

## Hyperosmolar Solution Effects in Guinea Pig Airways. III. Studies on the Identity of Epithelium-Derived Relaxing Factor in Isolated Perfused Trachea Using Pharmacological Agents

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Received March 14, 2003; accepted October 8, 2003

### ABSTRACT

Hyperosmolar challenge of airway epithelium stimulates the release of epithelium-derived relaxing factor (EpDRF), but the identity of EpDRF is not known. We examined the effects of pharmacological agents on relaxant responses of methacholine ( $3 \times 10^{-7}$  M)-contracted guinea pig perfused trachea to mucosal hyperosmolar challenge using D-mannitol. Responses were inhibited by gossypol ( $5 \times 10^{-6}$  M), an agent with diverse actions, by the carbon monoxide (CO) scavenger hemoglobin ( $10^{-6}$  M), and by the heme oxygenase (HO) inhibitor zinc (II) protoporphyrin IX ( $10^{-4}$  M). The HO inhibitor chromium (III) mesoporphyrin IX ( $10^{-4}$  M) was not inhibitory, and the HO activator heme-L-lysinate ( $3 \times 10^{-4}$  M) did not evoke relaxant responses. The CO donor tricarbonyldichlororuthenium (II) dimer ( $2.2 \times 10^{-4}$  M) elicited small relaxation responses. Other agents without an effect on responses included:

apyrase, adenosine, 6-anilino-5,8-quinolinequinone (LY83583), proadifen, (E)-3-[[[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl]-(3-(dimethylamino)-3-oxopropyl]thio)methyl]thio]-propanoic acid (MK 571), diphenhydramine, glibenclamide,  $\text{HgCl}_2$ , tetrodotoxin, nystatin,  $\alpha$ -hemolysin, 8-bromoguanosine 3',5'-cyclic monophosphothioate, Rp-isomer, 12-O-tetradecanoylphorbol-13-acetate, cholera toxin, pertussis toxin, thapsigargin, nifedipine,  $\text{Ca}^{2+}$ -free mucosal solution, hydrocortisone, and epidermal growth factor. Cytoskeleton inhibitors, including erythro-9-(2-hydroxy-3-nonyl)adenine, colchicine, nocodazole, latrunculin B, and cytochalasins B and D, had no effect on relaxation responses. The results suggest provisionally that a portion of EpDRF activity may be due to CO and that the release of EpDRF does not involve cytoskeletal reorganization.

The airway epithelium is involved in the regulation of airway reactivity to bronchoactive agents through the release of epithelium-derived relaxing factor (EpDRF). The existence of EpDRF has been demonstrated in vitro in coaxial bioassay preparations, in which EpDRF released from airway epithelium by methacholine (MCh) or histamine elicits relaxation of recipient vascular or other nonrespiratory smooth muscles (Ilhan and Sahin, 1986; Fernandes et al., 1989). Despite

several attempts using pharmacological agents, the chemical identity of EpDRF released by contractile agonists has not been established (Fernandes et al., 1989; Fernandes and Goldie, 1990; Spina et al., 1992).

In the guinea pig isolated perfused trachea preparation, application of hyperosmolar<sup>1</sup> solution to the mucosal surface elicits a relaxation of the airway smooth muscle, which is mediated by EpDRF (Munakata et al., 1988; Fedan et al., 1990). Evidence from two laboratories has indicated that the EpDRF released by hyperosmolar solution is neither a prostanoid nor nitric oxide (Munakata et al., 1990; Fedan et al.,

This work was supported, in part, by National Institutes of Health Grant 5-T32-GM07039 (to R.A.J.). Mention of brand name does not constitute product endorsement. This article is the third one of a series of four companion articles that report the effects of hyperosmolar solutions in guinea pig airways (Fedan et al., 2003; Johnston et al., 2003; Wu et al., 2003).

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

DOI: 10.1124/jpet.103.051664.

<sup>1</sup> Hypertonic solutions are those that cause cell shrinkage. Hyperosmolar solutions have osmolarity greater than that of the physiological extracellular solution. For simplicity, in this report we do not draw distinctions between the two terms when describing general phenomena.

