithelium more readily released EpDRF than depolarized in response to elevated mucosal tonicity. The epithelium can release EpDRF, as demonstrated by smooth muscle relaxation, when as little as 0.84 mOsm of NaCl was added to the IL surface of the trachea; however, the V_{ms} did not depolarize in response to hypertonicity until approximately 8.44 mOsm of added NaCl. If epithelial depolarization in response to hypertonicity signaled the release of EpDRF (Dortch-Carnes et al., 1999), we would have expected the epithelium to depolarize prior to every relaxation response; however, this was clearly not the case. Because of this discrepancy, it is tempting to speculate that two events are taking place. At low concentrations of hypertonicity, EpDRF release is independent of epithelial depolarization, a situation analogous to pharmacomechanical coupling. As EpDRF is released in response to higher concentrations of

hypertonicity, epithelial depolarization may be a necessary component to signal the release of greater quantities of EpDRF, a situation analogous to electromechanical coupling.

Alternatively, transepithelial depolarization may be quite, simply a coincident, epiphenomenon occurring in parallel with another signal (i.e., change in intracellular pH) that is more sensitive to osmolarity changes. It is quite possible that changes in intracellular pH, and not transepithelial depolarization, in response to elevated mucosal or serosal tonicity is the signal for the synthesis, release, and/or effects of EpDRF. This is a potential avenue of further investigation given the fact that exposure of cells to hyperosmotic solution causes cell alkalization (Miyata et al., 2000). In addition, the Na^+ channel blocker, amiloride, and the Cl^- channel blocker, DIDS, which attenuate EpDRF-induced smooth muscle relaxation, are also capable of inhibiting the Na^+/H^+ exchanger and the $\text{Cl}^-/\text{HCO}_3^-$ exchanger (Kreydiyyeh, 2000; Lamb et al., 2000); respectively. The actions of these pharmacological inhibitors on their respective targets would, without a doubt, profoundly influence intracellular pH.

This study also examined epithelial bioelectric reactivity to hypertonic NaCl in tracheas from saline- and LPS-treated animals. These experiments were done in the absence of MCh since smooth muscle contraction was not a necessary component of these experiments. In both treatment groups, the epithelium depolarized in response to extraluminally- and intraluminally-applied NaCl with the EL surface of the trachea being more sensitive to hypertonicity. This was in contrast to another study examining the effect of hypertonicity on airway epithelial bioelectric responses, in which cultured, human nasal epithelial cells depolarized only when hypertonic mannitol was added to the apical surface of the epithelium (Willumsen et al., 1994). This discrepancy may be attributed to species or regional differences in ion transport (Boucher et al., 1980). Tracheas from LPS-treated animals were also less sensitive to the effects of intraluminally-applied NaCl than those from saline-treated animals. Because the basal V_{ms} in tracheas from LPS-treated animals was significantly more hyperpolarized, it may be more difficult to depolarize the V_{ms} or the ion channels and/or transporters affected by hypertonicity could be less sensitive to the effect of IL hypertonicity following LPS-treatment. Overall, these

results demonstrate that the EL and IL surfaces of the trachea can function as an osmotic sensor, with the EL surface being more sensitive. In addition, LPS-treatment alters the ability of the IL surface to act as an osmotic sensor.

This study investigated the mechanism(s) behind the hyperpolarization of the V_{ms} following LPS-treatment by examining the effect of various pharmacological agents on the V_{ms} . Because LPS is known to up-regulate inducible nitric oxide synthase and cyclooxygenase, the effect of their inhibitors on the basal V_{ms} was examined. The small hyperpolarization of the V_{ms} by L-NAME in both treatment groups was not consistent with the reported effects of NO on ion transport. In rat colon and human airway epithelial cells, NO stimulated Cl^- secretion (Tamai and Gagginella, 1993; Kamosinska et al., 1997). If NO stimulates Cl^- secretion in our system, L-NAME, by virtue of its ability to inhibit NO synthesis, should have depolarized the V_{ms} which was clearly not the case. Sodium nitroprusside, a NO donor, depolarized the V_{ms} , which was not consistent with a previous report demonstrating its ability to stimulate Cl^- secretion (Tamai and Gagginella, 1993). Our results, however, were internally consistent. Since L-NAME hyperpolarized the V_{ms} in our system, we would have expected a NO donor such as sodium nitroprusside to depolarize the V_{ms} . From both of these experiments, however, we can conclude that NO makes an insignificant contribution to the generation of the V_{ms} in tracheas from either saline- or LPS-treated animals.

