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**Research Article**

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# Activation of Cl Secretion during Chemical Hypoxia by Endogenous Release of Adenosine in Intestinal Epithelial Monolayers

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## Abstract

**Intestinal ischemia is characterized by rapid early inhibition of absorptive function and the appearance of net secretion, although why active secretion persists in the setting of a mucosal energy deficit is unknown. The cryptlike epithelial line T84, a well-characterized model of intestinal $Cl^-$ secretion, develops a prominent increase in short-circuit current ($I_{sc}$, indicative of active $Cl^-$ transport) in response to "hypoxia" induced by metabolic inhibitors. The increased $I_{sc}$ is associated with the initial decrease in monolayer ATP content. The $I_{sc}$ is transient and disappears with progressive energy depletion, although graded degrees of ATP depletion induce a more sustained $I_{sc}$ response. Chromatographic analysis and secretory bioassays show that the $I_{sc}$ response to metabolic inhibitors is related to the endogenous release of adenosine into the extracellular space in quantities sufficient to interact locally with stimulatory adenosine receptors. Unlike its classical role as a metabolic feedback inhibitor, adenosine appears to function as an autocrine "feed-forward" activator of active intestinal $Cl^-$ secretion. These studies suggest a novel role for adenosine in the conversion of the gut from an absorptive to a secretory organ during ischemic stress, thus contributing to the initial diarrheal manifestations of intestinal ischemia. (*J. Clin. Invest.* 1995. 96:117–125.) Key words: electrolyte transport • ischemia • diarrhea • purine • adenosine triphosphate**

## Introduction

Early in the course of acute mesenteric ischemia, active salt, and nutrient absorption from the intestine is inhibited. Curiously, despite the sudden deprivation of oxygen and nutrients, secretion from the intestinal crypts persists and may, in fact, be stimulated (1–7). Net intestinal absorption is replaced by secretion, resulting in significant intraluminal fluid sequestration and occasionally diarrhea as an initial manifestation (8). The volume of fluid and electrolytes that accumulates in the intestinal lumen may contribute to the mucosal hypoperfusion by exacerbating systemic intravascular depletion (4, 7). The basis for the differential sensitivity of intestinal transport functions to ischemia is not immediately apparent, and the mechanism for the activation of secretion in the face of a mucosal energy deficit remains a matter of speculation. In animal models, the appearance of net secretion precedes the development of histologic evidence of intestinal infarction or gross increases in intestinal permeability and is associated with submucosal vascular stasis (1–3, 5). Thus, one cannot explain the massive fluid losses solely by increased filtration of plasma through damaged mucosa (5). Alternatively, it has been postulated that secretion could be evoked by a substance released locally during ischemia, although the existence of such a secretagogue has never been demonstrated (6, 7, 9, 10).

While investigating the effect of chemical metabolic inhibitors on epithelial permeability in the human intestinal cryptlike cell line T84, we unexpectedly observed a large $Cl^-$ secretory current that developed during the initial phases of cellular adenosine triphosphate (ATP) depletion (11), an event highly reminiscent of the early diarrheal response of native intestine to ischemia. In the present study, we report that this paradoxical secretory response is due to the endogenous release of adenosine from the epithelial monolayer in quantities sufficient to interact locally with cell surface adenosine receptors in autocrine fashion. Unlike its proposed role as an inhibitory feedback regulator in most tissues (12–17), adenosine appears to function as a "feed-forward" activator of intestinal epithelial $Cl^-$ secretion during metabolic stress. These experiments suggest a novel role for adenosine as an autocrine factor which may contribute to the initial diarrheal manifestations of intestinal ischemia.

## Methods

*Cell culture and buffers.* T84 cells obtained from American Type Culture Collection (ATCC, Rockville, MD) were grown to confluence on collagen-coated permeable supports for 12- or 24-well culture dishes (Costar Corporation, Cambridge, MA) and maintained until steady-state transepithelial electrical resistance (TER)[1] was achieved (18, 19). Flasks were passaged weekly, and monolayers for experiments were fed every 2–3 d. Over 400 monolayers (7–14 days postplating) composed of cells from passages 59 through 90 were used for these experiments. Monolayers were bathed in a Hepes-phosphate buffered Ringer's solution (HPBR) containing 135 mM NaCl, 5 mM KCl, 3.33 mM $NaH_2PO_4$, 0.83 mM $Na_2HPO_4$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM Hepes, pH 7.4. For control experiments, HPBR additionally contained 10 mM glucose as metabolic substrate; in subsets of monolayers, ATP depletion was achieved by removal of substrate and addition of chemical metabolic inhibitors from concentrated stock solutions as described below.

*Transepithelial transport.* Transepithelial potential difference, TER, and short-circuit current ($I_{sc}$) were determined in monolayers grown on

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1. *Abbreviations used in this paper:* ADA, adenosine deaminase; DOG, 2-deoxyglucose; HPBR, Hepes-phosphate buffered Ringer's solution; $I_{sc}$, short-circuit current; OLI, oligomycin A; TER, transepithelial electrical resistance.

0.33 or 1.0 $cm^2$ inserts using a dual voltage-current clamp (University of Iowa, Iowa City, IA) and apical and basolateral Ag/AgCl and calomel electrodes interfaced via "chopstick" KCl-agar bridges as reported previously (20–23). The $I_{sc}$ response in monolayers grown on either 0.33 or 1.0 $cm^2$ filter supports to chemical hypoxia and to exogenous secretagogues was indistinguishable (data not shown). In the T84 cell model, $I_{sc}$ has been demonstrated repeatedly to be an accurate measure of electrogenic $Cl^-$ secretion under a wide variety of experimental conditions (18, 19).