**ABBREVIATIONS:** EpDRF, epithelium-derived relaxing factor; MCh, methacholine; D-M, D-mannitol; MKHS, modified Krebs-Henseleit solution; ZnPP, zinc (II) protoporphyrin IX; CrMP, chromium (III) mesoporphyrin IX; Rp-8-Br-cGMPS, 8-bromoguanosine 3',5'-cyclic monophosphothioate, Rp-isomer; CO, carbon monoxide; HO, heme oxygenase;  $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ , tricarbonyldichlororuthenium (II) dimer; PK, protein kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; EGF, epidermal growth factor; LY83583, 6-anilino-5,8-quinolinequinone; MK 571, (E)-3-[[[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl]-(3-(dimethylamino)-3-oxopropyl]thio)methyl]thio]-propanoic acid.

1999; Johnston et al., 2003), although an inhibitory effect of hemoglobin but not of methylene blue or inhibitors of nitric oxide synthase suggested that EpDRF release by hyperosmolar solution has features resembling nitric oxide (Munakata et al., 1990; Fedan et al., 1999). It is not known whether the EpDRF released by contractile agonists in the coaxial bioassay preparation, and that released by hyperosmolar solution in the perfused trachea, are the same substance. It has been suggested that the epithelium may release two substances, one to which vascular smooth muscle is sensitive and the other to which airway smooth muscle is sensitive (Fedan et al., 1990).

Previous experiments have suggested (Fedan et al., 2003; Wu et al., 2003) that EpDRF is released in response to incremental changes in osmolarity as opposed to absolute osmolarity of solutions bathing the epithelium. Furthermore, the release of EpDRF is associated with changes in bioelectric activity of the epithelium (Dortch-Carnes et al., 1999; Wu et al., 2003). In the present study, a panel of agents were examined for their effects on relaxation responses to hyperosmolar solution, to gain insight into the identity of EpDRF. Some of these were chosen because they have been shown to interfere with cell volume regulation in other cell types (Lang et al., 1998), which is an important component of a cell's response to hyperosmolar challenge.

## Materials and Methods

**Animals.** These studies were conducted in facilities accredited fully by the Association for the Assessment and Accreditation of Laboratory Animal Care International and were approved by the institutional Animal Care and Use Committee. The animals were anesthetized with sodium pentobarbital (65 mg/kg i.p.) and sacrificed by thoracotomy and bleeding before removing the trachea. Other details of animal use have been given (Fedan et al., 2003).

**Isolated Perfused Trachea Preparation.** The isolated perfused trachea preparation was used to measure responses of the smooth muscle elicited by challenge of the epithelium with hyperosmolar solution, and other drug effects. This preparation permits separate application of agents to the mucosal (intraluminal) or serosal (extraluminal) surfaces of the trachea. The method has been described in detail previously (Fedan and Frazer, 1992).

**Effect of Various Agents on Relaxation Responses to Hyperosmolar Solution.** At the conclusion of the equilibration period, the perfused tracheas were contracted with extraluminally applied MCh ( $3 \times 10^{-7}$  M; approximate extraluminal  $EC_{50}$ ). At the plateau of the response, D-mannitol (D-M) was added to the intraluminal modified Krebs-Henseleit solution (MKHS) to evoke a control relaxation response. The concentration of D-M was 120 mosM for every agent except gossypol, in which case the D-M concentration was 160 mosM, to be consistent with a study by Teeter et al. (1988). The tracheas were then washed intraluminally and extraluminally with MKHS at 15-min intervals for 1.5 h. During this period the agent undergoing evaluation was added to the intraluminal and/or extraluminal baths for incubation periods described in Table 1. At the end of this period, the trachea was contracted a second time with extraluminal MCh, and D-M was readded to the intraluminal perfusing solution. Separate control tracheas served as time or vehicle controls. The vehicles in the volumes used to dissolve the agents had no effects, as verified in separate experiments ( $n = 4$  or greater for each vehicle; not shown).

Particular attention was paid to whether a given agent itself caused an effect when added to the preparation. In addition, some agents were examined for their ability to evoke responses by applying them directly to MCh-contracted preparations. These will be described under *Results*.

