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Hyperosmolar solution effects in guinea-pig airways. III. Studies on the identity of epithelium-derived relaxing factor in isolated, perfused trachea using pharmacological agents[1]

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Abbreviations: EpDRF, epithelium-derived relaxing factor; MKHS, modified Krebs-Henseleit solution; DIDS, 4,4'-diisothiocyano-2,2'-stilbene disulfonate; MCh, methacholine; HO, heme oxygenase; NO, nitric oxide; D-M, D-mannitol; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; EHNA, erythro-9-(2-hydroxyl-3-nonyl)adenine; ZnPP, zinc (II) protoporphyrin IX; CrMP, chromium (III) mesoporphyrin IX; PKG, protein kinase G; EGF, epidermal growth factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; $[Ru(Co)_3Cl_2]_2$, tricarbonyldichlororuthenium (II) dimer; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid

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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(3×10^{-7} M)-contracted guinea-pig perfused trachea to mucosal hyperosmolar challenge using D-mannitol. Responses were inhibited by gossypol (5×10^{-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 (3×10^{-4} M) did not evoke relaxant responses. The CO donor, tricarbonyldichlororuthenium (II) dimer (2.2×10^{-4} M) elicited small relaxation responses. Other agents without an effect on responses included: apyrase, adenosine, LY83583, proadifen, MK571, diphenhydramine, glibenclamide, $HgCl_2$, tetrodotoxin, nystatin, α-hemolysin, Rp-8-Br-cGMPS, 12-O-tetradecanoylphorbol-13-acetate, cholera toxin, pertussis toxin, thapsigargin, nifedipine, Ca^{2+}-free mucosal solution, hydrocortisone and epidermal growth factor. Cytoskeleton inhibitors, including erythro-9-(2-hydroxyl-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 co-axial bioassay preparations, in which EpDRF released from airway epithelium by methacholine (MCh) or histamine elicits relaxation of recipient vascular or other non-respiratory 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[2] 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 (NO; Munakata et al., 1990; Fedan et al., 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 NO (Munakata et al., 1990; Fedan et al., 1999). It is not known whether the EpDRF released by contractile agonists in the co-axial 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, 1999; Wu et al., 2003). In the present study, a panel of agents was 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.

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, ip) 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 previously in detail (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 (3×10^{-7} M; ~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, in order to be consistent with a study by Teeter and colleagues (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

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second time with extraluminal MCh, and D-M was re-added 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 in 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 RBI (Natick, MA). Proadifen (SKF525A) was from BIOMOL Research Labs, Inc. (Plymouth Meeting, PA). Erythro-9-(2-hydroxyl-3-nonyl)adenine (EHNA) was from Calbiochem (San Diego, CA). Rp-8-Br-cGMPS was from Axxora (San Diego, CA). All other drugs and reagents were from Sigma Chemical Co. (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, who kindly provided the procedure.

MKHS. MKHS 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), pH 7.4 (37°C); gassed with 95% O_2 - 5% CO_2. The osmolarity of MKHS was 281±5 mosM. $CaCl_2$ was omitted from Ca^{2+}-free MKHS.

Analysis of results. The results are expressed as mean ± SE; 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). While 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 which gives rise to CO, with zinc (II)

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protoporphyrin IX (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, chromium (III) mesoporphyrin IX (CrMP), was without effect on responses to D-M, even when present in both baths. If CO is released from epithelium in response to hyperosmolarity, the effect should be mimicked by CO. To test this possibility, the effects of the CO-releasing molecule, tricarbonyldichlororuthenium (II) dimer {$[Ru(Co)_3Cl_2]_2$}(Motterlini et al., 2002), were examined. $[Ru(Co)_3Cl_2]_2$ 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 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 was examined to determine if 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 (EDHF) are inhibited by the cytochrome P_{450} 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_1-histamine receptor antagonist, diphenhydramine, and the $CysLT_1$-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 (see Fedan et al., 2003 for review). 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-hydroxyl-3-nonyl)adenine (EHNA), a cytoskeleton inhibitor which 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 $\triangle P$ 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 re-organization and EpDRF release in epithelial cells.