*Monolayer ATP content.* After experimental manipulation, cells were extracted with ice cold 2% trichloroacetic acid and 2 mM EDTA, scraped with a rubber policeman, sonicated, and subjected to low-speed centrifugation. The supernatant was assayed for ATP content using a commercially available kit from BioOrbit (Turku, Finland) based on luciferin-luciferase chemiluminescence.

*Measurement of adenosine release from T84 monolayers.* The purine nucleotide adenosine was measured to a detection limit of 10.0±8.8 nM by reverse-phase high pressure liquid chromatography (HPLC). The chromatographic system included a model 802B Gilson Manometric Module (Middleton, WI), a model 811 Gilson Dynamic Mixer, a Rainin (Woburn MA) Rabbit-HP delivery system interfaced with a MacIntosh IICx computer, a Rainin C18 Microsorb-MV analytical column (4 mm I.D. × 100 mm length, 3 $\mu$m spherical particles, 100 Å pore size), and a Hewlett-Packard 1040A Variable Wavelength UV-Vis Detector (Fort Collins, CO) interfaced with a Hewlett-Packard 91538 computer. Samples (100 $\mu$l) were eluted isocratically with 14% aqueous methanol containing 58 mM $KH_2PO_4$ and 3.3 mM $(n\text{-}Bu)_4N^+$ $HSO_4$-(tetrabutylammonium hydrogen sulfate). The eluent was monitored at the adenosine absorbance maximum, 260 nm (0.001 absorbance units full scale, AUFS), at a flow rate of 1.0 ml-$min^{-1}$ at 2750 psi. A linear calibration curve for adenosine in HPBR in the ranges 10 to 1000 nM based on peak height was constructed ($r^2 = 0.982$). The identity of the adenosine signal was confirmed by signal enhancement following spiking of biological samples with authentic adenosine, by comparison with the retention time of known adenosine standards, and by demonstrating the secretagogue bioactivity of HPLC fractions containing the presumed adenosine peak. The adenosine peak could be readily distinguished from the peaks of other purine nucleotide degradation products, which were also identified by spiking and by comparison with standards. We concurrently measured the basolateral release of adenosine and the $I_{sc}$ in response to metabolic inhibition for monolayers grown on 1 $cm^2$ supports as a function of time. Controls at 5, 15, and 30 min in HPBR with glucose gave $I_{sc}$'s less than 5 $\mu$A-$cm^{-2}$, and HPLC analysis showed only low adenosine concentrations independent of time. Samples collected in the presence or absence of the adenosine deaminase (ADA) inhibitor deoxycoformycin (10 $\mu$M) contained identical concentrations of adenosine, indicating that there was no significant ADA activity in the extracellular buffer.

*Hypoxia-conditioned buffer experiments.* Monolayers grown on 0.33 $cm^2$ supports were exposed to HPBR containing 10 mM 2-deoxyglucose and 1 $\mu$M oligomycin A for 30 min, at which point the buffer bathing the basolateral surface was pooled and collected ("hypoxia-conditioned buffer"). Hypoxia-conditioned buffer was then applied to both surfaces of fresh, metabolically intact monolayers and $I_{sc}$ measurements were obtained as described above. In some experiments, adenosine deaminase (0.1 U/ml) was added to hypoxia-conditioned buffer, 30 min before applying this conditioned buffer to fresh monolayers.

*Lactate dehydrogenase (LDH) release.* After experimental manipulation, extracellular buffer was collected and LDH measured using a commercially available kit from Sigma Chemical Co. (St. Louis, MO) based on enzymatic oxidation of lactate and the simultaneous reduction of nicotinamide adenine dinucleotide, and an automated analyzer (COBAS; Roche Diagnostic System, Nutley, NJ). Total LDH was determined from 0.1% Triton X-100 extracts and LDH release expressed as percent of total LDH, as previously described (24).

*Materials and statistical analysis.* Deoxycoformycin was a gift of Parke-Davis (Ann Arbor, MI). All other chemicals were obtained from Sigma. Data are expressed as mean±SEM. Statistical analysis was by Student's *t* test for paired or unpaired variates, Mann-Whitney U test, and by 2-way analysis of variance (ANOVA), where appropriate, with $P < .05$ considered significant.

