Hypoxia Inhibits Cyclic Nucleotide-Stimulated Epithelial Ion Transport: Role for Nucleotide Cyclases as Oxygen Sensors

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ABSTRACT

Decreased oxygen delivery to cells (hypoxia) is prevalent in a number of important diseases. Little is known about mechanisms of oxygen sensing at the cellular level or about whether functional correlates of oxygen sensing exist. In this study, we examined the impact of hypoxia on stimulated epithelial ion transport function. T84 cells, a model of intestinal epithelia, were grown on permeable supports, exposed to hypoxia (range 1–21% O2) for periods of time between 0 and 72 h and assessed for stimulated ion transport. Hypoxia evoked a specific decrease in cyclic nucleotide-stimulated (cAMP and cGMP) but not Ca2+-stimulated transport. cAMP-stimulated Cl– secretion and direct elevation of cGMP by hypoxia (range >50% decrease) and reoxygenation resulted in partial recovery of the ion transport responses. Stimulated basal levels of both cAMP and cGMP were decreased in response to hypoxia, although intracellular ATP levels were unaltered under similar conditions. Exogenous addition of cobalt, nickel or manganese, all of which compete for oxygen binding on heme-containing proteins, mimicked hypoxia. Because guanylate cyclase is a heme protein, we measured the influence of cobalt on the activity of guanylate cyclase in purified plasma membrane preparations and found cobalt to inhibit stimulated cGMP levels in this cell-free system. Finally, pharmacological lowering of intracellular cGMP (using LY83583) resulted in decreased cAMP-stimulated Cl– secretion, and direct elevation of cGMP by (using 8-bromo-cGMP or dibutyryl-cGMP) restored this hypoxia-induced activity. We conclude that a potential oxygen-sensing mechanism of epithelial cells involves the cooperation of heme-containing proteins such as guanylate cyclase and that biochemical cross-talk between cAMP- and cGMP-stimulated pathways may be important in such responses.

Cells of the body are commonly exposed to decreased levels of oxygen, a condition termed hypoxia. The ability of cells to tolerate and adapt to acute, and sometimes severe, hypoxia is crucial to survival. Surprisingly little is known about the basic oxygen-sensing mechanisms and adaptive strategies that occur during hypoxia. Significant evidence indicates that cellular adaptation to hypoxia varies greatly across species boundaries and among cell types (Hochachka, 1986). Interestingly, most mammalian cells have limited ability to cope with oxygen deprivation and consequently are easily damaged by hypoxia (Stevens and Rodman, 1995).

Tissue hypoxia is commonly associated with a number of important diseases. Recent evidence from a number of laboratories indicates that the damaging effects of cellular hypoxia are likely not mediated by direct oxygen deprivation (Waxman, 1996). Rather, a new paradigm has evolved to indicate that hypoxia may “prime” cellular machinery for damage mediated by additional physiological stimuli (Waxman, 1996). Such physiological stimuli may include inflammatory cytokines (Clark et al., 1995; Colgan et al., 1996), bioactive lipids (Michiels et al., 1993), bacterial toxins (Waxman, 1996; Zund et al., 1996b) and reactive oxygen intermediates (Mertens et al., 1990). This universal “priming” effect of diverse signals suggests that responses elicited by hypoxia involve a basic cellular event common to a number of signal transduction pathways.

A potential mechanism of adaptation to hypoxia at the cellular level could involve the functional regulation of nucleotide cyclases, cellular enzyme systems that catalyze the conversion of intracellular ATP/GTP to cyclic AMP/GMP (Tausig and Gilman, 1995). Indeed, we (Zund et al., 1996b) and others (Ogawa et al., 1992; Stevens and Rodman, 1995; Tretyakov and Farber, 1995) have shown that hypoxia directly regulates intracellular levels of adenine nucleotides in a number of cell types. Moreover, our data indicated that pharmacological regulation of adenyl cyclase effectively reversed hypoxia-elicted cellular responses (Zund et al., 1996b), and others have shown that preservation of cAMP/
cGMP pathways are protective at the whole-organ level (Pinsky et al., 1994; Pinsky et al., 1993). The mechanism(s) by which hypoxia regulates cyclic nucleotide levels is at present poorly understood. Moreover, the generation of intracellular cyclic nucleotides, especially cGMP, involves heme-containing proteins (Stone and Marletta, 1995). Previous investigations by others have shown that molecular oxygen binding to hemoproteins may serve as a mechanism of sensing extracellular oxygen concentrations and, as such, could serve as a signal transduction pathway leading to gene activation (Goldberg et al., 1988). Moreover, it has been shown that extracellular cobalt and nickel can mimic hypoxia by binding within the porphyrin ring of heme and substituting for iron, thus locking heme in a deoxy state (Goldberg et al., 1988). Whether adenylyl or guanylyl cyclase serves to “sense” extracellular O₂ levels remains to be determined.