**Drugs and Reagents.** Zinc (II) protoporphyrin IX (ZnPP) and chromium (III) mesoporphyrin IX chloride (CrMP) were from Porphyrin Products (Logan, UT). MK 571 was from Cayman Chemicals (Ann Arbor, MI). Glibenclamide was from Sigma/RBI (Natick, MA). Proadifen (SKF525A) was from BIOMOL Research Laboratories (Plymouth Meeting, PA). Erythro-9-(2-hydroxyl-3-nonyl)adenine was from Calbiochem (San Diego, CA). Rp-8-Br-cGMPS was from Axxora (San Diego, CA). All other drugs and reagents were from Sigma-Aldrich (St. Louis, MO). The synthesis of heme-L-lysinate was modified from Tenhunen et al. (1987), according to J. S. Naik (University of New Mexico, Albuquerque, NM), who kindly provided the procedure.

**MKHS.** MKHS contained 113.0 mM NaCl, 4.8 mM KCl, 2.5 mM  $CaCl_2$ , 1.2 mM  $KH_2PO_4$ , 1.2 mM  $MgSO_4$ , 25.0 mM  $NaHCO_3$ , and 5.7 mM glucose, pH 7.4 (37°C); gassed with 95%  $O_2$ , 5%  $CO_2$ . The osmolarity of MKHS was  $281 \pm 5$  mosM.  $CaCl_2$  was omitted from  $Ca^{2+}$ -free MKHS.

**Analysis of Results.** The results are expressed as mean  $\pm$  S.E.;  $n$  is the number of separate experiments. The results were analyzed for differences using Student's *t* test for paired samples.  $p < 0.05$  was considered significant.

## Results

**Effects of Agents on Responses to Intraluminal Hyperosmolarity.** A number of agents were used to obtain pharmacological evidence for the identity of EpDRF by examining their ability to inhibit hyperosmolarity-induced relaxations or to mimic relaxation responses to hyperosmolar solution. The agents examined, the experimental conditions used, and their effects are summarized in Table 1.

ATP and UTP are released from epithelial cells and are involved in cell-to-cell communication (Homolya et al., 2000). ATP and its breakdown product adenosine relax airway smooth muscle; UTP is a weak relaxant (Fedan et al., 1993). The possibility that ATP or adenosine could mediate relaxation to hyperosmolar solution was tested using adenosine and apyrase, which catabolizes ATP. The concentration of apyrase used, 10 U/ml, inhibits intercellular communication mediated by the nucleotides (Homolya et al., 2000). Although both agents elicited contractile responses initially, neither inhibited relaxation to D-M. EpDRF is not likely to be an adenine nucleotide or breakdown product.

Recognition of the vascular and airway smooth muscle relaxant effects of carbon monoxide (CO; Maines, 1997; Villamor et al., 2000; Kinhult et al., 2001) prompted an examination of its possible role in hyperosmolarity-induced relaxation. Hemoglobin (ferrous), which scavenges CO with high affinity, evoked a modest contraction when added to the baths, which could suggest basal release of CO. Hemoglobin had no effect on the transient contractile phase of response to D-M (Fedan et al., 2003; not shown) but inhibited the relaxation to D-M (Fig. 1). Inhibition of heme oxygenase (HO), the enzyme that gives rise to CO, with ZnPP, also had no effect on the transient contraction but inhibited the relaxation response to D-M (Fig. 1). Identical findings were obtained when experiments with ZnPP were performed under dark conditions ( $n = 3$ ; Zygmunt et al., 1994; not shown). Inhibition by ZnPP occurred only when it was present in both the intraluminal and extraluminal baths. These findings provide provisional support for the release of CO in response to hyperosmolarity. However, a second inhibitor of HO, CrMP, was without effect on responses to D-M, even when present in both baths. If CO is released from epithelium in response to