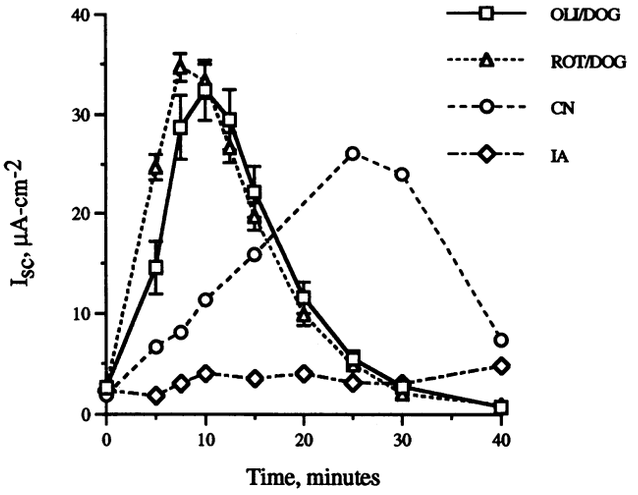


*Figure 1.* Effect of several different models of "chemical hypoxia" on transepithelial transport ($I_{sc}$ response) in T84 intestinal epithelial monolayers grown on permeable supports. Monolayers exposed to glucose-free HPBR containing 1 $\mu$M oligomycin A (OLI) and 10 mM 2-deoxyglucose (OLI/DOG, □, $n = 20$), 10 $\mu$M rotenone and 10 mM DOG (△, $n = 4$), and 10 mM NaCN in glucose-free HPBR (○, $n = 2$) all develop substantial increase in $I_{sc}$ which reaches a peak within 8–15 min. Monolayers exposed to 0.5 mM IA (◇, $n = 2$) do not develop an increase in $I_{sc}$. Control, untreated monolayers bathed in HPBR with 10 mM glucose (not shown) display a stable $I_{sc}$ of ~ 3 $\mu$A-$cm^{-2}$ for at least 90 min.

## Results

*Effect of metabolic inhibitors on transepithelial transport.* Unstimulated, control T84 monolayers grown to confluence on permeable supports display high TER and low spontaneous $I_{sc}$ in HPBR containing 10 mM glucose (e.g., TER = 910±60 Ω-$cm^2$ and $I_{sc}$ = 3.1±0.2 $\mu$A-$cm^{-2}$ for 15 representative monolayers). When T84 monolayers were transferred to glucose-free HPBR containing the mitochondrial inhibitor oligomycin A (OLI, 1 $\mu$M) and the non-metabolizable hexose 2-deoxyglucose (DOG, 10 mM), a sizable $I_{sc}$ invariably developed after an initial lag phase to reach peak values typically within 10 min of exposure to OLI/DOG (Fig. 1). The $I_{sc}$ induced by OLI/DOG was transient and declined to zero within 30–45 min of exposure to the metabolic inhibitors. The peak $I_{sc}$ achieved in response to OLI/DOG in glucose-free HPBR was 41.0±2.7 $\mu$A-$cm^{-2}$ (n = 29, $P < .0001$ compared to controls). Some degree of variability was observed in the length of "lag phase" before the appearance of the $I_{sc}$ (range: 3–10 min), the time to reach the peak $I_{sc}$ value (range: 8–15 min after exposure to OLI/DOG), and the total duration of the $I_{sc}$ response (range: 10–20 min); this variability may reflect differences in cell passage, monolayer age, or the timing of monolayer refeeding prior to experimentation. However, monolayers of identical age and passage exhibited remarkably uniform $I_{sc}$ responses to OLI/DOG. Consistent with previous findings (11), the $I_{sc}$ was inhibitable by 30 $\mu$M bumetanide (e.g., peak $I_{sc}$ = 4.5±0.2 $\mu$A-$cm^{-2}$, $n = 3$) and was not observed in $Cl^-$ free (gluconate)

*Table I. Effect of Metabolic Inhibitors on Transepithelial Transport in T84 Monolayers*

| Condition* | Peak $I_{SC}$, $\mu A\text{-}cm^{-2}$ | Time of peak, min§ | *n* |
|---|---|---|---|
| No inhibitors, 10 mM glucose | 3.1±0.17 | — | 15 |
| No inhibitors | 3.0±0.6 | — | 8 |
| OLI/DOG | 41.0±2.7‡ | 7.5–15 | 29 |
| DOG | 15.6±1.4‡ | 20 | 7 |
| OLI | 20.3±3.1‡ | 25–50 | 15 |
| CN | 22.5±1.7‡ | 15–25 | 5 |
| IA | 3.9±0.9 | — | 5 |
| ROT/DOG | 34.7±1.2‡ | 7.5 | 4 |

* Monolayers bathed in glucose-free HPBR with inhibitors added as indicated. OLI, oligomycin A, 1 $\mu$M; DOG, 2-deoxyglucose, 10 mM; CN, cyanide, 10 $\mu$M; IA, iodoacetate, 1 mM ROT, rotenone, 10 $\mu$M. ‡ $P < .0001$ compared with HPBR, 10 mM glucose. § Time to reach peak $I_{SC}$ after exposure to conditions indicated in first column.

buffer (peak $I_{sc}$ = 1.0±0.06 $\mu A\text{-}cm^{-2}$, $n$ = 3), indicating that the OLI/DOG-elicited $I_{sc}$ indeed reflects electrogenic $Cl^-$ secretion.

A similar $I_{sc}$ was elicited by KCN (10 mM) in glucose-free HPBR, by rotenone (10 $\mu$M) plus DOG, by OLI alone in glucose-free HPBR, and by DOG alone in glucose-free HPBR (Fig. 1 and Table I). Monolayers exposed to glucose-free HPBR without additional chemical metabolic inhibitors did not develop an increase in $I_{sc}$ over a 60-min observation period. Interestingly, when "chemical hypoxia" was induced by iodoacetate (IA, 0.01–1 mM) in glucose-free HPBR, no increase in $I_{sc}$ was observed.