Because L-NAME has muscarinic antagonist properties (Buxton et al., 1993), we investigated the effects of atropine, a nonspecific muscarinic receptor antagonist, on the basal V_{ms} in both treatment groups. The variable effects of atropine on the V_{ms} observed in this study were not consistent with another study which reported that atropine profoundly and rapidly depolarized the V_{ms} of rabbit trachea *in vivo* (Tamaoki et al., 1996). The variable effects of atropine seen in this study could have resulted from the antagonism of different muscarinic receptor subtypes, some of which may be more prevalent in one animal than another. This is a reasonable conclusion given the fact that this study has shown that at least two populations of muscarinic receptor subtypes exist in the guinea-pig tracheal epithelium which are capable of

initiating opposite electrophysiological effects. The fact that atropine does have an electrophysiological effect suggests that the nerves endings embedded in the airway wall are tonically releasing ACh, which exerts its effect by binding to epithelial muscarinic receptors. From our results with atropine, the true effect of L-NAME cannot be deciphered; however, a more rigorous, future study should use equieffective concentrations of atropine and L-NAME with respect to their muscarinic antagonist properties.

Unlike L-NAME, the electrophysiological effects of indomethacin reported in this study were consistent with other studies in the literature. Two prostaglandins in particular, D_2 and E_2 , have been shown to stimulate Cl^- secretion in several unrelated species (Tamaoki et al., 1992a,b; Saunders et al., 1993; Bourke et al., 1995). Indomethacin depolarized the V_{ms} in both treatment groups, but significantly more so in the LPS-treated group. The depolarizing effects of indomethacin were consistent with an inhibition of prostanoid-stimulated Cl^- secretion. The fact that indomethacin depolarized the V_{ms} to a greater extent in the LPS-treated group suggests that prostanoids contribute more to the generation of the basal V_{ms} following LPS-treatment and/or their effects were potentiated by changes in their receptor or post-receptor signaling. In addition, this suggests that prostanoid-stimulated Cl^- secretion may contribute to the significant hyperpolarization of the V_{ms} after LPS treatment.

The addition of amiloride to the IL surface of the trachea abolished the difference between the V_{ms} 's among the two treatment groups. This strongly suggests that the hyperpolarization of the V_{ms} following LPS-treatment was due to an increase in amiloride-sensitive Na^+ transport or Na^+ transport in general. An increase in Na^+ removal from the lumen, via these channels, would generate a more negative V_{ms} . Because the airway epithelium consists of numerous ion channels and transporters, alterations in any of them (Figure 6) could explain changes in the V_{ms} following LPS-treatment. For example, an increase in the number and/or activity of the $Na^+-K^+-ATPase$ would increase amiloride-sensitive Na^+ transport and subsequently hyperpolarize the V_{ms} . More systematic experiments which would take advantage of patch clamping and transepithelial resistance and I_{sc} measurements, and other ion channel and

transporter inhibitors would be necessary to determine the mechanism responsible for LPS-induced hyperpolarization of the V_{ms} . These results with amiloride also impact on the possible mechanism(s) underlying the potentiation of EpDRF-induced smooth muscle relaxation following LPS-treatment. If amiloride-sensitive Na^+ channels increase in number and/or activity following LPS-treatment, this may potentiate EpDRF-induced smooth muscle relaxation inasmuch as their inhibition significantly attenuates this response (Fedan et al., 1999).

The potentiation of EpDRF-induced smooth muscle relaxation following LPS-treatment was associated with *in vivo* airway hyporeactivity to inhaled, MCh aerosol while no differences in *in vitro* airway reactivity to MCh existed in isolated, perfused tracheas from saline- and LPS-treated animals. Over the years, there have been several, inconsistent reports concerning the effect of LPS-treatment on *in vitro* and *in vivo* guinea-pig airway reactivity. *In vivo*, some studies have reported hyperreactivity (Oosterhout et al., 1991), hyporeactivity (Folkerts et al., 1988), and no change (Fedan et al., 1995) in guinea-pig airway reactivity to contractile agonists. The same can be said for *in vitro* airway reactivity as well. Again, some studies have reported hyperreactivity to contractile agonists (ACh, histamine, arecoline) (Folkerts et al., 1989; Yamawaki et al., 1990; Oosterhout et al., 1991), hyporeactivity (Folkerts et al., 1988), and finally, others have shown no change (Fedan et al., 1995) in *in vitro* guinea-pig airway reactivity. In our study, there were no differences in airway reactivity to MCh in epithelium-intact and -denuded isolated, perfused tracheas. This is consistent with a previous study in our laboratory (Fedan et al., 1995) even though the 18 hr post-treatment time point was not examined. *In vivo*, LPS-treated guinea pigs were hyporeactive to the effects of inhaled, MCh aerosol. This is not consistent with the earlier, published reports from our laboratory, but again the 18 hr post-treatment time point was not examined which may account for the difference (Fedan et al., 1995). An acquired NO component does not seem to be responsible for the hyporeactivity to MCh following LPS-treatment in our study since inhaled, L-NAME aerosol was without effect on *in vivo* airway reactivity to MCh in LPS-treated animals.