TABLE 1  
Effect of agents on D-M-induced relaxation responses

Class/Agent/Osmolyte	Concentration	Bath	Incubation	n	Agent Effect	Effect on Response to D-M
	<i>M</i>		<i>min</i>			
Purine						
Apyrase	10 U/ml <sup>a</sup>	IL + EL	30	3	Small contraction	None
Adenosine	10 <sup>-4</sup>	IL	30	5	Contraction	None
CO system						
Hemoglobin	10 <sup>-6</sup>	IL + EL	30	6	Contraction	Inhibition <sup>c</sup>
ZnPP	10 <sup>-4</sup>	IL + EL	60	7	None	Inhibition <sup>c</sup>
	10 <sup>-4</sup>	IL	60	7	None	None
	10 <sup>-4</sup>	EL	60	6	Contraction	None
CrMP	10 <sup>-4</sup>	IL + EL	60	4	Contraction	None
[Ru(CO) <sub>3</sub> Cl <sub>2</sub> ] <sub>2</sub>	2.22 × 10 <sup>-4</sup>	IL	Addition <sup>b</sup>	2	Small, transient relaxation	
	2.22 × 10 <sup>-4</sup>	EL	Addition <sup>b</sup>	2	Small, transient relaxation	
LY83583	10 <sup>-5</sup>	IL + EL	30	5	None	None
Heme-l-lysinate	3 × 10 <sup>-4</sup>	IL + EL	Addition <sup>b</sup>	8	None	None
Rp-8-Br-cGMPS	10 <sup>-6</sup>	IL	30	1	None	None
	2 × 10 <sup>-6</sup>	IL	30	1	None	None
	10 <sup>-4</sup>	IL	30	1	None	None
Mediator						
Proadifen (SKF525A)						
D-M	5 × 10 <sup>-5</sup>	IL	30	7	Transient contraction	None
NaCl	5 × 10 <sup>-5</sup>	IL	30	7	Transient contraction	None
MK 571	10 <sup>-6</sup>	IL + EL	30	7	None	None
Diphenhydramine	10 <sup>-7</sup>	IL + EL	30	4	None	None
Thiorphan	10 <sup>-5</sup>	IL	30	3	None	None
Cytoskeleton disruptor						
EHNA	5 × 10 <sup>-4</sup>	IL + EL	30	4	None; inhibited MCh response	None
Colchicine	2 × 10 <sup>-4</sup>	IL	60	4	None	None
Nocodazole	2 × 10 <sup>-5</sup>	IL	30	5	None	None
Cytochalasin B	5 × 10 <sup>-7</sup>	IL	30	4	Inhibited MCh response	None
Cytochalasin D	5 × 10 <sup>-7</sup>	IL	30	4	None	None
Latrunculin B	5 × 10 <sup>-6</sup>	IL	30	4	See text; inhibited MCh response	Potentiation
Channel blocker						
Glibenclamide	10 <sup>-4</sup>	IL + EL	30	5	None	None
NPPB	10 <sup>-4</sup>	IL	60	8	Small relaxation	Inhibition
HgCl <sub>2</sub>	10 <sup>-4</sup> and 10 <sup>-5</sup>	IL	30	8	Inhibited MCh response	None
Tetrodotoxin	10 <sup>-6</sup>	IL + EL	30	4	None	None
Membrane permeability						
Nystatin						
D-M	2.6 × 10 <sup>-4</sup>	IL	60	6	Large contraction	Potentiation
KCl	2.6 × 10 <sup>-4</sup>	IL	60	6	Large contraction	Potentiation
α-Hemolysin	100 <sup>a</sup>	IL	30	4	None	None
Cell signaling						
TPA						
After MCh	10 <sup>-7</sup>	IL	30	2	None	None
	2 × 10 <sup>-7</sup>	IL	30	2	None	None
Before MCh	10 <sup>-7</sup>	IL	120	4	Sustained contraction	None
Cholera toxin	250 <sup>d</sup>	IL	120	4	None	None
Pertussis toxin	100 <sup>d</sup>	IL	30	5	None	None
Ryanodine	10 <sup>-6</sup>	IL	30	4	None	None
Thapsigargin + Ca <sup>2+</sup> -free MKHS	10 <sup>-6</sup>	IL	30 <sup>e</sup>	3	Contraction	None
Ca <sup>2+</sup> -free MKHS		IL	60	2	Increased baseline	None
Miscellaneous						
Gossypol	5 × 10 <sup>-6</sup>	IL + EL	60	5	None	Inhibition <sup>c</sup>
Hydrocortisone	10 <sup>-5</sup>	IL + EL	30	4	None	None
EGF	100 <sup>a</sup>	IL	60	4	None	None

EL, extraluminal; IL, intraluminal.

<sup>a</sup> Concentration in units per milliliter.

<sup>b</sup> These agents were applied to the MCh-contracted preparations to determine whether a response could be generated.

<sup>c</sup> See Fig. 1.

<sup>d</sup> Concentration in nanograms per milliliter.