We here examine the impact of hypoxia on functional aspects of cultured intestinal epithelial cells. Our results indicate that epithelial hypoxia specifically down-regulates stimulated electrogenic chloride secretion, the primary transport event responsible for mucosal hydration. Such hypoxia-induced alterations were specific for cyclic nucleotide agonists, were evident at the level of membrane channels/transporters and could be mimicked by exposing epithelia to cobalt. Moreover, both cobalt and hypoxia significantly diminished GC activity and could be partially reversed by the addition of exogenous cGMP. These data indicate a role for heme proteins, such as GC, in epithelial oxygen “sensing” and reveal significant cAMP/cGMP cross-talk during hypoxia.

Materials and Methods

Cell culture. T84 intestinal epithelial cells (passages 67–85) were grown and maintained as confluent monolayers on collagen-coated permeable supports as previously described in detail (Dharmasathaphorn and Madara, 1990). Monolayers were grown on 0.33-cm² ring-supported polycarbonate filters (Costar Corp., Cambridge, MA) unless otherwise noted, and they were used 6 to 12 days after plating as described previously (Madara et al., 1992a).

Epithelial cultures were exposed to hypoxia as described previously (Colgan et al., 1996). Growth media were replaced with fresh media equilibrated with hypoxic gas mixture, and cells were placed in the hypoxic chamber (Coy Laboratory Products, Ann Arbor, MI). Oxygen concentrations were as indicated (normoxia equal to 21% O₂), the balance being made up of nitrogen, carbon dioxide (constant 5% CO₂) and water vapor from the humidified chamber. Monolayers were monitored electrically in hypoxia by interfacing the voltage clamp from the outside through an airtight seal in the chamber.

Electrophysiological measurements. To measure agonist-stimulated SSC, trans epithelial potentials and resistance, we used a commercially available voltage clamp (Iowa Dual Voltage Clamps, Bioengineering, University of Iowa) interfaced with an equilibrated pair of calomel electrodes and a pair of Ag-AgCl electrodes, as described in detail elsewhere (Dharmasathaphorn and Madara, 1990). Cl⁻ secretory responses are expressed as a change in SSC (peak SSC minus base-line SSC; designated ΔSSC) necessary to maintain zero potential difference across the monolayer.

Isotope efflux and uptake assays. CI⁻ channel and K⁺ channel activity were monitored using 125I and ⁸⁶Rb efflux, respectively, on T84 cells grown on 1-cm² permeable supports, as described before (Colgan et al., 1994; Venglarik et al., 1990). Rate constants of efflux were calculated as \[\frac{\ln(R_f) - \ln(R_i)}{t_e} \] , where \(R_f\) is the percent of radioactivity remaining monolayer-associated at time \(t_e\), as reported elsewhere (Venglarik et al., 1990). Baseline-sensitive and bumetanide-insensitive components of ⁸⁶Rb uptake were used to determine Na⁺/K⁺2Cl⁻ cotransporter and Na⁺-K⁺-ATPase activity, respectively, as described elsewhere (Matthews et al., 1992). Results of ⁸⁶Rb uptake were corrected for the specific activity of K⁺ as described previously (Owen and Prastine, 1985).

Fluid transport assay. The methods for measuring monolayer fluid movement were adapted from those described by Smith and Welsh (Smith and Welsh, 1993) and as reported elsewhere (Zünd et al., 1996a). Cells were incubated in hypoxia or normoxia as described above for 24 h. In subsets of monolayers, the cAMP agonists forskolin (50 μM) and IBMX (100 μM) were added to the basolateral solution to promote fluid movement. The apical solution was collected and spun at high speed in an Eppendorf centrifuge, and the recovered fluid was weighed on a balance to determine volume.

Measurement of intracellular ATP. Confuent T84 monolayers on six well plates were exposed to the indicated experimental conditions and washed. After incubation, ATP was extracted from washed monolayers with ice-cold extraction buffer [2% trichloracetic acid and 2 mM EDTA], and lysates were sonicated and cleared by centrifugation at 10,000 × g for 5 min. ATP concentrations were determined using a luciferin/luciferase-based assay and a chemiluminometer (Chrono-log Corp., Havertown, PA) as previously described (Colgan et al., 1991).

