0022-3565/08/3261-186–195
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
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JPET 326:186–195, 2008

Vol. 326, No. 1
135871/3353522
Printed in U.S.A.

Hyperosmolarity-Induced Dilation and Epithelial Bioelectric Responses of Guinea Pig Trachea in Vitro: Role of Kinase Signaling

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Received December 21, 2007; accepted April 14, 2008

ABSTRACT

Exercise-induced airway obstruction is thought to involve evaporative water loss and hyperosmolarity of the airway surface liquid. Hyperosmolar challenge of the epithelium of isolated, perfused guinea pig trachea rapidly alters transepithelial potential difference (V_t), and it elicits smooth muscle relaxation mediated by epithelium-derived relaxing factor (EpDRF). In many cell types, protein kinases mediate responses to hyperosmolarity and regulatory volume increase. In this study, inhibitors were used to investigate the involvement of kinases and phosphatases in bioelectric responses of epithelium to hyperosmolarity and their possible relationship to EpDRF-mediated relaxation. After contraction of the perfused trachea with extraluminal methacholine, D-mannitol applied intraluminally (≤80 mosM) increased V_t and elicited dilation of the smooth muscle with a similar concentration-dependence; higher concentrations decreased V_t. In tracheas exposed to 30 mosM D-mannitol (~EC_{50}), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB 203580) and SKF 86002 [6-(4-fluorophenyl)-2,3-dihydro-5-(4-pyridyl)imidazo[2,1-*b*]thiazole] (p38 inhibitors) potentiated the dilation, whereas SP 600125 [anthra[1,9-*cd*]pyrazol-6(2*H*)-one-1,9-pyrazoloanthrone] and dicumarol [c-Jun NH_2-terminal kinase (JNK) inhibitors], chelerythrine [nonselective protein kinase C (PKC) inhibitor], and $NaAsO_2$ (mitogen-activated protein kinase stress inducer) and Na_3VO_4 (protein tyrosine phosphatase inhibitor) inhibited the hyperpolarization. Large increases in the phosphorylation of p38 and JNK occurred at concentrations higher than those needed to elicit functional responses. The phosphatidylinositol 3-kinase inhibitor 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY 294002) and Na_3VO_4 did not affect the V_t responses, but they inhibited methacholine-induced constriction; SP 600125 and dicumarol potentiated, and chelerythrine inhibited, methacholine-induced epithelial hyperpolarization. These results suggest that JNK, PKC, and phosphatase(s) are involved in hyperosmolarity-induced hyperpolarization of the tracheal epithelium but that p38 is involved in EpDRF-mediated relaxation.

The airway epithelium is a protective barrier against the external environment (Munakata et al., 1989; Hamilton et al., 2001), and it regulates the depth and composition of the airway surface liquid (ASL) (Widdicombe, 2002). Under physiological conditions, ASL osmolarity is approximately isosmolar with that of the interstitium (Boucher, 1999; Jayaraman et al., 2001; Tarran, 2004). During exercise and hyperventilation, evaporative water loss is thought to cause an increase in the osmolarity of the ASL (Anderson and Daviskas, 2000). In dogs, Freed and Davis (1999) observed increases in osmolarity of approximately 130 mOsM during hyperventilation with dry air, which was reduced to an ~40 mOsM increase after the end of hyperventilation. In nonasthmatic subjects, exercise causes a decrease in respiratory resistance due to bronchial dilation (Kagawa and Kerr, 1970; Silverman et al.,

This work was supported, in part, by a predoctoral fellowship from the Ohio Valley Affiliate of the American Heart Association (to Y.J.).
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.107.135871.

ABBREVIATIONS: ASL, airway surface liquid; D-M, D-mannitol; EpDRF, epithelium-derived relaxing factor; RVI, regulatory volume increase; MAP, mitogen-activated protein; JNK, c-Jun NH_2-terminal kinase; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; CaM-K-II, calmodulin kinase-II, MLCK, myosin light chain kinase; PI-3-K, phosphatidylinositol 3-kinase; MCh, methacholine; MKH, modified Krebs-Henseleit; SB 203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; PD 98059, 2′-amino-3′-methoxyflavone; U 0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; LY 294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; KN-62, 1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; DMSO, dimethyl sulfoxide; V_t, transepithelial potential difference; ΔP, inlet minus outlet perfusion pressure difference; R_t, transepithelial resistance; SKF 86002, 6-(4-fluorophenyl)-2,3-dihydro-5-(4-pyridyl)imidazo[2,1-*b*]thiazole; SP 600125, anthra[1,9-*cd*]pyrazol-6(2*H*)-one-1,9-pyrazoloanthrone; ML-7, 1-(5-iodonaphthalene-1-sulfonyl)homopiperazine.

2005). In asthmatic patients, however, exercise causes airway obstruction (Anderson and Daviskas, 2000), which is thought to be triggered by a 40 to 60 mOsM increase in the osmolarity of the ASL (Freed and Davis, 1999; Anderson and Kippelen, 2005; Hallstrand et al., 2005a,b; Anderson, 2006). Hyperosmolar stimulation of the airways, such as that induced with inhalation of the nonpermeant osmolyte D-mannitol (D-M), also evokes bronchoconstriction in asthmatic subjects (Porsbjerg et al., 2007). Studies using the guinea pig isolated, perfused trachea demonstrated that the airway epithelium is an osmometer that responds to very small increases in lumen osmolarity, within the range occurring during exercise, by releasing epithelium-derived relaxing factor (EpDRF) and relaxing the airway smooth muscle (Munakata et al., 1988; Fedan et al., 1990). EpDRF has not been isolated. Dilation responses of the perfused trachea triggered by challenge of the epithelium with hyperosmolar solution are not mediated by nitric oxide or prostanoids (Munakata et al., 1990; Johnston et al., 2004). The responses are partially inhibited by hemoglobin and zinc II protoporphyrin(IX) (Fedan et al., 2004b), which suggests that carbon monoxide contributes to the activity known as EpDRF. We have hypothesized that EpDRF participates in bronchodilation in normal individuals during exercise and that reduced secretion of EpDRF may contribute to obstruction in asthmatic subjects.

The relationship between bioelectric responses of the epithelium in response to hyperosmolar challenge and the ensuing relaxation has been under investigation. In fresh guinea pig tracheas mounted in Ussing chambers, elevation of osmolarity by 120 mOsM depolarized the epithelium and decreased transepithelial short-circuit current (Wu et al., 2004). In guinea pig isolated, perfused trachea, the depolarization preceded dilation (Dortch-Carnes et al., 1999). Blockade of epithelial Na^+ and Cl^- channels inhibited EpDRF-mediated airway relaxation, whereas blockade of Na^+,K^+,$2Cl^-$ cotransport had no effect (Fedan et al., 1999). The timing of the two responses and the effects of Na^+ and Cl^- channel blockade have suggested that the bioelectric and mechanical responses are linked.