In the previous study reporting *in vivo* airway hyporeactivity to histamine following LPS-treatment (Folkerts et al., 1988), the airways of these guinea pigs were infiltrated with large numbers of neutrophils and monocytes; however, there were no signs of pulmonary inflammation following LPS-treatment in our study. This was probably due to the fact that the guinea pigs in the previous study were exposed to LPS by inhalation and examined 24 hr post-treatment while our animals were given LPS i.p. and examined 18 hr post-treatment. The lack of pulmonary inflammation in our LPS-treated guinea pigs suggests that changes in airway reactivity are not necessarily accompanied by airway inflammation. The airway hyporeactivity following LPS-treatment does not seem to be due to an acquired NO component or alterations in the physical structure of the airways but may be due, at least in part, to an increase in the actions of EpDRF. *In vivo* changes in airway reactivity did not manifest themselves *in vitro* which suggests that the mechanism responsible for *in vivo* hyporeactivity to MCh did not involve the trachea or was lost *in vitro*.

This is not the first time that alterations in the actions of EpDRF have been accompanied by changes in *in vivo* and/or *in vitro* airway reactivity. For example, exposure to ozone causes a decrease in the actions of EpDRF which is associated with *in vivo* and *in vitro* airway hyperreactivity to MCh (Fedan et al., 2000). In addition, sensitization and challenge of guinea pigs with ovalbumin potentiates EpDRF-induced smooth muscle relaxation which is accompanied by *in vitro* airway hyporeactivity to MCh, but paradoxically, these animals are hyperreactive to MCh *in vivo* (Lawrence et al., 1996). Now, with the addition of our study, there are at least three instances in which changes in the actions of EpDRF are associated with *in vivo* and/or *in vitro* alterations in airway reactivity to MCh, suggesting a functional relationship between the two.

Although LPS-treatment did not have an effect upon *in vitro* airway smooth muscle reactivity to MCh, it did have a profound effect upon *in vitro* epithelial bioelectric reactivity to MCh. Biphasic concentration-response curves were generated in response to the EL- and IL-application of MCh in both treatment groups. Our results were consistent with the variable

electrophysiological effects produced by MCh in guinea-pig nasal epithelium (Suzuki et al., 1999). Suzuki et al. (1999) reported that MCh produced triphasic I_{sc} responses: a transient increase followed by a small decrease and then finally a sustained increased. The authors concluded that these changes were brought about by alterations in Cl^- secretion and amiloride-sensitive Na^+ transport (Suzuki et al., 1999). MCh, by binding to either, M_1 , M_3 , or M_5 muscarinic receptors increases cytosolic Ca^{2+} by coupling G_q -proteins (Janssen et al., 1998). Ca^{2+} in turn is capable of activating Ca^{2+} -activated Cl^- channels and/or Ca^{2+} -activated K^+ channels (Somlyo and Somlyo, 1994) which could subsequently alter the V_{ms} . In addition, the activation of M_2 and M_4 muscarinic receptors can open nonselective cation channels and inhibit current flow through Ca^{2+} channels and Ca^{2+} -activated K^+ channels by coupling G_i -coupled proteins (Roux et al., 1998). The activation of more than one channel was most likely responsible for these biphasic electrical responses, and it is not certain which muscarinic receptor subtypes were initiating these responses. M_1 , M_2 , and M_3 muscarinic receptors have been localized in guinea-pig lung (Mak and Barnes, 1990), and in the future it may be useful to repeat these experiments in the presence of selective, muscarinic receptor antagonists. The muscarinic receptors which were mediating these responses seem to be localized to the basolateral surface of the epithelium since MCh was more potent when added to the EL surface of the trachea. Thus, there seem to be two types of muscarinic epithelial receptors which promote opposite electrical responses in the guinea-pig tracheal epithelium. The high affinity receptor initiates hyperpolarization while the low affinity receptor mediates depolarization, and LPS-treatment seems to selectively alter the function of the high affinity receptor.