Measurement of cAMP/cGMP. Confuent T84 monolayers on six well plates were exposed to the indicated experimental conditions and washed. After incubation, cells were cooled to 4°C, and nucleotides were extracted from washed monolayers with extraction buffer [66% EtOH, 33% HBSS containing the phosphodiesterase inhibitors IBMX, 5 mM (Sigma Chemical Co., St. Louis, MO)]. Lysates were then cleared by spinning at 10,000 × g for 5 min and dried under vacuum to remove EtOH. Samples were rehydrated in water, and cAMP or cGMP was quantified using displacement ELISAs (both from Amersham, Arlington Heights, IL) according to the manufacturer’s instructions. Nucleotide levels were expressed as picograms of cGMP/cAMP per microgram of total protein.

Assay of plasma membrane GC activity. Plasma membranes for assay of GC activity were prepared as described previously (Parakos et al., 1996), with modifications. Briefly, T84 cells grown to confluence on six well plates were cooled to 4°C, washed with HBSS, and scraped from the surface with a Teflon spatula into homogenization buffer consisting of 0.34 M sucrose, 10 mM HEPES, 1 mM ATP, 0.1 M EDTA, 1 mM dithiothreitol and protease inhibitors (chymostatin, aprotinin and PMSF). Scraped cells were homogenized with a dounce homogenizer at 4°C, nuclear debris was removed by centrifugation at 1000 × g for 45 min, and the membrane pellet was resuspended in phosphate buffered saline. Protein concentrations were determined using the Bradford assay (Bradford, 1976), and 50 μg total protein was added to cGMP assay buffer (1 mM EDTA, 5 mM MgSO4, 3 U/ml creatine kinase, 5 mM creatine phosphate, 5 mM IBMX, 1.5 mM GTP, pH 7.4) in the presence or absence of heat-stable enterotoxin from E. coli (STA, 100 ng/ml) and in the presence or absence of CoCl₂ (333 μM) or FeCl₃ (333 μM) for 15 min at 37°C. The reaction was terminated by incubation at 90°C for 10 min, samples were centrifuged at 14,000 × g for 5 min and cGMP from supernatants was measured as described above.

Pharmacological alterations of intracellular cGMP in epithelia. In subsets of experiments, intracellular cGMP levels were specifically diminished using 6-anilino-5,8-quinolinedione (LY383583, Biomol Inc., Plymouth Meeting, PA), which decreases intracellular cGMP but not cAMP levels (Schmidt et al., 1985), or elevated using 8-bromo-cGMP or dibutyryl-cGMP (Sigma). In both cases, normoxic or hypoxic epithelial monolayers were preexposed to agent for 30 min at 37°C, washed into HBSS containing equivalent concentrations of agent and assessed for forskolin (1 μM)-stimulated Cl⁻ secretion, as described above.
Data presentation. Electrophysiological fluid transport and cGMP data were compared by two-factor ANOVA, Student’s t test or Wilcoxon’s signed rank test, where appropriate. Values are expressed as the mean and S.E.M. of n monolayers from at least three separate experiments.

Results

Hypoxia down-regulates epithelial ion transport and fluid movement. As shown in figure 1, exposure of T84 epithelial monolayers to hypoxia resulted in a time- (fig. 1A, T$_{1/2}$~8 h, P < .001) and O$_2$-dependent (fig. 1B, EC$_{50}$ ~ 7% O$_2$, P < .001) decrease in cAMP-stimulated (forskolin, 1 μM) Cl$^{-}$ secretion. Similarly, basal unstimulated SCC levels were significantly diminished in hypoxic epithelia (1.74 ± 0.17 μA/cm$^2$) compared with normoxic controls (2.38 ± 0.16 μA/cm$^2$, P < .01; n = 34). Based on measurement of TER, and as we have shown previously (Colgan et al., 1996), such results were not explained by cellular toxicity (fig. 1A inset, P = not significant). Intracellular levels of ATP were not significantly diminished up to 24 h of hypoxia (ATP levels of 107 ± 18% and 94 ± 12% of normoxia control for 12 and 24 h, respectively, n = 3, P = not significant), but longer periods of hypoxia resulted in decreased ATP (68 ± 14% and 36 ± 7% of normoxia controls for 48 and 72 h; n = 3, P < .025). On the basis of these data, we selected 1% O$_2$ for a period of 24 h as our standard hypoxia exposure. Figure 1C demonstrates the influence of hypoxia on transepithelial fluid transport, the functional result of epithelial electrogenic Cl$^{-}$ secretion (Powell, 1987). As can be seen, epithelial exposure to hypoxia (1% O$_2$, 24 h) in the presence of forskolin (1 μM, 24 h) decreased overall fluid transport by 61 ± 6% compared with normoxia (P < .025). No measurable differences were observed in unstimulated fluid transport.