Extracellular hyperosmolarity causes shrinkage in epithelium and other cells (Lang et al., 1998). The EpDRF-mediated relaxation is not linked to cell shrinkage (Fedan et al., 2004a, 2007a,b). Many cells, but not airway epithelial cells, undergo a compensatory regulatory volume increase (RVI) in the presence of raised osmolarity, resulting from activation of the Na^+,K^+,$2Cl^-$ cotransporter and other ion transport systems, causing an uptake of Na^+, Cl^-, and water to restore cell volume. The preponderance of studies of the effects of hyperosmolarity have been focused on pathways involved in RVI and long-term regulation of cell volume, have used supraphysiological osmolarities and long incubation periods, and have limited relevance for understanding the rapid, early events in EpDRF release in response to small increments in osmolarity. These studies, however, have revealed that several signaling pathways are involved in RVI, including mitogen-activated protein (MAP) kinases, p38 [c-Jun NH_2-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK)], protein kinase C (PKC), calmodulin kinase-II (CaM-K-II), myosin light chain kinase (MLCK), and phosphatidylinositol 3-kinase (PI-3-K) (Shrode et al., 1998; Puddicombe and Davies, 2000; Sheikh-Hamad and Gustin, 2004; Zhao et al., 2004). p38 and JNK are also involved in the release of inflammatory mediators from epithelium after hyperosmolar challenge (Hashimoto et al., 1999a,b; Furuichi et al., 2002).

We hypothesized that one or more of these kinases may play a role in EpDRF release and bioelectric responses of the epithelium to hyperosmolar challenge. The guinea pig perfused trachea preparation was used to compare the osmolar concentration dependencies of epithelial bioelectric responses and EpDRF release. We found that only p38 modulates EpDRF-mediated relaxant responses, whereas JNK, PKC, and phosphatase(s) participate in epithelial bioelectric responses to methacholine (MCh) and hyperosmolarity. Small increases in osmolarity that are relevant to exercise and that maximally release EpDRF do not elevate phosphorylation to the degree caused by supraphysiological hyperosmolar challenges.

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 they were approved by the Institutional Animal Care and Use Committee. Male guinea pigs (550–700 g), HsdPoc:DH, from Harlan (Indianapolis, IN), monitored free of endogenous viral pathogens, parasites, and bacteria, were used in all experiments. The animals were acclimated before use, and they were housed in filtered ventilated cages on α-Dri virgin cellulose chips and hardwood β-chips as bedding. They were provided HEPA-filtered air, and Teklad 7006 diet and tap water ad libitum, under controlled light cycle (12 h of light) and temperature (22–25°C) conditions. The animals were anesthetized with sodium pentobarbital (65 mg/kg i.p.), and then they were sacrificed by thoracotomy and bleeding before removing the trachea.

Materials. Modified Krebs-Henseleit (MKH) solution, pH 7.4, at 37°C contained 113 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 5.7 mM glucose, and it was saturated with 95% O_2 and 5% CO_2. The osmolarity of MKH was 281 ± 5 mOsM. Acetyl-β-methylcholine chloride (MCh) and D-M (Sigma-Aldrich, St. Louis, MO) solutions were prepared in saline. Kinase inhibitors (SB 203580, SKF 86002, SP 600125, dicumarol, PD 98059, U 0126, chelerythrine, LY 294002, KN-62, and ML-7) were from Calbiochem-Novabiochem (La Jolla, CA), and they were dissolved in dimethyl sulfoxide (DMSO). $NaAsO_2$ (Mallinckrodt Laboratory Chemicals, Phillipsburg, NJ) and Na_3VO_4 (Sigma-Aldrich) were prepared in MKH solution. Radioimmunoprecipitation assay cell lysis buffer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) contained 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 0.004% sodium azide in Tris-buffered saline, to which phenylmethanesulfonyl fluoride, sodium orthovanadate, and protease inhibitors were added according to the manufacturer's directions. Laemmli protein sample buffer and 10% SDS-polyacrylamide gels were from Bio-Rad (Hercules, CA). Phosphorylated and nonphosphorylated p38 and JNK control proteins and their primary antibodies, horseradish peroxidase-linked anti-rabbit secondary antibody, and β-actin antibody were from Cell Signaling Technology Inc. (Beverly, MA). Bicinchoninic acid protein assay reagents and Western blot stripping buffer were from Pierce Chemical (Rockford, IL). Enhanced chemiluminescence solutions were from GE Healthcare (Piscataway, NJ).

Perfused Trachea Preparation. The guinea pig isolated, perfused trachea preparation, which allows agents to be applied separately to the mucosal (intraluminal) or serosal (extraluminal) surfaces of the trachea (Munakata et al., 1988, 1990; Fedan et al., 1990), was used to measure simultaneously epithelial bioelectric and smooth muscle mechanical responses induced by various agents (Dortch-Carnes et al., 1999). With this preparation, agents can be added to either bath to target epithelium or smooth muscle. A 4.2-cm

segment of guinea pig trachea was removed, and it was mounted at its in situ length onto a plastic perfusion holder that allows indwelling cannulae with side-holes to be inserted into the lumen from either end of the trachea. The cannulae were made of stainless steel, and they were coated with nail polish to provide electrical insulation. MKH solution in the intraluminal and extraluminal baths was kept at 37°C and saturated with 95% O_2 and 5% CO_2. The holder was placed into an extraluminal bath filled with MKH solution. The tracheal lumen was perfused with MKH solution from the intraluminal bath at a constant rate (20 ml/min) using a pump; the transmural pressure was adjusted to zero. The inlet minus outlet perfusion pressure difference (ΔP), a fifth power function of tracheal diameter (Munakata et al., 1988, 1990), was measured through the side-holes in the indwelling cannulae that were connected to a differential pressure transducer. Contraction of the smooth muscle increases ΔP (constriction), whereas relaxation of the smooth muscle decreases ΔP (dilation). Voltage electrodes (silver/AgCl) were in continuity via a bridge (filled with 4% agar/saline) with the intraluminal and extraluminal baths to record transepithelial potential difference (V_t). The inner voltage electrode consisted of the side-holes of the indwelling cannula that had been inserted into the proximal end of the trachea; the apertures were 6 mm from the point of entry of MKH into the lumen of the trachea. The outer voltage electrode consisted of an MKH solution-filled glass tube placed close (1 cm) to the tracheal wall that was in continuity via a 4% agar/saline bridge with a silver/AgCl electrode. Both voltage and current electrodes were connected to a voltage/current clamp amplifier (DVC-1000; WPI, Sarasota, FL). Thus, the preparation allowed simultaneous monitoring of both V_t and ΔP in the same trachea.

Concentration-Response Curves for Hyperosmolar D-M-Induced V_t and ΔP Responses. The tracheal preparation was equilibrated by perfusing MKH solution for 2.5 to 3 h to allow V_t and ΔP to become stable. To prepare the trachea for dilation responses, the smooth muscle was contracted by adding MCh (3×10^{-7} M, $\sim EC_{50}$) to the extraluminal bath. MCh elicited epithelial hyperpolarization. When the MCh-induced V_t and ΔP responses reached their plateaus, the osmolarity of the perfused solution was elevated cumulatively with stepwise additions of D-M to the intraluminal bath.

Effects of Kinase and Phosphatase Inhibitors on V_t and ΔP. The guinea pig genome has not been sequenced, and genetic gain or loss of function models are not available for this species. Therefore, a pharmacological approach was taken to investigate the involvement of kinases and phosphatases in hyperosmolarity-induced responses.

To avoid variability between animals and shipments, control responses and the effects of the inhibitors were compared using a paired design protocol wherein responses were measured in both the absence and presence of an agent in each trachea. After the equilibration period, the tracheas were incubated with vehicle for 30 min in the intraluminal bath. MCh was then added to the extraluminal bath. When the constriction and hyperpolarization reached their plateaus, the osmolarity of intraluminal perfused MKH solution was elevated by adding 30 mOsM D-M; this concentration approximates the EC_{50} value for hyperosmolarity-induced relaxation responses (Fedan et al., 2004a). After the D-M-induced V_t and ΔP responses became stable, the preparation was washed with fresh MKH solution to remove all agents. Ninety minutes after the baselines were re-established, the trachea was incubated with a test agent in the intraluminal bath for 30 min, after which the trachea was challenged again with MCh and D-M in the presence of the agent. The possible effects of DMSO on the two sets of responses to MCh and D-M were evaluated in a separate group of control preparations.