Because OLI (without DOG) in glucose-replete HPBR did not induce an increase in $I_{sc}$ whereas OLI in glucose-free HPBR elicited a sizable secretory current, subsets of monolayers were exposed to OLI in HPBR containing graded concentrations of glucose (0.1–10 mM) in order to further characterize the conditions necessary to produce the secretory response. As shown in Fig. 2, the presence of small concentrations of metabolic substrate prolonged the lag phase, decreased the peak current achieved, and prolonged the duration of the secretory response. For example, in HPBR containing 0.1 mM glucose, the peak secretory current did not appear for 30 min, and the peak $I_{sc}$ was ~ 80% that achieved with OLI/DOG, but the secretory response was substantially prolonged, persisting for at least 1 h.

TER typically showed a biphasic response to "chemical hypoxia." The initial increase in $I_{sc}$ was invariably associated with a rapid fall in TER. After monolayers reached peak $I_{sc}$, TER usually began to return toward control values as the $I_{sc}$ dissipated. Subsequently, TER began to decrease again, ultimately settling at ~ 50% of control values within 60–90 min. In many experiments, however, the transient recovery of TER was not seen, and TER continued to slowly decline. We previously showed that the eventual decrease in TER is due to enhanced transjunctional permeability (11). The initial rapid decrease correlates closely with the appearance of the $I_{sc}$, and thus may largely reflect opening of transcellular pathways for passive ion movement. Monolayers treated with iodoacetate failed to produce an $I_{sc}$ response but displayed a profound fall

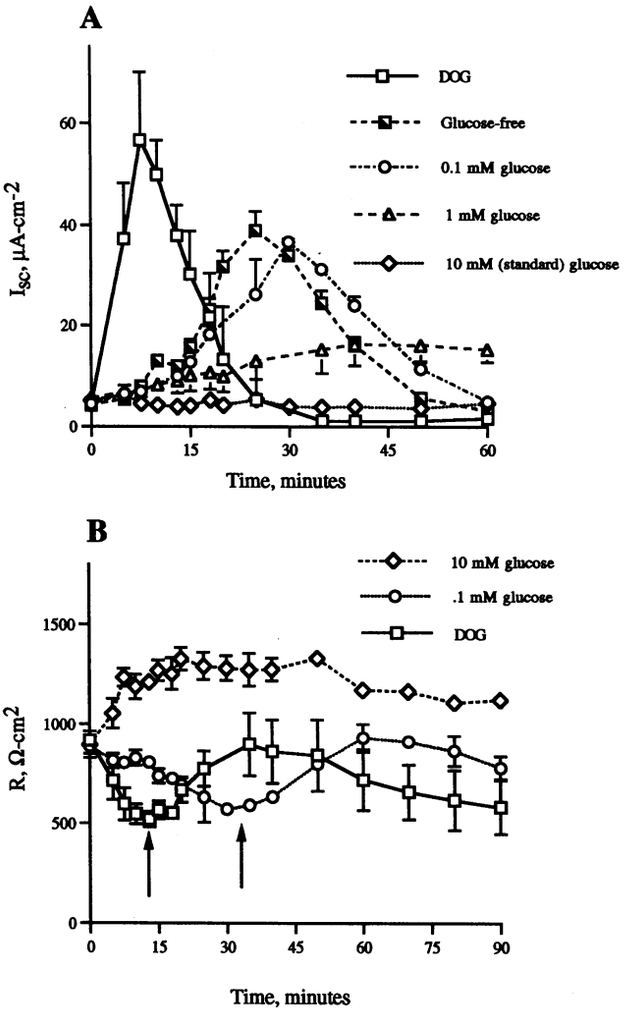


*Figure 2.* (*A*) $I_{sc}$ response of T84 monolayers to 1 $\mu$M oligomycin (OLI) with graded amounts of metabolic substrate. Earliest and highest peak $I_{sc}$ is seen with glucose-free HPBR containing 10 mM 2-deoxyglucose (DOG, □). $I_{sc}$ response is delayed in onset and reduced in peak value for glucose-free HPBR without DOG (◪) or for HPBR containing 0.1 mM glucose (○). With 1 mM glucose (△), a smaller, more sustained $I_{sc}$ response is seen which is substantially delayed in onset. No $I_{sc}$ response is observed with OLI in standard 10 mM glucose HPBR (◇). Data points are from representative experiment performed in triplicate, with similar results in two additional experiments. (*B*) Transepithelial resistance (TER) response during ATP depletion in T84 monolayers; same experiment as in A. Monolayers exposed to OLI/DOG (□) show biphasic response with initial decrease in TER correlated with appearance of maximal secretory current (*arrow*) followed by transient increase associated with dissipation of $I_{sc}$. With prolonged exposure to OLI/DOG, TER eventually continues to decrease slowly over a 90-min observation period (note difference in time scale from *A*). Monolayer exposed to OLI and 0.1 mM glucose-containing HPBR (○) shows similar biphasic response, with initial fall in TER again correlating with peak $I_{sc}$ (*arrow*). Control monolayers in OLI plus 10 mM HPBR (◇) show initial small increase in TER, with stable values over 90 min of observation.

in TER, suggesting that maintenance of cellular energy stores is essential for junctional integrity in T84 cells, consistent with our earlier report (11) and the data of others (25).