Epithelial electrogenic Cl$^{-}$ secretion can be stimulated by multiple agonists mediated by elevations in cAMP, cGMP or Ca$^{2+}$ (Barrett, 1993). As shown in table 1, we used a panel of Cl$^{-}$ secretagogues acting through different mechanisms to determine agonist specificity for hypoxia-elicited down-regulation of Cl$^{-}$ secretion. Epithelial exposure to hypoxia significantly diminished both cAMP-stimulated (forskolin, 8-bromo-cAMP, adenosine, 5’ AMP, VIP) and cGMP-stimulated (S7a) Cl$^{-}$ secretion but did not influence Ca$^{2+}$-stimulated Cl$^{-}$ secretion (carbachol, ionomycin). Such results indicate that hypoxia-induced responses are specific for cyclic nucleotide pathways of electrogenic chloride secretion.

We next determined whether hypoxia-elicited decreases in epithelial ion transport were evident at the level of channels and transporters distal to second messenger generation. To do this, we performed studies of $^{125}$I efflux and $^{86}$Rb uptake (in the presence and absence of bumetanide, an inhibitor of the Na$^{+}$-K$^{+}$-2Cl$^{-}$ cotransporter) (Madara et al., 1992b) after epithelial exposure to hypoxia (1% O$_2$, 24 h) or normoxia (21% O$_2$, 24 h). As shown in figure 2A, preexposure of epithelial monolayers to hypoxia significantly decreased forskolin-stimulated $^{125}$I efflux from T84 monolayers (P < .01). Such hypoxia-induced decreases were present at each time-point assessed (minutes 1–4) and were maximal at t = 4 min (36 ± 4% decreased compared with normoxia). Unstimulated efflux rate constants for $^{125}$I were not different for monolayers exposed to hypoxia compared with normoxia (rate constants of 0.027 ± 0.003 and 0.33 ± 0.004 min$^{-1}$ for normoxia and hypoxia, respectively, at $t = 4$ min, P = not significant).
TABLE 1
Hyoxia specifically down-regulates cyclic nucleotide-stimulated Cl\textsuperscript{−} secretion

T84 intestinal epithelial cells were grown to confluence on 0.33-cm\textsuperscript{2} permeable supports and exposed to normoxia (21% O\textsubscript{2}, 24 h) or hypoxia (1% O\textsubscript{2}, 24 h). Monolayers were washed and stimulated with indicated agonists at indicated concentrations. Values represent ΔSSC in response to each agonist for 8 to 12 monolayers in each condition.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Mechanism of Action</th>
<th>Concentration</th>
<th>ΔSSC (μA/cm\textsuperscript{2})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Normoxia (21% O\textsubscript{2})</td>
</tr>
<tr>
<td>Forskolin</td>
<td>cAMP</td>
<td>10 μM</td>
<td>45 ± 5.1</td>
</tr>
<tr>
<td>8-Bromo-cAMP</td>
<td>cAMP</td>
<td>1 mM</td>
<td>61 ± 10.8</td>
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<tr>
<td>Adenosine</td>
<td>cAMP</td>
<td>2 μM</td>
<td>28 ± 4.8</td>
</tr>
<tr>
<td>5′ AMP</td>
<td>cAMP</td>
<td>2 μM</td>
<td>36 ± 3.5</td>
</tr>
<tr>
<td>VIP</td>
<td>cAMP</td>
<td>1 nM</td>
<td>55 ± 11.0</td>
</tr>
<tr>
<td>STa</td>
<td>cGMP</td>
<td>100 ng/ml</td>
<td>7 ± 0.8</td>
</tr>
<tr>
<td>Carbachol</td>
<td>Ca\textsuperscript{2+}</td>
<td>100 μM</td>
<td>35 ± 12.1</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>Ca\textsuperscript{2+}</td>
<td>2 μM</td>
<td>54 ± 18.2</td>
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* Values significantly decreased compared with normoxia, P < .01.

Fig. 2. Hypoxia attenuates cAMP-stimulated Cl\textsuperscript{−} channel activity, Na-K-2Cl cotransporter activity and NaK-ATPase activity in T84 cells. Intestinal epithelial monolayers were exposed to normoxia (21% O\textsubscript{2}) or hypoxia (1% O\textsubscript{2}) for 24 h. A) After two 1-min washout periods, sequential 1-min rate constants of forskolin-stimulated \textsuperscript{125}I efflux from T84 cells were determined as described elsewhere (Venglarik \textit{et al.}, 1990). Asterisks indicate significant difference from normoxia control (P < .025). Data are pooled from 8 to 9 monolayers each, and results are expressed as the mean ± S.E.M. B) Forskolin (10 μM)-stimulated Na-K-2Cl cotransporter (bumetanide-sensitive component) and Na-K-ATPase (bumetanide-insensitive component) activity were measured by \textsuperscript{86}Rb uptake assays in the presence of 10 μM bumetanide. \textsuperscript{86}Rb uptake values were corrected for the indicated periods of time (0–240 min) and examined for forskolin (10 μM)-stimulated Cl\textsuperscript{−} secretion (measured as SSC). Data are pooled from four monolayers from duplicate experiments each, and results are expressed as the mean ± S.E.M.