The choice of the inhibitors and the concentrations to be used was decided after a search of the literature for investigations describing the kinetic properties of the agents (Table 1). When available, two structurally different agents were used to lessen the likelihood of interpreting findings caused by nonspecific agent effects. Concentrations were chosen that were ca. 1 order of magnitude above the reported EC_{50} values for enzyme inhibition for the reason that the concentrations used in intact epithelial cells would need to be higher than those used in broken cell preparations. Chelerythrine was used as a broad-spectrum inhibitor of the family of PKCs in these experiments to gauge the potential scope of PKC involvement.

Western Blots. In these experiments, tracheal segments were challenged with D-M in the absence of MCh. Two animals were required for each experiment. Tracheas were removed, placed in MKH solution, cleaned, and cut longitudinally into three strips. In a paired design, the strips from each animal were randomly divided into three experimental groups to give a total of two strips for each condition, one from each animal: 1) controls, incubated in MKH solution; 2) D-M-exposed (20, 40, 80, 160, 240, and 320 mOsM was added to MKH solution; each strip was exposed to a single concentration); and 3) $NaAsO_2$-exposed (0.5 mM in MKH solution; the concentration of $NaAsO_2$ was increased in these experiments), as a positive control. Each condition was investigated at least four times.

TABLE 1
Properties of the kinase and phosphatase inhibitors and $NaAsO_2$

Enzyme	Agent	EC_{50}	Conc. Used[a]	Mechanism	References
			μM		
p38	SB 203580	0.6	30	Competitive binding in the ATP pocket	Woo et al. (2005)
	SKF 86002	1	30	Competitive binding in the ATP pocket	Prabhakar et al. (1993); Pelaia et al. (2005)
JNK	SP 600125	0.19	30	Competes with ATP to inhibit the enzyme	Woo et al. (2005)
	Dicumarol	5	15	Blocks stress signaling by the inhibition of quinone reductase	Cross et al. (1999)
ERK	PD 98059	2	50	Inhibits activation of ERK by blocking upstream enzyme mitogen-activated protein kinase kinase	English and Cobb (2002); Woo et al. (2005)
	U 0126	0.5	30	Inhibits activation of ERK by blocking upstream enzyme mitogen-activated protein kinase kinase	English and Cobb (2002); Hirst et al. (2002)
PKC	Chelerythrine	0.66	20	Competitive binding to the phosphate acceptor of the enzyme	Herbert et al. (1990)
PI-3-K	LY 294002	1.4	50	Blocks the ATP-binding site of the enzyme	Vlahos et al. (1994)
CaM-K-II	KN-62	1	20	Binds directly to the calmodulin binding site of the enzyme	Tokumitsu et al. (1990); Massé and Kelly (1997)
MLCK	ML-7	0.3	30	Inhibits the binding of ATP to MLCK	Shen et al. (2002); Göggel and Uhlig (2005)
Phosphatase	Na_3VO_4	30–50	300	Analog to phosphate intermediates	Gordon (1991); Pugazhenthi et al. (1996)
Stress-activated protein kinases	$NaAsO_2$	60	300	Stress inducer (activates stress-sensitive protein kinases)	Ludwig et al. (1998); Flatman and Creanor (1999)

[a] Concentrations (Conc.) commonly reported in literature were used in this study.

After a 2-h equilibration period in MKH (37°C), the strips were exposed to MKH, D-M, or $NaAsO_2$ for 20 min at 37°C. At the end of this period, the strips were transferred into ice-cold MKH solution. At 0°C, the epithelium was scraped off with a scalpel blade, collected and centrifuged (600*g*) for 3 min at 4°C; the pellets were sonicated in 50 μl of radioimmunoprecipitation assay buffer. Protein concentrations were measured with the bicinchoninic acid assay.

Samples (20 μg of protein per well) were loaded on a 10% SDS-polyacrylamide gel together with phosphorylated and nonphosphorylated p38 or JNK standards, and then they were subjected to electrophoresis. The proteins were then transferred to a nitrocellulose membrane. After blocking nonspecific protein binding by incubating the membrane in phosphate-buffered saline with 0.1% Triton X-100 and 5% nonfat milk at room temperature for 1 h, the membrane was incubated overnight at 4°C with phosphorylated p38 or JNK primary antibodies, followed by incubation with the horseradish peroxidase-linked anti-rabbit secondary antibody at room temperature for 1 h. The membrane was washed in phosphate-buffered saline, and the blots were incubated with enhanced chemiluminescence solution for 1 min and exposed to Kodak BioMax XAR film. To determine the total amount of p38 and JNK proteins on the blots, the membrane was stripped and reprobed with nonphosphorylated p38 or JNK antibodies. β-Actin was included to assess the accuracy of sample loading.

Data Analysis. The epithelial bioelectric responses were quantified as V_t in millivolts. Responses to MCh were quantified as the increase in ΔP, in cm H_2O. D-M-induced dilation responses were normalized as a percentage of the MCh response. The EC_{50} values for V_t and ΔP responses to D-M were derived from concentration-response curves using least-squares analysis of four-parameter logit curve fits (SigmaPlot version 9; Systat Software, Inc., Point Richmond, CA), and they are given along with 95% confidence intervals. Other results are presented as means ± S.E. Differences were analyzed statistically using Student's paired *t* test (SigmaStat version 3.1; Systat Software, Inc.). $p < 0.05$ was considered significant. Bands on Western blots were scanned with a General Electric Molecular Dynamics scanner, and pixel intensity was quantified (ImageQuant 5.2 software; Molecular Dynamics). The effects of agents on the phosphorylated and total amounts of p38 and JNK were plotted as -fold increases above the levels observed in paired, unstimulated control samples.

Results

Concentration-Response Relationships for Epithelial Bioelectric and Mechanical Responses Induced by Hyperosmolarity. Basal V_t in these experiments was -12.1 ± 0.6 mV ($n = 88$).

Concentration-response curves for V_t and ΔP responses induced by raising intraluminal osmolarity with D-M were obtained from extraluminal MCh-contracted preparations (Fig. 1). Hyperpolarization and dilation responses were obtained at very low D-M concentrations, i.e., the threshold was 1–3 mOsM (Fedan et al., 2004b), attesting to the sensitivity of the epithelium to increases in osmolarity. Elevations in D-M up to ~80 mOsM caused hyperpolarization. The maximum dilation response was essentially reached at ~80 mOsM. The hyperpolarizing and relaxant responses exhibited the same concentration dependence; the EC_{50} values for hyperpolarization [16.6 (13.0–21.2) mOsM] and dilation [24.4 (19.2–31.3) mOsM] were not significantly different. The congruence of the two concentration-response curves suggests that the bioelectric and dilation responses to hyperosmolarity were closely, if not causally, linked. In addition, the EC_{50} values were in a range that is pertinent to the increase in ASL osmolarity that accompanies exercise. At concentrations greater than 80 mOsM, a transition occurred and the bioelectric response of the epithelium became converted from hyperpolarization to depolarization, whereas the dilation response was not increased appreciably. This reversal in the bioelectric response was not an artifact of the concentration-response determination, because elevations in osmolarity above 80 mOsM with a single addition evoke depolarization (data not shown; Dortch-Carnes et al., 1999; Wu et al., 2004). On the basis of these results, a D-M concentration of 30 mOsM, which produced consistent dilation and hyperpolarization responses, was used to investigate the effect of the inhibitors.