<sup>e</sup> Added in the presence of Ca<sup>2+</sup>-free MKHS, which had been 60 min earlier.

hyperosmolarity, the effect should be mimicked by CO. To test this possibility, the effects of the CO-releasing molecule tricarbonyldichlororuthenium (II) dimer ([Ru(CO)<sub>3</sub>Cl<sub>2</sub>]<sub>2</sub>) (Matterlini et al., 2002) were examined. [Ru(CO)<sub>3</sub>Cl<sub>2</sub>]<sub>2</sub> elicited small, transient relaxations when applied to the intraluminal and extraluminal baths. CO stimulates guanylate cyclase, leading to the formation of cyclic GMP and activation of protein kinase G (PKG; Brann et al., 1997; Maines, 1997), which could relax

smooth muscle (Furchgott and Jothianandan, 1991). Under this scenario, the relaxant effect of released CO should be inhibited by blocking guanylate cyclase. The guanylate cyclase inhibitor LY83583 when added bilaterally, and the PKG inhibitor (Homer and Wanstall, 2000) Rp-8-Br-cGMPS added intraluminally, had no effect on their own and did not affect relaxation responses to hyperosmolarity. The HO substrate heme-L-lysinate has been reported to

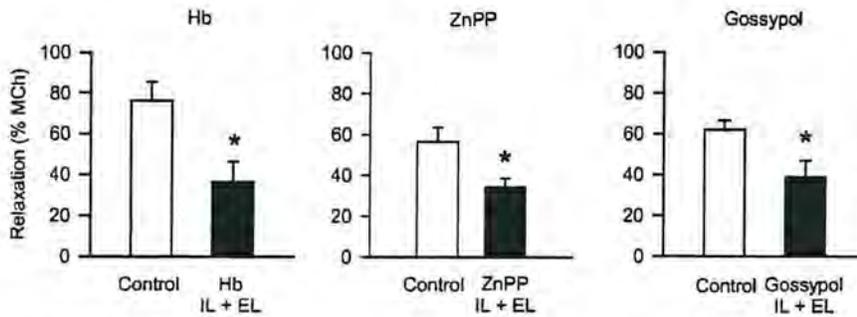


Fig. 1. Effect of  $10^{-6}$  M hemoglobin (Hb; 30 min; left),  $10^{-4}$  M ZnPP (60 min; middle), and  $5 \times 10^{-6}$  M gossypol (60 min; right) on relaxation responses elicited with D-M. The preparations had been contracted first with  $3 \times 10^{-7}$  M MCh. The agents were incubated in the intraluminal (IL) and extraluminal (EL) baths.  $n = 6, 7,$  and  $5$  for hemoglobin, ZnPP, and gossypol, respectively. The changes shown here were not seen in vehicle control experiments run in parallel. \*, significantly less than control.

evoke CO-like relaxation and hyperpolarization in mesenteric arteriolar smooth muscle from hypoxic rats that is blocked by ZnPP (Naik and Walker, 2002). However, administration of heme-L-lysinate to the intraluminal and extraluminal baths to drive CO synthesis from heme caused no response.

A series of agents were examined to determine whether responses to hyperosmolarity could involve a mediator known or postulated to exist in other organ systems or processes. Responses of vascular muscle to endothelium-derived hyperpolarizing factor are inhibited by the cytochrome P<sub>450</sub> inhibitor proadifen (SKF525A; Eckman et al., 1998). This agent, however, did not antagonize responses to D-M or hyperosmolar NaCl, suggesting that arachidonic acid epoxides are not mediators of the response. Because histamine and leukotrienes are viewed to be important mediators in exercise-induced asthma, the effects of the H<sub>1</sub>-histamine receptor antagonist diphenhydramine and the CysLT<sub>1</sub>-receptor antagonist MK 571 were examined, even though there is little likelihood that these contractile substances would mediate relaxation. These blockers had no effect, suggesting that these substances do not serve as intermediaries of the response to hyperosmolar solution, at least in vitro.