In addition to significantly decreasing rate constants of efflux from epithelial monolayers, preexposure of T84 cells to hypoxia attenuated \textsuperscript{86}Rb uptake (fig. 2B) as measured by bumetanide-sensitive (a measurement that largely reflects activity of the Na-K-2Cl cotransporter) (Matthews \textit{et al.}, 1992) and bumetanide-insensitive (activity of the Na-K-ATPase, which also corresponds to the ouabain-sensitive component of \textsuperscript{86}Rb uptake) (Matthews \textit{et al.}, 1992) \textsuperscript{86}Rb uptake. Hypoxia reduced forskolin-stimulated cotransporter activity by 49% (76.6 ± 6.3 vs. 37.1 ± 4.37 mmol K\textsuperscript{+}/mg/min for control and hypoxia-exposed monolayers, respectively; n = 12, P < .01) and Na\textsuperscript{+}-K\textsuperscript{−}-ATPase activity by 54% (20.9 ± 2.45 vs. 9.7 ± 1.91 mmol K\textsuperscript{+}/min for control and hypoxia-exposed monolayers, respectively; n = 12, P < .025). Such attenuation in membrane transporter activity is comparable to the decrease in SCC observed after hypoxia (fig. 1). These data indicate that decreased electrogenic Cl\textsuperscript{−} secretion induced by hypoxia is also evident at ion transport events downstream of second messenger generation.

Partial reversibility by reoxygenation. To determine the reversibility of the inhibitory effects of hypoxia on cAMP-stimulated chloride secretion, we reoxygenated cells before measurement of forskolin-stimulated ion transport. Such conditions of reoxygenation (up to 4 h) were not toxic to epithelia (determined by measurement of transepithelial resistance, data not shown). T84 cells exposed to hypoxia alone for 24 h had diminished ability to secrete Cl\textsuperscript{−} secretion (measured as SCC). Data are not shown). T84 cells exposed to hypoxia alone for 24 h had diminished ability to secrete Cl\textsuperscript{−} secretion (measured as SCC). Data are not shown). T84 cells exposed to hypoxia alone for 24 h had diminished ability to secrete Cl\textsuperscript{−} secretion (measured as SCC). Data are not shown).
normally recovered to 57.8 ± 2.2% of normoxic controls by 240 min (P < .001; n = 4), which indicates that such hypoxia-elicited diminutions are at least partially reversible with reoxygenation.

Role for nucleotide cyclases. The foregoing data indicate that hypoxia-elicited decreases in electrogenic Cl− secretion are specific for cyclic nucleotide-stimulated responses. We next examined whether hypoxia directly influenced cellular GC and/or AC activity by measuring intracellular levels of cGMP and cAMP, respectively. As shown in figure 4, T84 monolayer exposure to conditions that result in diminished Cl− secretion (hypoxia at 1% O2, 24 h, see fig. 1) resulted in decreased basal levels of cGMP (52.2 ± 12.4% of normoxia; P < .04, n = 4) and decreased STa-stimulated cGMP (24.4 ± 14.9% of normoxic control; P < .05, n = 7). Such responses were not specific for GC, because both basal (61.0 ± 5.1% of normoxia; P < .001, n = 6) and stimulated (1 μM forskolin) cAMP (48.8 ± 5.8% of normoxia; P < .005, n = 6) were also inhibited by hypoxia.