Effects of Kinase and Phosphatase Inhibitors on V_t and ΔP. The purpose of these experiments was to ascertain the roles of kinases in EpDRF-mediated relaxation and bioelectric responses to hyperosmolarity.

Experiments were first conducted to establish a no-effect concentration for DMSO. DMSO had no effect on ΔP, but it increased V_t by 0.6 ± 0.1 mV at concentrations as low as 0.1%. After investigating progressively lower concentrations, it was found that DMSO at a concentration of ≤0.04% had no effect.

Next, the effects of the intraluminally added kinase and phosphatase inhibitors on ΔP and V_t were investigated. Of the six MAP kinase inhibitors examined (SB 203580, SKF 86002, SP 600125, dicumarol, PD 98059, and U 0126), only SB 203580 affected V_t, eliciting a small depolarization (0.8 ± 0.3 mV). This small response not only could reflect modulation of ion transport by p38 but also it could represent a nonspecific effect, because SKF 86002, the other inhibitor of p38, had no effect. SB 203580, dicumarol, and PD 98059 decreased ΔP slightly (0.06 ± 0.02, 0.46 ± 0.13, and 0.16 ± 0.04 cm H_2O, respectively) but, compared with responses to MCh, these effects were negligible. The other three MAP kinase inhibitors had no effect on ΔP. Chelerythrine, LY 294002, KN-62, and ML-7, decreased V_t by 1.5 ± 0.3, 3.9 ± 0.6, 0.6 ± 0.1, and 0.6 ± 0.5 mV, respectively. The magnitude of these effects suggests that PKC and PI-3-K are involved in the regulation of ion transport, whereas the roles of CaM-K-II and MLCK are minor.

In contrast, the protein tyrosine phosphatase inhibitor Na_3VO_4 and the kinase activator $NaAsO_2$ increased V_t by 0.7 ± 0.2 and 1.7 ± 0.7 mV, respectively. The nonselective PKC inhibitor chelerythrine increased ΔP by 0.2 ± 0.1 cm H_2O, a negligible effect; the other kinase inhibitors had no effect on ΔP. These findings indicate together that V_t is regulated by some phosphorylation pathways but that this regulation is of little consequence to smooth muscle tone.

Effects of Kinase and Phosphatase Inhibitors on MCh-Induced Epithelial Hyperpolarization and Dilation Responses. Because some kinase and phosphatase inhibitors affected V_t, we investigated whether bioelectric responses of the epithelium to MCh are also influenced by these agents. Extraluminally applied MCh (3×10^{-7} M) caused constriction and hyperpolarized the epithelium. None of the MAP kinase inhibitors (SB 203580, SKF 86002, SP 600125, dicumarol, PD 98059, and U 0126) affected the contractile response to MCh ($n = 7$, 6, 10, 6, 6, and 6, respectively; data not shown). Intraluminally applied LY 294002 (Fig. 2A) and Na_3VO_4 (Fig. 2B) inhibited contractile responses to MCh. Apparent effects of chelerythrine and $NaAsO_2$ did not reach

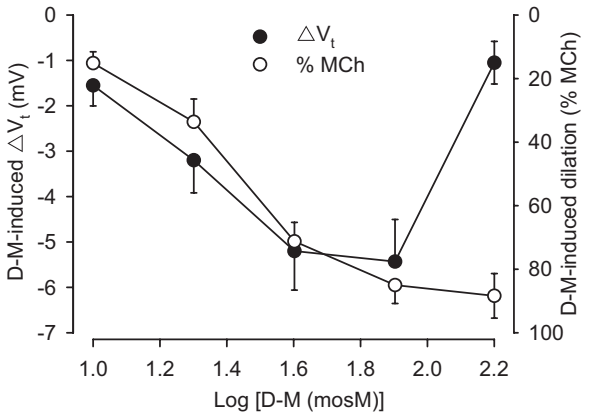

Fig. 1. Cumulative concentration-response relationships for D-M-induced bioelectric and mechanical responses of perfused trachea. ΔV_t, changes in V_t from the baseline value of -11.3 ± 2.8 mV. Dilation, percentage of dilation of 3×10^{-7} M MCh-induced constriction (% MCh). $n = 6$. D-M induced concentration-dependent dilation with the maximal response occurring at ~80 mOsM. In concentrations ≤80 mOsM, D-M hyperpolarized the epithelium. The two curves were concordant in their concentration dependence at concentrations of ~80 mOsM or less. Above 80 mOsM, the bioelectric response became depolarization.

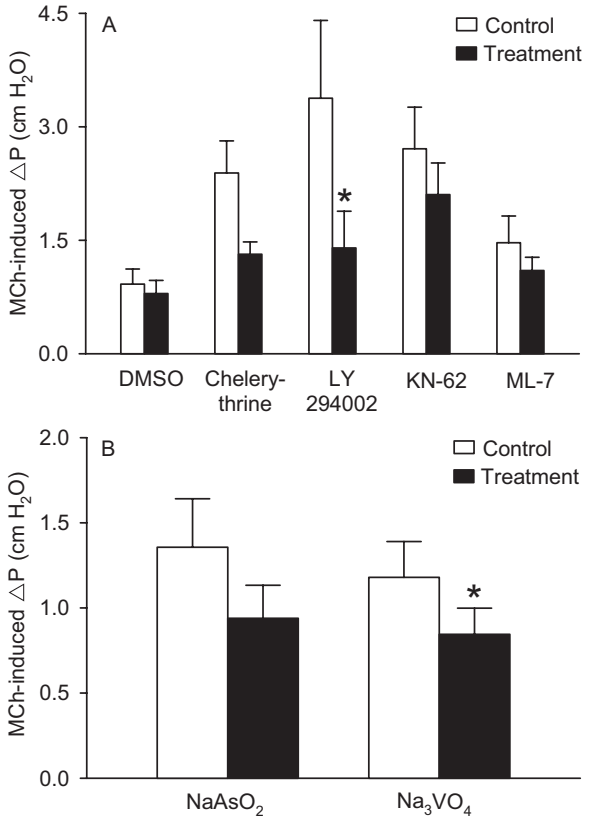

Fig. 2. Effects of kinase inhibitors (A) and $NaAsO_2$ and Na_3VO_4 (B) on MCh-induced constriction of the perfused trachea. Each agent was examined in tracheas from separate groups of animals using a paired design. The open bars depict the constriction responses to MCh in the absence of the inhibitor or agent; the filled bars represent constriction responses obtained in the presence of the inhibitor or agent. A, kinase inhibitors. For DMSO, the results indicate that there were no differences in two control responses obtained in the presence of the vehicle. LY 294002 ($n = 6$) inhibited the constriction responses (*, $p < 0.05$ compared with control). KN-62 and ML-7 had no effect ($n = 6$ for each). The effect of chelerythrine did not reach statistical significance even when sample size was increased ($n = 8$; $p = 0.052$). B, Na_3VO_4 ($n = 6$) inhibited contractile responses to MCh (*, $p < 0.05$ compared with control), whereas the effect of $NaAsO_2$ ($n = 9$) was not significant ($p = 0.093$ compared with control).

significance; KN-62 and ML-7 had no effect. Because they were added to the intraluminal bath and the epithelium constitutes a 400 to 800-fold diffusion barrier for agents (Fedan and Frazer, 1992), these findings indicate that the inhibitory effects of LY 294002 and Na_3VO_4 on smooth muscle responses were indirect and mediated by the epithelium.