Measurement of LDH release during chemical hypoxia indi-

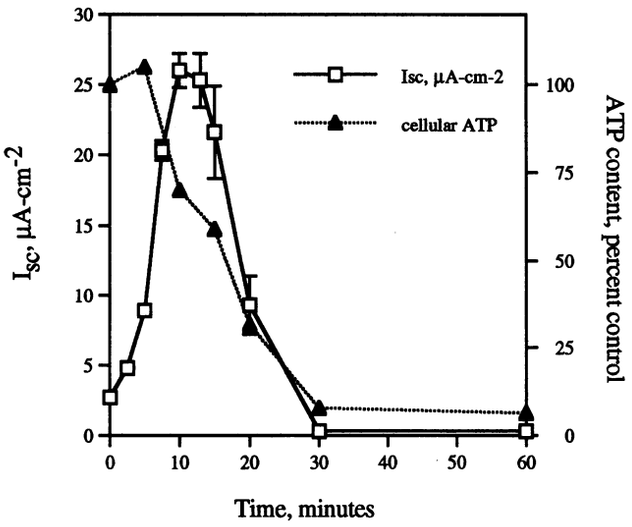


*Figure 3.* Comparison of time course of monolayer ATP depletion with $I_{sc}$ response to "chemical hypoxia" induced by 1 μM oligomycin A and 10 mM 2-deoxyglucose. $I_{sc}$ data (□) are mean±SEM for four monolayers. ATP content (▲) from duplicate monolayers of same passage treated in parallel ($n$ = 14), expressed as percentage of control for monolayers bathed in HPBR with 10 mM glucose. Control monolayers contained 41.2±3.6 nmol ATP-mg protein$^{-1}$ ($n$ = 10).

cated the absence of gross monolayer toxicity (e.g., 2.5±0.2% vs. 2.2±0.1% for monolayers exposed to OLI/DOG vs. standard HPBR for 60 min, respectively, each $n$ = 3). Moreover, short-term chemical hypoxia induced by OLI/DOG was largely reversible, based on functional recovery of a cAMP secretory response. For example, monolayers treated for 20 min with OLI/DOG (a period sufficient for completion of the hypoxia-induced $I_{sc}$ response) showed no $I_{sc}$ response to 10 μM forskolin. However, a substantial response to this agonist was restored after a 30-min recovery period in HPBR containing 10 mM glucose (38.3±3.3 μA-cm$^{-2}$, $n$ = 3).

*Effect of metabolic inhibitors on monolayer ATP content.* The ATP content of T84 monolayers bathed in glucose-replete (10 mM) HPBR devoid of metabolic inhibitors remained constant for up to 1 h (data not shown). In contrast, the ATP content of monolayers bathed in glucose-free HPBR containing OLI/DOG was rapidly depleted, reaching ~ 5% of control values by approximately 30 min (Fig. 3). The $I_{sc}$ induced by these conditions reached a peak at a point where monolayer ATP content was approximately 70% of control values; the $I_{sc}$ subsequently declined in parallel with progressive ATP depletion and was abolished as ATP content reached its nadir. Not surprisingly, monolayers depleted of ATP in this fashion displayed no secretory response to subsequent addition of 10 μM forskolin. Lesser degrees of ATP depletion were achieved in monolayers exposed to OLI in HPBR containing graded concentrations of glucose; for example, the ATP content of monolayers exposed to OLI in HPBR containing 0.5 mM glucose declined to only 69±2.1% control after 60 min, associated with a small $I_{sc}$ (8.9±0.9 μA-cm$^{-2}$, $n$ = 6). Substantial ATP depletion was also induced by IA in glucose-free HPBR, although, as noted above, IA did not induce a secretory current.

*Mechanism of activation of $Cl^-$ secretion by metabolic inhibitors.* Our initial hypothesis, based on earlier reports suggesting that reactive oxygen metabolites evoked $Cl^-$ secretion in T84 monolayers by a $Ca^{2+}$-dependent mechanism (26), was that ATP depletion would perturb intracellular $Ca^{2+}$ homeostasis, leading to an increase in intracellular [$Ca^{2+}$], and thereby activate $Ca^{2+}$-dependent ion transport pathways (e.g., basolateral $K^+$ channels). However, we found that the $I_{sc}$ elicited by OLI/DOG in glucose-free buffer was not significantly altered by a number of manipulations that inhibit the secretory response to typical $Ca^{2+}$-mediated agonists: i.e., by reducing extracellular $Ca^{2+}$ to 0.1 mM (peak $I_{sc}$ = 83±10% of that of monolayers bathed in standard 1 mM $Ca^{2+}$, $n$ = 12), by prior exposure to the $Ca^{2+}$-ATPase inhibitor thapsigargin (90±9%, $n$ = 8), or by treatment of monolayers with $La^{3+}$ with or without thapsigargin (90±11% and 102±4%, respectively, $n$ = 4).

Given these findings, we considered the possibility that the secretory response to metabolic inhibitors could be due to an endogenous substance released into the extracellular space by the cell monolayers during ATP depletion. In support of this, we found that exposure of a control monolayer to the buffer obtained from an OLI/DOG treated monolayer evoked a rapid increase in $I_{sc}$ (28.5±2.3 μA-cm$^{-2}$ at 1 min, 45.3±4.4 at 10 min, $n$ = 4), indicating that hypoxia-conditioned buffer (from an ATP-depleted monolayer) possessed a factor that could immediately initiate a secretory response from metabolically intact cells without the typical 3–10-min lag phase observed prior to the beginning of the $I_{sc}$ response induced by nonconditioned OLI/DOG buffer. We then found that incubation of this hypoxia-conditioned buffer with adenosine deaminase (ADA, 0.1 U/ml) for 30 min essentially abolished its prosecretory effects on control monolayers (i.e., $I_{sc}$ = 5.9±0.4 μA-cm$^{-2}$ at 10 min, $n$ = 8). This finding suggested that extracellular adenosine might be responsible for the "hypoxic" $I_{sc}$.