Application of hyperosmolar solution to airway epithelium results in cell shrinkage (for review, see Fedan et al., 2003). Cell volume changes and the resulting compensatory changes in ion transport activity (Rehn et al., 1998) involve the cellular cytoskeleton (Lang et al., 1998). Inasmuch as the relaxant and bioelectric responses to hyperosmolarity are affected by inhibition of ion transport (Fedan et al., 1999; Wu et al., 2003), we examined the effects of cytoskeleton disruptors. Erythro-9-(2-hydroxy-3-nonyl)adenine, a cytoskeleton inhibitor that also inhibits phosphodiesterase II and adenosine deaminase, inhibited the contraction to MCh but had no effect on relaxation to D-M. Furthermore, colchicine, nocodazole, cytochalasin B, and cytochalasin D had no effect when added to the baths, nor did they affect relaxation to D-M. Latrunculin B had several effects: 1) it caused a transient contraction in half the preparations, 30 to 40% of the size of the MCh-induced contraction, and a slight decrease in basal  $\Delta P$  in the remaining tracheas; 2) it subsequently inhibited the contraction to MCh; and 3) it potentiated the relaxant response to D-M. Thus, the results obtained using these six blockers failed to reveal a definitive link between cytoskeleton reorganization and EpDRF release in epithelial cells.

The ATP-sensitive K<sup>+</sup>-channel blocker glibenclamide had no effect itself nor did it affect relaxation responses to D-M. It has been observed previously that glibenclamide had no effect on EpDRF release (Tamaoki et al., 1997). The Cl<sup>-</sup> channel blocker 5-nitro-2-(3-phenylpropylamino)benzoic acid in-

hibited significantly the relaxant response to D-M, in the manner described earlier for 4,4'-diisothiocyano-2,2'-stilbene disulfonate (Fedan et al., 1999).

Cell shrinkage in response to hypertonic challenge is associated with cellular water loss (Lang et al., 1998) through aquaporins (Agre et al., 2002). We evaluated the effects of HgCl<sub>2</sub>, an aquaporin inhibitor, to determine whether blockade of water movement could affect the responses of the epithelium to hyperosmolarity. This experiment was done in two ways: in the first, the trachea was incubated with HgCl<sub>2</sub> in the mucosal bath for 30 min before MCh was added; in the second, HgCl<sub>2</sub> was added to the bath at the plateau of the MCh-induced contraction. Two concentrations of HgCl<sub>2</sub> were studied. The higher one ( $10^{-4}$  M), which is used widely in studies investigating aquaporins, led to rapidly occurring complex responses of uncontracted tracheas and blunted contractions to MCh. In the contracted tracheas, administration of HgCl<sub>2</sub> led to a reduction in the contraction. It was thus difficult to determine quantitatively whether HgCl<sub>2</sub> affected the response to D-M; however, relaxation responses were still observed. Using the lower concentration of HgCl<sub>2</sub>,  $10^{-5}$  M, the contractile effects were delayed in onset and smaller in magnitude using both protocols. The effect of HgCl<sub>2</sub> was, once again, difficult to quantify because of the deleterious effect of the metal, but relaxation responses to D-M still occurred. In Ussing chamber experiments (D. Wu and J.S. Fedan, unpublished observations) on guinea pig cultured epithelial cells, HgCl<sub>2</sub> ( $10^{-4}$  M) applied to the mucosal or serosal baths caused a rapidly occurring, complex-shaped decrease in short circuit current ( $I_{sc}$ ) and a decrease in trans-epithelial resistance ( $R_t$ ;  $n = 4$ ). In freshly isolated guinea pig tracheal segments,  $10^{-5}$  M HgCl<sub>2</sub> applied to the mucosal bath increased or decreased  $I_{sc}$  and decreased  $R_t$  ( $n = 4$ ). It would seem that epithelial aquaporins may play a role in the epithelium-smooth muscle axis and affect muscle activity, but the role of aquaporins in the D-M-induced relaxation was judged to be small or nonexistent.

Nystatin, which increases cation permeability of cell membranes (Akaike and Harata, 1994), and  $\alpha$ -hemolysin, a pore-forming protein (Panchal et al., 2002), were used to permeabilize the epithelial apical membrane. Upon addition to the bath, nystatin evoked a large and long-lasting contraction, which faded somewhat over 30 to 45 min, but not to baseline. We take this as evidence that cation permeability in the epithelium was increased, but the mechanism of the contraction is not known. The total force achieved upon the addition of MCh was higher than the control response, and the relaxation response to D-M was potentiated significantly. If nystatin affected apical membrane K<sup>+</sup> permeability, responses to hyperosmolarity achieved by addition of KCl to the bath

should differ from those obtained with D-M; however, relaxation responses to KCl also were potentiated.  $\alpha$ -Hemolysin had no effect when added to the intraluminal bath and did not affect the relaxation response. Thus, a nonselective increase in cation permeability or pore formation at the apical epithelial membrane did not inhibit the relaxation response to hyperosmolarity.