Role for heme in epithelial oxygen sensing. A number of previous studies have indicated that metal ions can mimic hypoxia. Such studies are exemplified by basic understanding of erythropoietin regulation by hypoxia (Goldberg et al., 1988), which indicates that cellular exposure to metal ions such as cobalt, nickel and manganese mimic conditions of hypoxia. To investigate whether such metal ions would recapitulate hypoxia-elicited down-regulation of electrogenic Cl− secretion, we exposed T84 epithelia to CoCl2, NiCl2, MnCl2 or FeCl2 (concentration range 0–500 μM) under normoxic conditions (8 h of exposure) and assessed stimulated Cl− secretion (forskolin, 1 μM). As shown in figure 5, CoCl2 (P < .01, EC50 = 290 ± 59 μM) but not FeCl2 (P = not significant) dose-dependently inhibited forskolin-stimulated Cl− secretion. Similarly, both NiCl2 and MnCl2 (250 μM) inhibited cAMP-dependent SCC responses to 1 μM forskolin (44 ± 6% and 32 ± 6% decrease, respectively; P < .01, n = 8–11). Such results were not explained by cellular toxicity (fig. 5A inset, measured as a TER, an accurate and sensitive measure of epithelial viability (Dharmasathaphorn and Madara, 1990)). Moreover, much as in hypoxia (table 1), cobalt-elicited decreases in ion transport were specific for cAMP- (forskolin) and cGMP- (STa) but not Ca++-mediated (carbachol) agonists (fig. 5B). When compared with normoxia (ΔSCE = 43 ± 3.1 μA/cm²), less than additive decreases in ion transport were observed when epithelia were exposed to a combination of CoCl2 and hypoxia (16 h of hypoxia, plus 8 h of hypoxia and 250 μM CoCl2, 64 ± 8% decrease) as compared with cobalt alone (43 ± 6% decrease) or hypoxia alone (40 ± 4% decrease).

Finally, because CoCl2 but not FeCl2 mimicked hypoxia in intact epithelia, we determined whether CoCl2 would inhibit stimulated GC activity in a cell-free assay. As shown in figure 5C, STa-stimulated cGMP from purified plasma membrane preparations were inhibited significantly by the addition of CoCl2 (P < .025) but not FeCl2, at concentrations shown to inhibit in intact epithelia (333 μM). Addition of FeCl2 alone significantly elevated cGMP generation (51% increase over control), although not to the level of FeCl2 and STa (84% increase over control). Notably, no elevation in cGMP was observable over baseline using the soluble GC agonist sodium nitroprusside (cGMP of 250 ± 29 vs. 184 ± 15 fmol/μg cytosolic protein for control vs. 3 mM sodium nitroprusside, respectively; n = 3, P = not significant). These results, which are consistent with others (Currie et al., 1992), indicate that the particulate form of GC is predominant in T84 cells. Taken together, these data indicate that decreases in electrogenic Cl− secretion elicited by hypoxia can be mimicked in normoxic condition by the metal ions that bind to heme proteins, a result that suggests a role for heme moieties as epithelial oxygen sensors.

Interaction between cGMP and cAMP pathways during hypoxia. Unlike other cell types, cAMP and cGMP do not elicit opposing effects in intestinal epithelia, and in fact, epithelial cAMP and cGMP share a number of components in pathways for stimulation of epithelial Cl− secretion (Forte et al., 1992; Huott et al., 1988). Our data indicate a role for heme (fig. 5), and because GC activity involves heme protein(s) (Stone and Marletta, 1995), we determined whether pharmacological inhibition of cGMP elevation would influence cAMP-mediated Cl− secretion. As shown in figure 6A, addition of the cGMP inhibitor Ly 83583 (concentration range 0–30 μM) dose-dependently decreased forskolin-stimulated Cl− secretion (IC50 = 4.0 ± 0.7 μM; P < .001). In addition, Ly 83583 (10 μM) inhibited stimulated Cl− secretion over a range of forskolin concentrations (decrease of...
7.5%, 19.2%, 71.4% and 78.4% compared with no Ly 83583 control for forskolin concentrations of 0.001, 0.01, 0.1 and 1 μM, respectively). Ca**+**-stimulated (carbachol, 100 μM) Cl− secretion was not inhibited by Ly 83583 (ΔSSC = 10.6 ± 2.5 vs. 13.1 ± 2.4 for epithelial exposed to 10 μM LY83583 control, P < .001), which indicates specificity of Ly 83583 in cyclic nucleotide-mediated responses.

Incubation of epithelia with Ly 83583 resulted in decreased generation of cGMP (29.8 ± 9.2% decrease in basal cGMP at 10 μM, P < .025 compared with control) but not cAMP (8.9 ± 5.4% decrease, P = not significant compared with control). Furthermore, LY83583 did not influence baseline TER (fig. 6A, P = not significant). We next determined whether direct elevation of intracellular cGMP (i.e., bypassing GC) by using dibutyryl-cGMP or 8-bromo-cGMP would reverse hypoxia-elicited attenuation of forskolin-stimulated Cl− secretion. As shown in figure 6B, exposing epithelia to a combination of hypoxia and dibutyryl- or 8-bromo-cGMP resulted in significantly increased forskolin-stimulated SSC (P < .05 for 1 mM 8-bromo- or 1 mM dibutyryl-cGMP compared with analog-free controls). At these concentrations (0.1 and 1 mM), cGMP analogs alone did not stimulate epithelial Cl− secretion from either normoxic or hypoxic epithelia (data not shown). Notably, dibutyryl-cGMP (1 mM) also enhanced forskolin-stimulated Cl− secretion in normoxic epithelia (ΔSSC = 47.9 ± 3.7 and 64.9 ± 3.6 for no analog and 1 mM dibutyryl-cGMP, respectively, P < .05). These data suggest that hypoxia-induced decreases can, at least in part, be reversed by direct elevation of intracellular cGMP and indicate significant cross-talk between cAMP and cGMP at the level of ion transport in epithelia.