In further support of this notion, we found that the secretory response to OLI/DOG was abolished by the adenosine receptor antagonist 8-phenyltheophylline (Fig. 4) and was significantly attenuated by ADA ($n$ = 2). Furthermore, also as shown in Fig. 4, the hypoxic $I_{sc}$ was completely prevented by prior treatment of monolayers for 48 h with media containing 3 μM 5′-(N′-ethylcarboxamido) adenosine (NECA, a nonmetabolizable analogue of adenosine). Such treatment has been shown by others to virtually abolish the secretory response of T84 cells to exogenous adenosine (27).

Taken together, these functional data strongly supported the hypothesis that the $I_{sc}$ response to metabolic inhibition was due specifically to the release of adenosine by the hypoxic epithelial cells. Direct evidence of the accumulation of adenosine in the extracellular buffer was then obtained by chromatographic analysis of buffer supernatant collected after varying periods of exposure to OLI/DOG (Fig. 5 *A*). We observed the time-dependent appearance of three major peaks. The peak with the longest retention time corresponded to adenosine. The middle peak co-eluted with authentic samples of inosine, hypoxanthine, and xanthine, and may therefore represent any combination of these three purine degradation products. Because each of these is inactive as a secretagogue in T84 cells in concentrations up to 5 μM (data not shown), we did not pursue complete characterization of this peak. The peak with the shortest retention time also did not show secretagogue activity (see below) and was not identified. The amount of adenosine released after a 30-min exposure to OLI/DOG (~ 1 nmol for a 1 cm$^2$ insert, $n$ = 5) represented ~ 10% of the original ATP pool (~10 nmol/cm$^2$). Adenosine was released into both the basolateral and apical

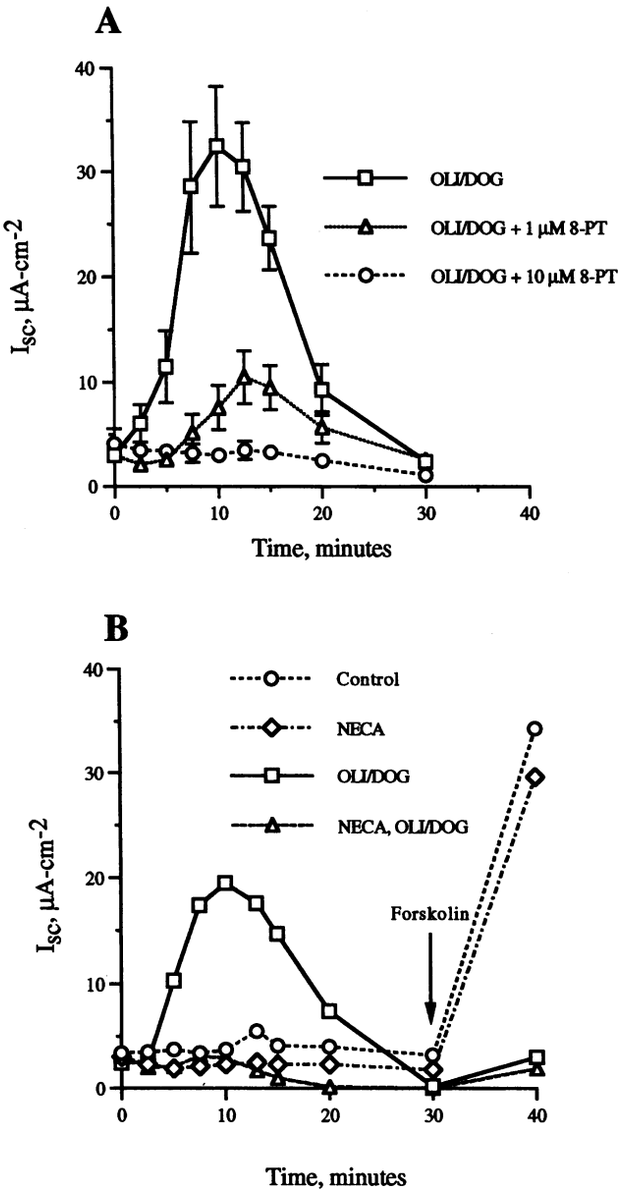


*Figure 4.* (*A*) $I_{sc}$ response to "chemical hypoxia" induced by oligomycin A and 2-deoxyglucose (OLI/DOG) is largely attenuated by 1 μM 8-phenyltheophylline (8-PT) and abolished by 10 μM 8-PT. Mean±SEM for $n$ = 6–8 each group. (*B*) Monolayers pre-treated for 48 h with 3 μM 5′-(N′-ethylcarboxamido) adenosine (NECA) do not show an $I_{sc}$ response to OLI/DOG. Control monolayers (○) and NECA-treated monolayers (◇) display identical $I_{sc}$ responses to the cAMP agonist forskolin (10 μM, added at *arrow*), indicating general preservation of secretory responses in NECA-treated cells. While control monolayers exposed to OLI/DOG (□) show typical $I_{sc}$ response, no such response is seen in NECA-treated cells (△). OLI/DOG-treated monolayers (with or without prior NECA) do not respond to forskolin after 30 min (*arrow*), indicating depletion of functional energy stores. All $n$ = 3, SEM too small to appear in figure.

buffers; the amount of adenosine released apically was ~ 25% of that released basolaterally (data not shown).