Gossypol, an agent with diverse pharmacological effects, had no effect when added to both baths or on the transient contraction, but it caused a significant reduction in the relaxation to D-M (Fig. 1).

In guinea pigs, application of hypertonic solution to airways results in activation of sensory nerve ending reflexes (Fox et al., 1995). The possibility that local axon reflexes might have been activated by hyperosmolarity to cause the release of a relaxant mediator(s) from such nerve endings is not likely, inasmuch as the response to D-M was unaffected by tetrodotoxin.

Agents that inhibit cell signaling were examined for their effects. When applied at the plateau of the MCh-induced contraction, activation of PKC with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) had no effect itself on the response to D-M. Added before MCh, however, TPA elicited a sustained contraction but the response to D-M was unaffected. Likewise, cholera and pertussis toxins, which catalyze the ADP-ribosylation of G proteins, elicited no response upon challenge of the epithelium and did not modify the response to D-M. Thus, it is unlikely that EpDRF release involves protein kinases C or A.

Intraluminal  $\text{Ca}^{2+}$ -free MKHS and thapsigargin ( $10^{-6}$  M) were used to deplete intracellular  $\text{Ca}^{2+}$  stores. Intraluminal  $\text{Ca}^{2+}$ -free MKHS raised basal tone, and addition of thapsigargin caused a substantial contraction. D-M-induced relaxation was not affected by intraluminal  $\text{Ca}^{2+}$ -free MKHS alone or in combination with thapsigargin. Although interfering with  $\text{Ca}^{2+}$  in the epithelium may influence the modulatory effect of the epithelium on smooth muscle tone, neither intracellular nor extracellular  $\text{Ca}^{2+}$ , at the mucosal surface, are required for EpDRF release.

The anti-inflammatory glucocorticoid hydrocortisone had no effect itself and did not alter D-M-induced relaxation responses.

Hyperosmolar challenge induces epidermal growth factor (EGF) expression in smooth muscle cells (Koh et al., 2001), and EGF pathways may be involved in the response of cells to hyperosmolar stress (Sheikh-Hamad et al., 2000). However, activation of EGF receptors with ligand had no effect and did not influence the response to D-M.

## Discussion

Challenge of the epithelium with hyperosmolar solution releases EpDRF. To gain insight into the nature of EpDRF and the mechanisms involved in its release, we examined the effects of a diverse array of agents on D-M-induced relaxations. Only hemoglobin, ZnPP, and gossypol had inhibitory effects, and the inhibition was not complete.

HO is expressed in the epithelium and nerves of human and guinea pig large airways (Undem et al., 1996; Donnelly and Barnes, 2001) and may modulate reactivity of airway smooth muscle to agonists, such as carbachol (Samb et al., 2002). In addition, HO is thought to be involved in protection

of liver and kidney cells from hyperosmolar stress (Lordnejad et al., 2001; Tian et al., 2001). The increase in basal tone upon addition of hemoglobin, as well as the inhibition of relaxant responses to hyperosmolarity, which confirms the findings of Munakata and coworkers (Munakata et al., 1990), is evidence in support of a role of CO in the unstimulated trachea and in the relaxation response to hyperosmolar solution. Likewise, HO inhibition with ZnPP also inhibited the relaxant responses. Such findings in other tissues have been taken as evidence of a physiological role of CO (Wang et al., 1997; Rattan and Chakder, 2000). ZnPP was effective only when it was present in both the intraluminal and the extraluminal baths, and CrMP, which is more selective for HO than nitric-oxide synthase in biochemical studies (Appleton et al., 1999), was without effect when present in both baths. The reasons for the different effects of ZnPP and CrMP are not known. The requirement for ZnPP to be present in both baths could signify that the enzyme inhibitor reached a sufficient intracellular concentration only under this condition. If this is correct, then perhaps CrMP did not reach a sufficient level to inhibit the enzyme, perhaps because it cannot penetrate into the epithelial cells as readily as ZnPP. We could identify no investigations describing the cellular uptake characteristics of these two HO inhibitors. Canning and Fischer (1998) noted that HO II is present in airway neurons, but that ZnPP did not affect cholinergic contractions of guinea pig trachea. In preliminary experiments, we tested the possibility that hemoglobin and ZnPP together might result in an additive inhibitory effect; however, the two agents precipitated when present in the same bath. Caution is needed when using protoporphyrin compounds to identify a role of CO, because they have inhibitory effects that are unrelated to inhibition of HO (Grundemar and Ny, 1997).