**Discussion**

The studies outlined here complement a growing literature regarding tight regulation of cellular responses during conditions of hypoxia. These studies highlight the important relationship between hypoxia and intracellular levels of cyclic nucleotide second messengers and reveal that coordinated endpoint function (electrogenic Cl− secretion) can be used as a marker of a hypoxia-elicited phenotype. Two novel observations are noteworthy. First, studies using cobalt to mimic hypoxia revealed that a heme moiety may serve as an epithelial oxygen sensor. Second, at the level of the epithelium, biochemical cross-talk pathways between the second messengers cAMP and cGMP are important during such responses.

Mucosal surfaces are lined by a monolayer of epithelia that provides tissue barrier and vectorial ion transport functions (Powell, 1981; Powell, 1987). Although epithelia are exposed to hypoxia in a number of disease states, only limited information is available about the direct impact of hypoxia on epithelial function. Previous studies demonstrated that renal tubule epithelia are, when compared with endothelia, quite sensitive to hypoxia and are rapidly and reversibly damaged (Tretyakov and Farber, 1995; Zimmerman et al., 1991). Furthermore, we have recently demonstrated that exposure of intestinal epithelia (T84 cells) to hypoxia modulates neutrophil-epithelial interactions and induces production and basolateral release of the proinflammatory cytokine interleukin-8 (Colgan et al., 1996). Thus our present results of decreased epithelial ion transport in response to hypoxia may serve as a mechanism of damping fluid loss (the endpoint function of electrolytic Cl− secretion) (Powell, 1987) during periods of mucosal hypoxia.

A number of previous studies, exemplified by original work with erythropoietin (Beru et al., 1986; Goldberg et al., 1988; Schuster et al., 1989; Tsujiya et al., 1993), have demonstrated a direct role for heme proteins in “sensing” extracellular oxygen concentrations. Such studies were substantially aided by the observation that a hypoxia-elicited phenotype can be mimicked in normoxia using cobalt, nickel or manganese, but not using iron (Goldberg et al., 1988). Although the exact mechanism of cobalt action on heme proteins has not been elucidated, a proposed model suggests that cobalt substitutes for ferrous iron within the porphyrin ring and locks heme into a deoxy state (Goldberg et al., 1988). Given that: a) both cobalt and metal ions specifically attenuate cyclic nucleotide-stimulated (but not Ca**+**-stimulated) Cl− secretion (fig. 5; table 1), b) in the absence of significant decreases in intracellular ATP, hypoxia decreases cAMP and cGMP (fig. 4) and c) exogenous addition of cGMP partially reverses the hypoxia phenotype (fig. 6), a potential target for cobalt and hypoxia in epithelia is heme moieties associated with cyclic nucleotide signal transduction pathways, such as GC. On the basis of our observation that reoxygenation results in...
6–12 monolayers from three experiments). System, diminishes O₂ from an ambient environment with initial differences in the models. Hypoxia, as defined in our lar ATP (normal glucose levels and only gradually depletes intracellularly.

cells pretreated (30 min) with 1 mM dibutyryl cGMP or 8-Br cGMP were hypoxia for 24 h before measurement of SCC responses to forskolin (1 μM). Hypoxia significantly inhibited responses to forskolin (P < .01), and cells pretreated (30 min) with 1 mM dibutyryl cGMP or 8-Br cGMP were significantly greater than in control monolayers (P < .05 in each case, n = 6–12 monolayers from three experiments).

Fig. 6. Inhibition of cGMP elevation attenuates cAMP-stimulated Cl⁻ secretion, and exogenous cGMP partially reverses hypoxia-induced decrease in Cl⁻ secretion. A) T84 monolayers were exposed to a range of concentrations of the inhibitor LY83583 for 30 min before measurement of SSC responses to forskolin (1 μM). Base-line TER was also measured (right vertical axis). B) T84 monolayers were exposed to normoxic or hypoxic for 24 h before measurement of SCC responses to forskolin (1 μM). Hypoxia significantly inhibited responses to forskolin (P < .01), and cells pretreated (30 min) with 1 mM dibutyryl cGMP or 8-Br cGMP were significantly greater than in control monolayers (P < .05 in each case, n = 6–12 monolayers from three experiments).