The time course of adenosine release by T84 monolayers into the basolateral buffer and the $I_{sc}$ response of the same monolayers is shown in Fig. 5 *A*. The onset of the $I_{sc}$ response occurred synchronously with the appearance of adenosine in the extracellular buffer (Fig. 5 *B*), and the adenosine concentration thereafter increased in concert with the $I_{sc}$. Comparison of the secretory responses of metabolically inhibited monolayers with the dose-response of metabolically intact monolayers to exogenous application of adenosine (Fig. 5 *C*) suggests that the amount of adenosine released into the bulk solution during treatment with OLI/DOG is sufficient to account for the magnitude of the observed $I_{sc}$. Additionally, in control monolayers and hypoxic monolayers before the appearance of the $I_{sc}$, the concentration of extracellular adenosine was at or below the threshold level for inducing a sizable $I_{sc}$ in metabolically intact cells, also as suggested by comparison with Fig. 5 *C*.

We caution, however, that comparison of the adenosine dose-response curve in Fig. 5 *C* with the adenosine release curve and associated $I_{sc}$ in Fig. 5 *A* is not simple. For example, the secretory response to endogenously released adenosine in cells with rapidly falling ATP levels is probably different than the secretory response to exogenous adenosine in cells with stable ATP levels. Moreover, precise correlation of the $I_{sc}$ response with the time course of adenosine release into the extracellular bulk solution is complicated by the fact that the concentration of adenosine in the bulk solution probably does not reflect the concentration of adenosine in the boundary region of the plasma membrane (in the immediate vicinity of high-affinity membrane adenosine receptors) under these non-equilibrium conditions. Specifically, the local concentration of adenosine in the immediate vicinity of the plasma membrane, where adenosine receptor density is high, is likely to be higher than in bulk solution. One expects this in general for paracrine or autocrine factors, as dilution into a large extracellular volume serves to limit the action of such substances to the immediate local environment.

We tested this notion with experiments in which the volume of the extracellular buffer was doubled or tripled, thereby proportionately decreasing the concentration of adenosine in the bulk solution. If the increase in buffer volume does not affect the rate of adenosine release at the plasma membrane surface, then this volume increase should have only limited impact on the adenosine concentration in the microenvironment of the plasma membrane. Consequently, this volume change should have little impact on the observed $I_{sc}$. As expected, this manipulation did not substantially alter the peak $I_{sc}$ induced by OLI/DOG treatment (mean peak $I_{sc}$ = 23.9, 20.9, and 22.0 μA-cm$^{-2}$ for basolateral buffer volumes of 0.4, 0.8, and 1.2 ml, respectively, for $n$ = 2 in each group). More profound dilution and rapid stirring of the extracellular buffer reduced but did not eliminate the $I_{sc}$ response. The peak $I_{sc}$ in OLI/DOG-treated monolayers mounted in modified Ussing chambers (in which a 4-mL chamber volume was rapidly circulated by a gas-lift apparatus was 8.8±1.5 μA-cm$^{-2}$ compared to 3.6±1.4 for untreated controls ($P < .03$ for $n$ = 6).

To further support the hypothesis that the hypoxic $I_{sc}$ is due to endogenously released adenosine, fractions of HPLC effluent were collected, lyophilized, and redissolved in 200 μl HPBR. Secretagogue bioactivity was found to be confined to the fraction corresponding to the adenosine peak. To determine whether the adenosine identified by HPLC in the OLI/DOG supernatant derived from extracellular hydrolysis of AMP or ADP, monolayers were incubated in OLI/DOG buffer containing the 5′-ectonucleotidase inhibitor α, β-methylene-ADP; the $I_{sc}$ evoked was unaffected by such treatment (46.8±4.1 μA-cm$^{-2}$, $n$ = 3).