SP 600125 and dicumarol potentiated the hyperpolarization to MCh (Fig. 3A), whereas chelerythrine inhibited this response (Fig. 3B). Hyperpolarization in response to MCh is thought to involve primarily activation of Cl^- secretion (Wu et al., 2004; Fedan et al., 2007b), a possible downstream target of these pathways. The remaining kinase inhibitors, as well as $NaAsO_2$ and Na_3VO_4 ($n = 9$ and 6, respectively; data not shown), had no effect on bioelectric responses to MCh.

Effects of Kinase and Phosphatase Inhibitors on D-M-Induced Epithelial Hyperpolarization and Smooth Muscle Dilation Responses. We next investigated the involvement of kinase pathways on epithelial bioelectric and dilation responses to D-M. SP 600125 and dicumarol (Fig. 4A), chelerythrine (Fig. 4B), and $NaAsO_2$ and Na_3VO_4 (Fig. 5) added to the intraluminal bath inhibited the increase in V_t. The remaining kinase inhibitors had no effect (Fig. 4, A and B). In contrast to its lack of effect on MCh-induced responses, $NaAsO_2$ completely inhibited hyperpolarization in response to D-M (Fig. 5). These findings suggest that the JNK and PKC pathways as well as phosphatases, but not ERK, are intermediaries in the bioelectric responses of the epithelium to D-M.

The p38 inhibitors SB 203580 and SKF 86002 potentiated dilation responses to D-M (Fig. 6A). The other kinase inhibitors [SP 600125, dicumarol, PD 98059, and U 0126 (Fig. 6A); chelerythrine, LY 294002, KN-62, and ML-7 (Fig. 6B)] as well as $NaAsO_2$ and Na_3VO_4 ($n = 9$ and 6, respectively, data not shown), had no effect on dilation responses. Thus, signaling through p38 is involved in the dilation response but not the bioelectric response to hyperosmolarity.

Epithelial p38 and JNK Phosphorylation Induced by Hyperosmolarity. The above-mentioned results indicated that p38 is involved in D-M-induced EpDRF release but not bioelectric responses of the epithelium, whereas the converse is true for JNK and PKC, and the remaining kinases are little involved in the development of either response. Thus, we investigated phosphorylation of p38 in the EpDRF release context. Because two JNK inhibitors had given evidence suggesting an integral role of JNK in epithelial ion transport, JNK was included in these experiments. In the absence of pharmacological evidence for their involvement, immunoblotting of ERK and the other kinases was not performed.

Using human cultured bronchial epithelial cell to explore the role of p38 in hyperosmolarity-induced release of cytokines, Hashimoto et al. (1999b) observed little, if any, phospho-p38 in unstimulated cells. In contrast, at the outset of this study, we observed high levels of phospho-p38 when the epithelium was scraped off and centrifuged at room temperature, and little further increase occurred in response to D-M. This outcome was not encountered to the same degree with JNK. The warmer temperatures had been used to minimize disruption of ion gradients which, itself, might alter catalytic activity. We determined after performing extensive preliminary experiments that the phospho-p38 background

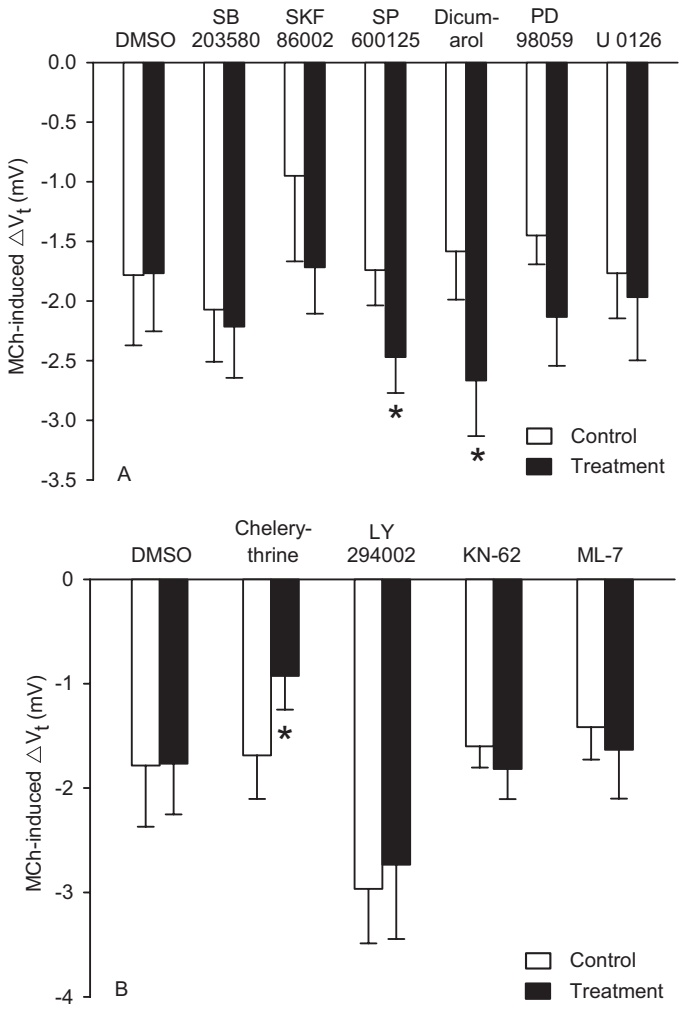

Fig. 3. Effects of kinase inhibitors on MCh-induced hyperpolarization. Each agent was examined in tracheas from separate groups of animals using a paired design (see Fig. 2 legend). A, MAP kinase inhibitors. SP 600125 and dicumarol (n = 10 and 6, respectively) potentiated the hyperpolarization response to MCh (*, $p < 0.05$ compared with control). The effects of DMSO and SB 203580, SKF 86002, PD 98059, and U 0126 (n = 6, 7, 6, 6, and 6, respectively) were not significant. B, other kinase inhibitors. Chelerythrine (n = 8) inhibited the hyperpolarization responses (*, $p < 0.05$ compared with control). DMSO, LY 294002, KN-62, and ML-7 had no effect. n = 6 for each.