The CO donor  $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$  failed to elicit large relaxation responses of the magnitude caused by D-M, but weak relaxations did occur. This could have resulted from a failure of the compound to liberate appreciable CO under our experimental conditions or from an insensitivity of the guinea pig tracheal muscle to respond to CO. The effect of  $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$  in our experiments provides support, albeit weak, that hyperosmolar solution liberates CO from the epithelium.

The lack of inhibition of relaxant responses by the guanylate cyclase inhibitor LY83583 and the PKG inhibitor Rp-8-Br-cGMPs, and the inability of HO substrate heme-L-lysinate to evoke relaxation, is evidence against the notion that CO is an important substance in the regulation of airway diameter in the guinea pig trachea. Future experiments will examine the efficacy of pure CO gas as a relaxant.

These results suggest that EpDRF is scavenged by hemoglobin (Munakata et al., 1990) and that its production may be linked in some way to HO activity. However, the issue of whether CO is a component of EpDRF has not been resolved unequivocally by our experiments. It is feasible that CO plays a secondary role in airway relaxation.

Gossypol inhibited the relaxation to hyperosmolar D-M, in agreement with the preliminary observation of Teeter et al. (1988). The mechanism by which this effect occurred is not clear, because gossypol inhibits endothelium-dependent relaxation factor-mediated relaxation (Radermacher et al., 1990), lipoxygenase (Kulkarni and Sajan, 1997), phospholipase  $A_2$  (Soubeyrand et al., 1997), protein kinase C (Pelosin et al., 1990), gap junctions (Ye et al., 1990), and  $\text{Ca}^{2+}$  chan-

nels (Sgaragli et al., 1993; Bai and Shi, 2002). Of relevant importance, gossypol inhibits channels and transporters that are involved in cell volume regulation, i.e., taurine channels (Ballatori et al., 1995), myo-inositol uptake (Strange et al., 1993) and volume-activated  $\text{Cl}^-$  channels that are also sensitive to 4,4'-diisothiocyano-2,2'-stilbene disulfonate (Gschwentner et al., 1996; Szucs et al., 1996), an agent that blocks EpDRF-induced relaxation (Fedan et al., 1999). It is tempting to speculate that our results are explained by inhibition of epithelial  $\text{Cl}^-$  channels. Gossypol could represent the starting point for exploration of agents that block EpDRF release and/or action. It is very interesting that gossypol did not affect MCh- and histamine-induced relaxation responses of rat aortic strips placed in coaxial arrangements inside guinea pig tracheal tubes (Fernandes and Goldie, 1990), which supports the postulate that the epithelium may release two EpDRF substances, one that is released by the receptor-acting agonists and affects nonairway smooth muscle, and one that is released by hyperosmolar solution which affects airway smooth muscle (Fedan et al., 1990).

In summary, our experiments led to two main conclusions. First, CO plays a role, albeit minor, in EpDRF-mediated airway smooth muscle relaxation in response to hyperosmolar solution. This conclusion is somewhat provisional because evidence obtained with some agents argues against it. Additional investigation is required to understand in greater detail the precise mechanisms of action, and effectiveness, of some of the agents used, i.e., CrMP and heme-L-lysinate. Second, evidence was obtained that the release of EpDRF by hyperosmolar solution does not involve reorganization of the epithelial cytoskeleton, even though shrinkage of the epithelium occurs in response to hyperosmolarity. Experiments currently underway will define whether the cytoskeleton disruptors interfere with cell volume changes in response to hyperosmolar solutions under these conditions.

#### Acknowledgments

We are grateful to Deborah C. Sbarra and Nicole Diotte for technical assistance, and J. Naik (University of New Mexico) for providing the protocol for the preparation of heme-L-lysinate.

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