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The ATP-sensitive K^+-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^- channel blocker, NPPB, inhibited significantly the relaxant response to D-M, in the manner described earlier for 4,4'-diisothiocyano-2,2'-stilbene disulfonate (DIDS; 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 $HgCl_2$, an aquaporin inhibitor, to determine if 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 $HgCl_2$ in the mucosal bath for 30 min before MCh was added; in the second, $HgCl_2$ was added to the bath at the plateau of the MCh-induced contraction. Two concentrations of $HgCl_2$ 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 $HgCl_2$ led to a reduction in the contraction. It was thus difficult to determine quantitatively whether $HgCl_2$ affected the response to D-M; however, relaxation responses were still observed. Using the lower concentration of $HgCl_2$, 10^{-5} M, the contractile effects were delayed in onset and smaller in magnitude using both protocols. The effect of $HgCl_2$ 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, $HgCl_2$ (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 transepithelial resistance (R_t; $n = 4$). In freshly-isolated guinea-pig fresh tracheal segments, 10^{-5} M $HgCl_2$

applied to the mucosal bath increased or decreased I_{sc} and decreased R_t (n = 4). It would appear 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 α-hemolysin, a pore-forming protein (Panchal et al., 2002), were employed 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^+ 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. α-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 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 which inhibit cell signaling were examined for their effects. When applied at the plateau of the MCh-induced contraction, activation of protein kinase C (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-ribosylaton 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 Ca^{2+}-free MKHS and thapsigargin (10^{-6} M) were used to deplete intracellular Ca^{2+} stores. Intraluminal Ca^{2+}-free MKHS raised basal tone, and addition of thapsigargin caused a substantial contraction. D-M-induced relaxation was not affected by intraluminal Ca^{2+}-free MKHS alone or in combination with thapsigargin. While interfering with Ca^{2+} in the epithelium may influence the modulatory effect of the epithelium on smooth muscle tone, neither intracellular or extracellular 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.

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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 co-workers (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 co-workers (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, $[Ru(CO)_3Cl_2]_2$, 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 $[Ru(CO)_3Cl_2]_2$ 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 or not CO is a component of EpDRF has not been resolved unequivocally by our

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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 and co-workers (Teeter et al., 1988). The mechanism by which this effect occurred is not clear, as 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 Ca^{2+} channels (Sgaragli et al., 1993; Bai and Shi, 2002). Of relevant importance, gossypol inhibits channels and transporters which are involved in cell volume regulation, i.e., taurine channels (Ballatori et al., 1995), *myo*-inositol uptake (Strange et al., 1993) and volume-activated Cl^- channels which are also sensitive to DIDS (Gschwentner et al., 1996; Szucs et al., 1996), an agent which blocks EpDRF-induced relaxation (Fedan et al., 1999). It is tempting to speculate that our results are explained by inhibition of epithelial 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 co-axial 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 non-airway 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 employed, i.e., CrMP and heme-L-lysinate. Evidence was obtained that the release of EpDRF by hyperosmolar solution does not involve re-organization 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.

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Footnotes

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[1]Mention of brand name does not constitute product endorsement. This paper is the third one of a series of four companion papers which report the effects of hyperosmolar solutions in guinea-pig airways (Fedan et al., 2003; Johnston et al., 2003; Wu et al., 2003).

[2]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 will not draw distinctions between the two terms when describing general phenomena.

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Figure Legends

Fig 1. Effect of 10^{-6} M hemoglobin (Hb; 30 min; left panel), 10^{-4} M ZnPP (60 min; middle panel), and 5×10^{-6} M gossypol (60 min; right panel) on relaxation responses elicited with D-M. The preparations had been contracted first with 3×10^{-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.

TABLE 1

Effect of agents on D-M-induced relaxation responses.