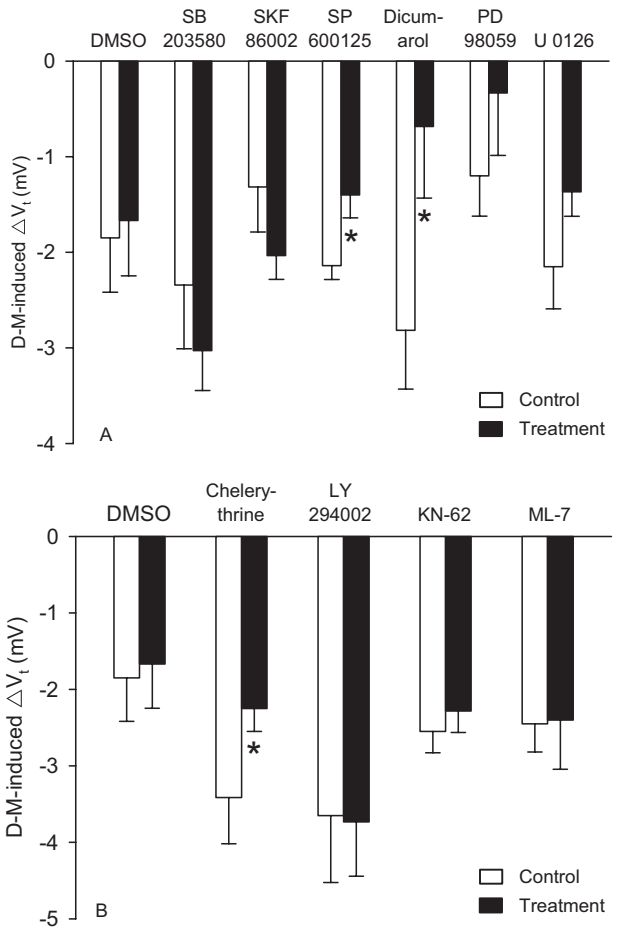

Fig. 4. Effects of kinase inhibitors on D-M(30 mOsM)-induced hyperpolarization. Each agent was examined in tracheas from separate groups of animals using a paired design (see Fig. 2 legend). A, MAP kinase inhibitors: SP 600125 and dicumarol (n = 10 and 6, respectively) inhibited the hyperpolarization responses (*, $p < 0.05$ compared with control). The effects of DMSO, SB 203580, SKF 86002, PD 98059, and U 0126 (n = 6, 7, 6, 6, and 6, respectively) were not significant. B, other kinase inhibitors. Chelerythrine (n = 8) inhibited the hyperpolarization responses (*, $p < 0.05$ compared with control). DMSO, LY 294002, KN-62, and ML-7 (n = 6 for each) had no effect.

was reduced greatly but not eliminated by immersing the tracheal strips into ice-cold MKH solution at the end of the challenge with D-M, and preparing cell extracts at 4°C. Nevertheless, basal phospho-p38 was not reduced to the low levels reported by Hashimoto et al. (1999b).

D-M stimulated a concentration-dependent increase in the phosphorylation of p38 (Fig. 7) and JNK II (Fig. 8). For p38, the increase in phospho-p38 ranged from 1.4 to 1.7-fold at D-M concentrations ≤80 mOsM; these changes were not significant. Significant increases (up to 4-fold) in phospho-p38 were observed at the two highest D-M concentrations. Similar results were seen with JNK, with significant increases in phosphorylation occurring at the three highest concentrations. The -fold increases in phospho-JNK were appreciably higher than obtained for p38. The amounts of p38 and JNK proteins were unaffected by D-M (Figs. 7 and 8). To investigate the extent of phosphorylation of these two enzymes stimulated by D-M challenge against the levels that the cells

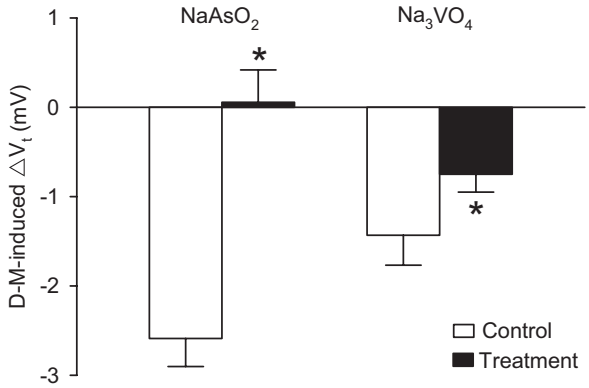

Fig. 5. Effects of $NaAsO_2$ and Na_3VO_4 on D-M(30 mOsM)-induced hyperpolarization. Each agent was examined in tracheas from separate groups of animals using a paired design (see Fig. 2 legend). Both $NaAsO_2$ and Na_3VO_4 inhibited the hyperpolarization responses (*, $p < 0.05$ compared with control). n = 9 and 6 for $NaAsO_2$ and Na_3VO_4, respectively. The inhibition by $NaAsO_2$ was complete.

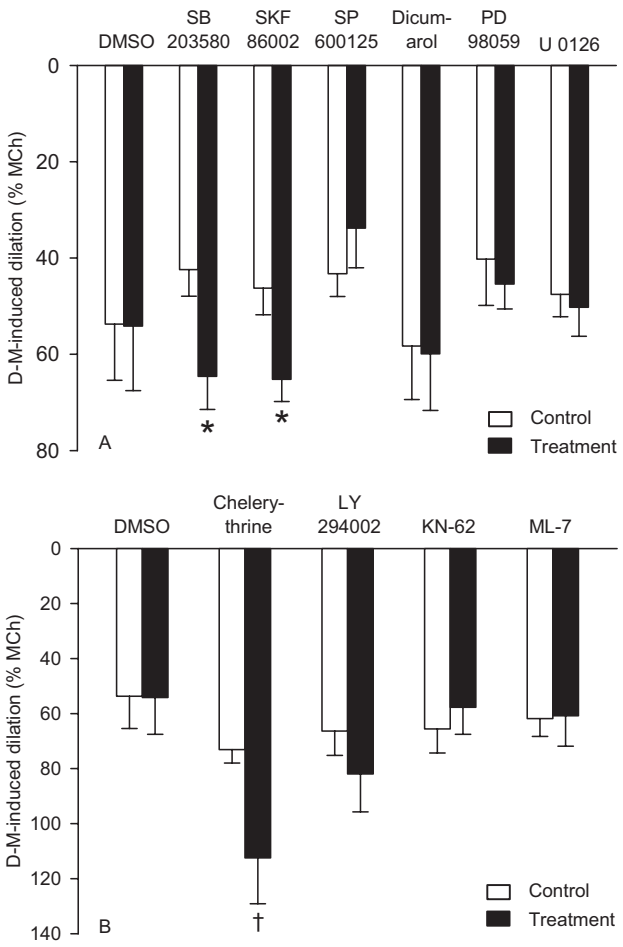

Fig. 6. Effects of kinase inhibitors on D-M(30 mOsM)-induced dilation. Each agent was examined in tracheas from separate groups of animals using a paired design (see Fig. 2 legend). A, MAP kinase inhibitors SB 203580 and SKF 86002 (n = 7 and 6, respectively) potentiated the dilation responses (*, $p < 0.05$ compared with control). DMSO, SP 600125, dicumarol, PD 98059, and U 0126 (n = 6, 10, 6, 6, and 6, respectively) had no effect. B, other kinase inhibitors. DMSO, LY 294002, KN-62, and ML-7 (n = 6 for each) had no effect. The effect of chelerythrine was not significant (p = 0.065; n = 8).

might be potentially capable of producing in response to a nonspecific stress stimulus, the effects of $NaAsO_2$ (0.5 mM) were examined as a positive control. A stress-inducing agent, $NaAsO_2$ has multiple sites of action on protein kinases and phosphatases (Ludwig et al., 1998). $NaAsO_2$ stimulated strongly the phosphorylation of p38 and JNK II (Figs. 7 and 8). Interestingly, $NaAsO_2$ also caused an increase in immunostaining of total p38 protein (Fig. 7), which suggests that de novo protein synthesis had occurred. However, inasmuch as the epithelium was incubated with $NaAsO_2$ for only 20 min, this increase in immunostaining is unexplained. $NaAsO_2$ also caused an increase in JNK II phosphorylation. Unlike p38, the total amount of JNK protein was not markedly changed (Fig. 8).