Since LPS was used as a tool to alter the basal bioelectric properties of guinea-pig tracheal epithelium, the results of this study have implications with respect to certain pathophysiological conditions in which LPS is involved, such as sepsis, ARDS, as well as gram-negative pulmonary infections. Our study has shown that LPS-treatment alters the actions of EpDRF on the airway smooth muscle, and this may potentially be happening under a variety of LPS-induced disease states. At present, EpDRF's only known function is to relax the airway

smooth muscle; however, it may be an important mediator of other unknown, physiological functions. That is, these other potential functions of EpDRF may be altered in such a way by LPS that it contributes to the disease process. EpDRF may, when present at high or low levels, be directly toxic to various cell types such that it inhibits their normal function or enhances the release of toxic substances from cells. Conversely, high or low levels of EpDRF may protect cells from the insulting effects of LPS in such a way as to prevent further damage.

In conclusion, the results of this study suggest that the effects of EpDRF on the airway smooth muscle are associated with changes in the electrical activity of the airway epithelium; however, there is not always a direct correlation between the two events. In addition, this study has shown that perturbations in the effects of EpDRF are associated with changes in airway reactivity, demonstrating that EpDRF exerts a powerful, modulatory influence on airway smooth muscle tone.

Conclusions

The initial reports by Raeburn and Fedan (1989), Dortch-Carnes et al. (1999), and Fedan et al. (1999), suggesting that the synthesis, release, and/or effects of EpDRF were functionally linked to the electrical activity of the airway epithelium, have been confirmed by the further characterization of the airway epithelial bioelectric mechanisms associated with the effects of EpDRF in this study.

Fedan et al. (1999) previously reported that apical Na^+ channels are involved in hypertonicity-induced smooth muscle relaxation *via* EpDRF, and this study has shown that these channels are also involved in the epithelial bioelectric response to elevated mucosal tonicity. These data suggest that the mechanical and bioelectric events are functionally linked; however, the exact role of these channels in mediating these responses is unclear. Despite the fact that inhibitors of basolateral $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporters, basolateral $\text{Na}^+ - \text{K}^+ - \text{ATPases}$, and apical Cl^- channels altered the response to elevated mucosal tonicity, their effects were different, in some respects, from their actions on EpDRF-induced smooth muscle relaxation, which makes their overall role in these two responses difficult to discern.

This study has also demonstrated that perturbations in the basal electrical activity of the epithelium following LPS-treatment are associated with alterations in EpDRF-induced smooth muscle relaxation and its associated bioelectric events as well as airway reactivity. This further supports the notion that the synthesis, release, and/or effects of EpDRF are linked to the electrical activity of the epithelium. Interestingly, the results seen with LPS have reinforced the importance of amiloride-sensitive Na^+ channels in mediating EpDRF-induced smooth muscle relaxation since LPS-induced changes in epithelial electrophysiology appear to be due alterations in these Na^+ channels. At the most fundamental level, apical Na^+ channels may control airway reactivity by modulating the synthesis, release, and/or effects of EpDRF.

Future Directions

The two, separate studies contained within this project have provided the groundwork for a more thorough investigation of the airway epithelial bioelectric mechanisms associated with the effects of EpDRF in guinea-pig isolated trachea. In fact, this study has left many loose ends which could be tied up with future experiments.

First, the effects of other hypertonic solutions (i.e., KCl, sucrose, urea, etc.), on epithelial bioelectric responses could be examined to determine if the epithelial response to hypertonic challenge is osmolyte dependent. In addition, the effects of ion channel and transporter inhibitors on the epithelium's bioelectric response to these hypertonic solutions could also be investigated for similar reasons. In addition, these experiments could be repeated with LPS-treated tissue to determine how this would influence the epithelial bioelectric responses to hypertonic solution.

Since it appears that alterations in amiloride-sensitive Na^+ channels play a significant role in the hyperpolarization of the V_{ms} following LPS-treatment, it would be interesting to examine these channels at the molecular level. Channel protein or mRNA levels may change after LPS-treatment which could be examined by Western and Northern blotting, respectively. In addition, channel kinetics could be investigated through patch clamping. The effect of amiloride and the other ion channel and transporter inhibitors on LPS-induced alterations in EpDRF-induced smooth muscle relaxation could also be investigated. Finally, it would be interesting to see if epithelial depolarization is potentiated following LPS-treatment in response to the IL-application of D-mannitol, urea, and KCl, which could shed more light on the relationship between epithelial depolarization and the degree of EpDRF-induced smooth muscle relaxation.

Although it was not a major focus of this study, it would be extremely interesting to distinguish which epithelial muscarinic receptor subtypes were responsible for the biphasic electrical responses to MCh, not only in saline-treated animals but also in those treated with LPS. The use of selective, muscarinic receptor antagonists could give insight into the receptor

subtypes involved. In addition, it would be of interest to distinguish why LPS-treatment only affects the bioelectric reactivity of some muscarinic receptor subtypes and not others. This could be accomplished through an examination of receptor binding, coupling, expression, etc.

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