Class/Agent/Osmolyte	Concentration (M)	Bath	Incubation (min)	*n*	Agent Effect	Effect on response to D-M
Purine						
Apyrase	10 U/ml[a]	IL + EL	30	3	Small contraction	None
Adenosine	10^{-4}	IL	30	5	Contraction	None
CO system						
Hemoglobin	10^{-6}	IL + EL	30	6	Contraction	Inhibition[c]
ZnPP	10^{-4}	IL + EL	60	7	None	Inhibition[c]
	10^{-4}	IL	60	7	None	None
	10^{-4}	EL	60	6	Contraction	None
CrMP	10^{-4}	IL + EL	60	4	Contraction	None
$[Ru(CO)_3Cl_2]_2$	2.22×10^{-4}	IL	Addition[b]	2	Small, transient relaxation	—
	2.22×10^{-4}	EL	Addition[b]	2	Small, transient relaxation	—
LY83583	10^{-5}	IL + EL	30	5	None	None
Heme-L-lysinate	3×10^{-4}	IL + EL	Addition[b]	8	None	—
Rp-8-Br-cGMPS	10^{-6}	IL	30	1	None	None
	2×10^{-6}	IL	30	1	None	None

	10^{-4}	IL	30	1	None	None
Mediator						
Proadifen (SKF525A)						
D-M	5×10^{-5}	IL	30	7	Transient contraction	None
NaCl	5×10^{-5}	IL	30	7	Transient contraction	None
MK571	10^{-6}	IL + EL	30	7	None	None
Diphenhydramine	10^{-7}	IL + EL	30	4	None	None
Thiorphan	10^{-5}	IL	30	3	None	None
Cytoskeleton disruptor						
EHNA	5×10^{-4}	IL + EL	30	4	None; inhibited MCh response	None
Colchicine	2×10^{-4}	IL	60	4	None	None
Nocodazole	2×10^{-5}	IL	30	5	None	None
Cytochalasin B	5×10^{-7}	IL	30	4	Inhibited MCh response	None
Cytochalasin D	5×10^{-7}	IL	30	4	None	None
Latrunculin B	5×10^{-6}	IL	30	4	See text; inhibited MCh response	Potentiation
Channel blocker						
Glibenclamide	10^{-4}	IL + EL	30	5	None	None
NPPB	10^{-4}	IL	60	8	Small relaxation	Inhibition
$HgCl_2$	10^{-4} and 10^{-5}	IL	30	8	Inhibited MCh response	None
Tetrodotoxin	10^{-6}	IL + EL	30	4	None	None

Membrane permeability						
Nystatin						
D-M	2.6×10^{-4}	IL	60	6	Large contraction	Potentiation
KCl	2.6×10^{-4}	IL	60	6	Large contraction	Potentiation
α-Hemolysin	100[a]	IL	30	4	None	None
Cell signaling						
TPA						
After MCh	10^{-7}	IL	30	2	None	None
	2×10^{-7}	IL	30	2	None	None
Before MCh	10^{-7}	IL	120	4	Sustained contraction	None
Cholera toxin	250[d]	IL	120	4	None	None
Pertussin toxin	100[d]	IL	30	5	None	None
Ryanodine	10^{-6}	IL	30	4	None	None
Thapsigargin + Ca^{2+}-free MKHS	10^{-6}	IL	30[e]	3	Contraction	None
Ca^{2+}-free MKHS	—	IL	60	2	Increased baseline	None
Miscellaneous						
Gossypol	5×10^{-6}	IL + EL	60	5	None	Inhibition[c]
Hydrocortisone	10^{-5}	IL + EL	30	4	None	None
EGF	100[d]	IL	60	4	None	None

[a]Concentration in Units/ml. [b]These agents were applied to the MCh-contracted preparations to determine if a response could be generated. [c]See Fig. 1.

[d]Concentration in ng/ml. [e]Added in the presence of Ca^{2+}-free MKHS, which had been 60 min earlier.

Fig. 1
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