Discussion

The present investigation was conducted to understand the link between epithelial bioelectric responses and EpDRF release and the role of epithelial protein phosphorylation in response to hyperosmolar challenge of guinea pig tracheal epithelium. This was accomplished using intraluminally applied pharmacological inhibitors of kinases that have been implicated in other cell types to be involved in responses to hyperosmolarity and cell volume regulation. Our results indicate that kinases are involved at several points in the epithelium-smooth muscle relationship (Table 2): 1) in the constriction response to MCh, at the level of the epithelium (PI-3-K and perhaps PKC), 2) in the EpDRF-mediated dilation response (p38), and 3) in the epithelial bioelectric responses to MCh and hyperosmolarity (PKC and JNK). Because p38 inhibition potentiated hyperosmolarity-induced dilation, it would seem that p38 activation in epithelial cells leads to an inhibition of EpDRF release by downstream effectors.

The epithelium is very sensitive to small increases in luminal osmolarity. The concentration-response curves for hyperpolarization and dilation at concentrations of D-M lower than 80 mOsM were coincident, suggesting that a link exists between the two types of responses over an osmolar concentration range that is relevant to changes in the ASL during exercise. However, above 80 mOsM D-M, depolarization ensued even though dilation continued to occur. Earlier, a sin-

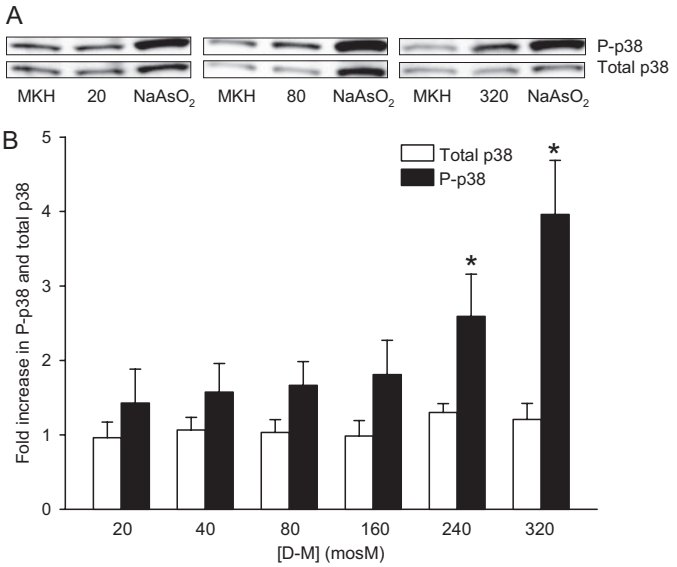

Fig. 7. Phosphorylation of p38 induced by hyperosmolarity. A, representative examples of immunoblots obtained after exposure of epithelium to 20, 80, and 320 mOsM D-M and 0.5 mM $NaAsO_2$ (n = 4 for each concentration). For each experiment, two tracheas were used; each was divided into three longitudinal strips, and one strip from the tracheas of two animals was pooled for each condition, allowing a paired design within each gel for -fold increase calculations. Immunostaining of β-actin was also performed as a loading control; this is not shown because -fold increases were compared between ± D-M conditions. Challenge with D-M for 20 min concentration-dependently increased phosphorylation of p38 (P-p38) compared with unstimulated strips incubated in MKH solution (left-most lanes). $NaAsO_2$ induced p38 phosphorylation that exceeded that caused by any concentration of D-M; it also increased the total amount of p38 staining. B, concentration-response relationships for the effects of D-M on p38 and P-p38. This figure summarizes the results obtained from densitometric scanning of 24 individual gels, from which three representative examples are shown in A. Shown here are the mean -fold increases in band intensity measured in the presence versus the absence of D-M. Comparison of -fold changes (±D-M) over the concentration range was done using paired analysis. Although evident after densitometric analysis of each gel, the mean data obscured the 1.4 to 1.7-fold increases in P-p38 after challenge with ≤80 mOsM D-M. Above 80 mOsM, significant elevations in P-p38 occurred ($p < 0.05$, total p38 versus P-p38). There was no effect of D-M treatment on total p38 protein. n = 4 for each concentration.

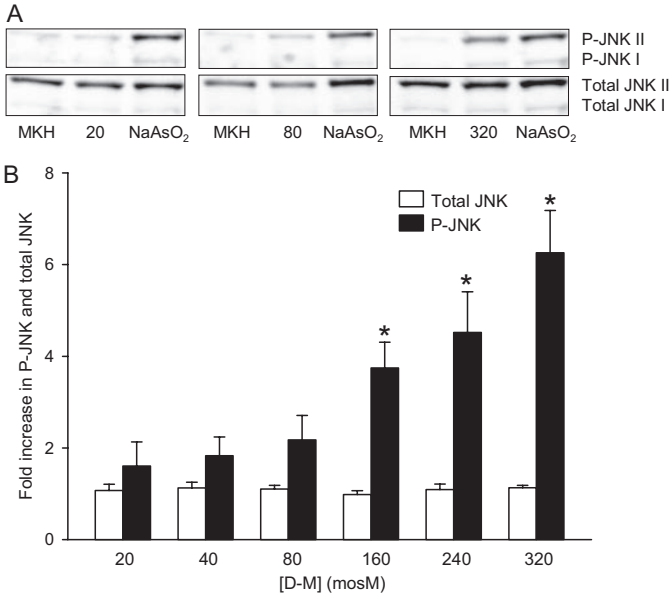

Fig. 8. Phosphorylation of JNK induced by hyperosmolarity. A, representative examples of immunoblots obtained after exposure of epithelium to 20, 80, and 320 mOsM D-M and 0.5 mM $NaAsO_2$. $n = 4$ for each concentration. The same protocols and analysis described in the legend of Fig. 7 were used in this experiment to investigate the effects of D-M on total and phosphorylated JNK (JNK I and II, and P-JNK I and II, respectively). Basal levels of P-JNK II were relatively low, and those for P-JNK I were barely detectable. Challenge of the epithelium with D-M for 20 min increased the phosphorylation of JNK II; JNK I was phosphorylated to a lesser extent. $NaAsO_2$ increased P-JNK II appreciably, but it had a smaller effect on P-JNK I; in contrast to p38, $NaAsO_2$ had no effect on total JNK I and II proteins. The elevation in P-JNK II caused by the higher concentrations of D-M were comparable with those elicited by $NaAsO_2$. B, concentration-response relationships for the effects of D-M on JNK and P-JNK. This figure summarizes the results obtained from densitometric scanning of 24 individual gels from 24 separate experiments, from which three examples are shown in A. Over the range of D-M concentration of ≤80 mOsM, the phosphorylation of JNK II increased gradually by 1.6 to 2.2-fold. At concentrations of 80 mOsM D-M and higher, the increase in the levels of P-JNK II was substantial ($p < 0.05$, total JNK versus P-JNK). There was no effect of D-M treatment on total JNK I and II proteins. $n = 4$ for each concentration.

gle challenge with 240 mOsM sucrose was reported to depolarize the epithelium (Dortch-Carnes et al., 1999).