a rapid (~30 min), albeit partial, reversal of the hypoxia-mediated effect, it is unlikely that a significant reduction in enzyme level is responsible for the attenuation we observed in chloride secretion. Matthews et al. have recently demonstrated that conditions consistent with chemical hypoxia—namely, the use of metabolic inhibitors—resulted in extra-cellular loss of adenosine and, ironically, stimulation of electrogenic Cl⁻ secretion (Matthews et al., 1995). We have not observed the generation of spontaneous currents in epithelia exposed to hypoxia. Carveryer experiments of conditioned media derived from hypoxic epithelia to normoxic monolayers have not consistently resulted in generation of a Cl⁻ secretory response (data not shown). Discrepancies between these results are probably explained by the substantial differences in the models. Hypoxia, as defined in our system, diminishes O₂ from an ambient environment with normal glucose levels and only gradually depletes intracellular ATP (>24 h, see “Results”). The Matthews et al. model disrupted electron transport in low-glucose conditions and achieved depletion of cellular ATP levels by greater than 90% within 30 min. Thus it is possible that low levels of biologically undetectable adenosine are released over a longer period of time in our model. We have not directly addressed this issue.

The present results do not reveal the source of hemoglobin responsible for epithelial oxygen sensing. Evidence is provided that the hemoglobin moiety of GC and/or other heme molecules within cyclic nucleotide signal transduction may provide oxygen-sensing qualities. This issue is complicated by the fact that multiple forms of GC exist, including soluble GC, particulate GC and intestinal GC (Currie et al., 1992; Schulz et al., 1990). In a result consistent with previous reports (Currie et al., 1992), minimal soluble GC was observed in T84 cells. Although the various forms of GC are heme proteins, it is not known whether the active enzyme directly binds oxygen. Some evidence indicates that the heme of isolated bovine soluble GC, in fact, has the unique feature of not binding oxygen (Stone and Marletta, 1994). Additionally, Waldman et al. have demonstrated that specific porphyrins can differentially activate particulate and soluble GC (Waldman et al. 1984). With regard to our data, because hypoxia/cobalt inhibited Cl⁻ secretory responses to STa in intact cells (fig. 5; table 1), and because STa-stimulated cGMP from purified plasma membranes was inhibited by cobalt (fig. 5), it is likely that hypoxia influences at least the plasma membrane fraction of GC.

The present work lends insight into potential biochemical cross-talk pathways between cGMP and cAMP with regard to epithelial ion transport. Notably, hypoxia-elicited decreases in ion transport were specific for cyclic nucleotide- (cAMP and cGMP) but not calcium-stimulated ion transport (table 1), a result that suggests some degree of similarity in signaling. The observed decreases in forskolin-stimulated ion transport after specific inhibition of GC (fig. 6) directly implies significant cAMP/cGMP cross-talk at the level of epithelial ion transport, and direct elevation of intracellular cGMP in hypoxic cells normalized cAMP-stimulated responses. Unlike other cell systems, cGMP and cAMP signaling in epithelia do not appear to be antagonistic (Barrett, 1993), and in fact, some evidence suggests that shared pathways exist. Our data indicate that hypoxia inhibits cAMP signal transduction at all levels proximal (adenylate cyclase) and distal (inhibition of cAMP analog response; see table 1) to cAMP generation. Also, because this inhibitory effect is at least in part dependent on decreased cellular cGMP and is mimicked by cobalt, our results indicate a multifaceted epithelial response to hypoxia involving inhibition of regulatory heme proteins. A potential site for inhibition of cAMP responses dependent on cGMP includes protein kinase A, which is cross-activated by cGMP. Another potential target includes specific cAMP phosphodiesterases, which have been shown to be negatively regulated by cGMP (Acker, 1994). Evidence for such a pathway includes studies demonstrating that T84 cells do not possess specific cGMP-dependent protein kinases, so it is likely that responses to STa (i.e., elevation of cGMP) are mediated by cross-activation of protein kinase A (Forte et al., 1992); this would explain the lack of response to cGMP analogs alone and the enhanced responses to forskolin in both hypoxic and normoxic epithelia. Finally, a third candidate for such regulation is the cystic fibrosis transmembrane regulator (CFTR),
which is regulated by both cAMP and cGMP pathways during
electrogenic Cl− secretion (Barrett, 1993). T84 cells, used in
these experiments, have a well-defined CFTR (Gregory et al.,
1990). These findings indicate that pathophysiologically rel-


evant conditions such as hypoxia may directly influence intracellular signaling events, resulting in altered endpoint functional responses such as ion transport.

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