The transformation in the bioelectric responses to D-M is not understood; a graded continuum of bioelectric response of one type would be expected. At D-M concentrations ≤80 mOsM, the hyperpolarization is likely to result from rapid (within seconds) apical Cl^- and basolateral K^+ secretion as the cells shrink (Russell, 2000). Upon shrinkage, most cells readjust their volume, principally through activation of Na^+,K^+,$2Cl^-$ cotransport (O'Neill, 1999). However, airway epithelial cells challenged with hyperosmolarity do not undergo RVI (Willumsen et al., 1994; Fedan et al., 2007a,b). At D-M concentrations ≥80 mOsM, apical D-M (120 mOsM) rapidly decreased short-circuit current (Wu et al., 2004), suggesting that the driving force for transport, the Na^+,K^+-pump, was inhibited. However, depolarization after inhibition of the Na^+,K^+-pump with ouabain is slow in onset and completion compared with the rapid depolarization triggered by high concentrations of D-M. Another explanation is epithelial Na^+ absorption was decreased by large elevations in osmolarity. This notion is supported by the finding that high levels of osmolarity (using NaCl) decreased V_t of human nasal epithelium as a result of a decrease in Na^+ conductance (Hebestreit et al., 2007). The phenomenon is also supported by findings in other cell types. For example, in rat hepatocytes, Na^+ channel conductance was increased in response to 80 mOsM and higher concentrations of sucrose (Wehner et al., 2000). The depolarization at D-M concentrations ≥80 mOsM could result from a decrease in transepithelial resistance (R_t). However, in the Ussing chamber (Wu et al., 2004) and the perfused trachea apparatus (Y. Jing and J. S. Fedan, unpublished observations), D-M did not decrease R_t. Further studies will be necessary to establish the mechanisms involved in the bioelectric responses to low and high concentrations of D-M.

We investigated the possible roles of kinases and phosphatases in epithelial signaling involved in the regulation of V_t and ΔP and responses to MCh and D-M. Of the six MAP kinase inhibitors, only SB 203580 decreased basal V_t; because SKF 86002 did not produce the same effect, the effect of SB 203580 might not involve p38. Chelerythrine, LY 294002, KN-62, and ML-7 decreased V_t. In contrast, $NaAsO_2$ and Na_3VO_4 increased basal V_t. By increasing protein phosphorylation, the effects of $NaAsO_2$ and Na_3VO_4 on ion transport should be opposite those of the kinase inhibitors. Thus, these results suggest that ion transport of unstimulated epithelial cells is regulated by PKC, PI-3-K, CaM-K-II, and MLCK.

TABLE 2

Summary of the effects of the agents investigated in this study on baseline, and bioelectric (V_t) and mechanical (ΔP) responses to MCh and D-M
This table summarizes only effects that were significant.

Response	MAP Kinase Inhibitor						Other Kinase Inhibitors and Agents					
	p38		JNK		ERK		PKC	PI-3-K	CaM-K-II	MLCK	Stress Inducer	Phosphatase Inhibitor
	SB203580	SKF86002	SP600125	Dicumarol	PD98059	U 0126	Chelerythrine	LY294002	KN-62	ML-7	$NaAsO_2$	Na_3VO_4
V_t (mV)												
Basal	Depol						Depol	Depol	Depol	Depol	Hyper	Hyper
MCh			Hyper Potent	Hyper Potent			Hyper Inhibit					
D-M			Hyper Inhibit	Hyper Inhibit			Hyper Inhibit				Hyper Inhibit	Hyper Inhibit
ΔP (cm H_2O)												
Basal	Dilat			Dilat	Dilat		Constr					
MCh								Inhibit				Inhibit
D-M	Potent	Potent										

Depol, depolarization or decrease in V_t; Hyper, hyperpolarization or increase in V_t; Potent, potentiation; Inhibit, inhibition; Dilat, dilation of trachea; Constr, constriction of trachea.

Administered intraluminally, the MAP kinase inhibitors were generally without effect on the constriction responses to MCh. However, evidence was obtained that PI-3-K, phosphatases, and, possibly, PKC, may regulate reactivity of the muscle to MCh indirectly at the level of the epithelial cells.

MCh-induced epithelial hyperpolarization in dog (Phillips et al., 2002) and guinea pig tracheal epithelium (Wu et al., 2004) results primarily from Cl^- secretion and activation of the Na^+,K^+,$2Cl^-$ cotransporter (Wu et al., 2004). MCh activated JNK in Chinese hamster ovary cells, and the activation of JNK was negatively regulated by PKC (Wylie et al., 1999). This suggests that PKC and JNK could have differential regulatory effects on MCh-induced responses. These findings help to explain the diverse effects of SP 600125, dicumarol, and chelerythrine on the MCh-induced hyperpolarization. PKC down-regulation of the activity of JNK was also observed in Rat-1 fibroblasts (Cadwallader et al., 1997). Because the activity of the Na^+,K^+,$2Cl^-$ cotransporter is modulated by phosphorylation, PKC and JNK may regulate responses to MCh at this locus (Flatman, 2002). The recent findings that Ste20-related proline-alanine-rich kinase (SPAK) and oxidative stress response 1 (OSR1) may be the primary regulatory kinases of the Na^+,K^+,$2Cl^-$ cotransporter (Gagnon et al., 2006) could suggest that PKC may be involved in a secondary but related capacity.

SP 600125, dicumarol, and chelerythrine inhibited D-M-induced hyperpolarization. The mechanism may also have involved inhibition of the Na^+,K^+,$2Cl^-$ cotransporter and secondary secretion of Cl^- and K^+, because hyperosmolar sucrose induced activation of the Na^+,K^+,$2Cl^-$ cotransporter through the regulation of JNK and PKC in endothelial and epithelial cells, respectively (Klein et al., 1999; Liedtke and Cole, 2002). Inhibition of hyperpolarization by SP 600125 and dicumarol occurs not only through inhibition of transcellular ion transport but also involves decreases in R_t (Y. Jing and J. S. Fedan, unpublished observations).

The effects of $NaAsO_2$ and Na_3VO_4 may have resulted from their interactions with multiple kinase pathways involved in the regulation of the Na^+,K^+,$2Cl^-$ cotransporter (Zagórska et al., 2007). Inhibition of the D-M-induced hyperpolarization by $NaAsO_2$ involves decreases in R_t (Y. Jing and J. S. Fedan, unpublished observations). Although it inhibits the Na^+,K^+-pump (Dafnis and Sabatini, 1994), which would lead to depolarization, Na_3VO_4 increased V_t. Na_3VO_4 had no effect on R_t (Y. Jing and J. S. Fedan, unpublished observations); its inhibition of hyperpolarization could result from decreased transcellular ion transport.

p38 inhibition led to potentiation of EpDRF-mediated dilation responses. Immunoblotting experiments were performed to obtain biochemical evidence to support the pharmacological evidence for an involvement of p38 in EpDRF release. An important observation was the high levels of phosphorylated p38 in unstimulated epithelium, compared with what is observed in epithelial cell culture (Hashimoto et al., 1999b). A question that we are unable to answer is whether this represents the constitutive level of phosphorylation or an artifact of tissue handling. Nevertheless, the immunoblotting results suggested that there are small increases in phospho-p38 over the concentration range in which D-M initiates hyperpolarization and dilation. It is unequivocal that large increases in phosphorylation occurred at supraphysiological concentrations associated with depolarization. Is it possible that only small increases in phospho-p38 are required to modulate EpDRF release over osmolar concentrations that are pertinent to exercise? If the high basal levels of phospho-p38 do reflect the influence of experimental manipulations, the -fold increases in phosphorylation at the lower D-M concentrations would have been greater.

In conclusion, our results indicate that physiologically relevant increases in luminal osmolarity elicit epithelial hyperpolarization and smooth muscle dilation; the two responses share the same osmolarity concentration dependence. p38 plays a modulatory, inhibitory role in the EpDRF-mediated dilation. As such, p38 inhibition could be a useful therapeutic approach for preventing obstruction during exercise without affecting epithelial ion transport. Hyperosmolarity-induced hyperpolarization may be regulated by JNK, PKC, and phosphatase, but these pathways seem not to be involved in the regulation of EpDRF release.

Acknowledgments

We are grateful to Janet A. Dowdy for expert technical assistance.

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