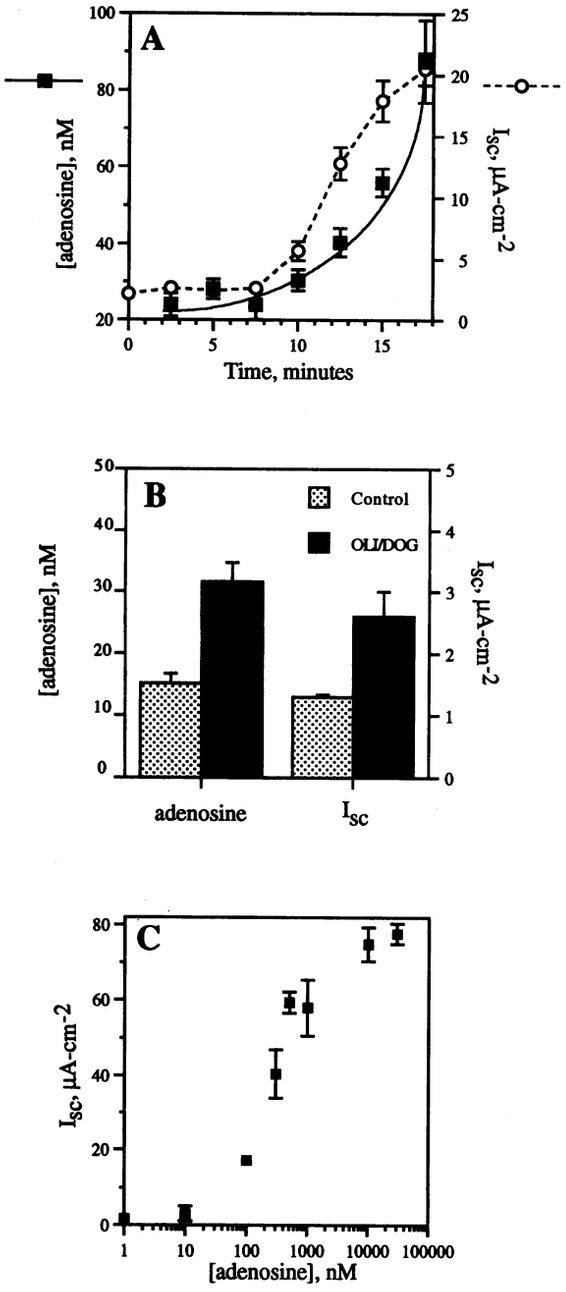


*Figure 5.* (*A*) Comparison of time-dependent adenosine release into basolateral-side buffer during chemical hypoxia (oligomycin A and 2-deoxyglucose, OLI/DOG) and comparison with $I_{sc}$ response. Monolayers grown on 1 $cm^2$ permeable supports were washed in glucose-free HPBR and then transferred to wells containing 0.375 mL HPBR with OLI/DOG at time zero; 0.3 mL of the same solution was added to the apical well. The basolateral buffer was collected and analyzed for adenosine content by HPLC (mean±SEM for $n$ = 3–12 monolayers at each time point). The left-sided vertical axis indicates in nM the concentration of adenosine released into basolateral buffer and corresponds to the filled squares. The curve is drawn by eye. The right-side vertical axis represents $I_{sc}$ in μA-$cm^{-2}$ and corresponds to the open circles which indicate the $I_{sc}$ response in the same monolayers (mean±SEM for $n$ = 4–22 at each time point). Note that these monolayers display a lag phase of nearly 10 min prior to developing an $I_{sc}$ response to OLI/DOG. (*B*) Adenosine concentration in basolateral buffer and $I_{sc}$ response with respect to matched controls at the time of onset of the hypoxic $I_{sc}$ at $t$ = 10 min. Monolayers on 1 $cm^2$ supports were washed in HPBR without glucose and transferred to wells containing 0.375 mL HPBR with OLI/DOG, with 0.3 ml of the same solution added apically. Controls were washed with HPBR plus glucose and placed in HPBR with glucose at identical volumes. At the 10 min time point, $I_{sc}$ (indicated on right-sided vertical axis) began to rise in the OLI/DOG group (■) but not the controls (▨) and was uniformly higher in OLI/DOG monolayers compared with control ($n$ = 6, $P$ = 0.02, Mann-Whitney U-test). At this 10 min time point, the basolateral buffer from these monolayers was immediately collected for HPLC analysis. Adenosine concentration (left-sided vertical axis) in the basolateral buffer from these two groups of monolayers is shown ($n$ = 6, $P$ < .02, unpaired Student's $t$ test). Thus, the extracellular adenosine concentration is two-fold elevated over controls at the time of onset of the $I_{sc}$ response to OLI/DOG. (*C*) Dose-response of $I_{sc}$ to exogenous application of adenosine in metabolically intact T84 monolayers grown on 1 $cm^2$ supports. Adenosine at the indicated concentrations was applied to both the apical and basolateral buffer and steady state (8–10 min) $I_{sc}$ values were recorded ($n$ = 3–4 for each point). Threshold level for activation of $I_{sc}$ by exogenous adenosine is ~ 20–30 nM.

Nitrobenzylthioinosine (NBTI, 1 μM), an inhibitor of facilitated membrane transport of purine nucleosides, did not greatly attenuate the $I_{sc}$ evoked by OLI/DOG (44±11 vs. 31±3.9 μA-$cm^{-2}$ for OLI/DOG vs. OLI/DOG plus NBTI, $n$ = 3, n.s.), suggesting that the appearance of adenosine in the extracellular space occurs largely by free diffusion of adenosine from the cytosol rather than facilitated transport. However, NBTI itself proved to be a weak secretory agonist (peak $I_{sc}$ ~ 15 μA-$cm^{-2}$, $n$ = 3). Finally, we determined that monolayers treated with IA did not accumulate substantial adenosine in the extracellular buffer even after 60 min (data not shown); as noted above, monolayers treated by IA invariably failed to develop a significant increase in $I_{sc}$. Furthermore, no $I_{sc}$ could be elicited by the combination of IA plus deoxycoformycin (10 μM) to inhibit ADA ($n$ = 2).

## Discussion

The biologic effects of adenosine are many, reflected by the widespread distribution of adenosine receptors throughout endothelial, mesothelial, and epithelial cell systems (28). Representing a product of stepwise ATP dephosphorylation, adenosine has long been viewed as a regulatory link that serves to balance cellular energy supply and demand (12–17, 29–31). Thus, according to this hypothesis, a decrease in cellular energy availability due to hypoxia or increased cellular ATP consumption leads to the intracellular accumulation of ATP metabolites; adenosine formed in this manner can traverse cell membranes through passive or facilitated diffusion and then interact with cell surface receptors to restore energy balance (28). Local responses to adenosine are mediated by two major subclasses of receptors, $A_1$ and $A_2$. As a general rule, the higher-affinity $A_1$ receptor, negatively coupled to adenylate cyclase via an inhibitory G protein, mediates events that inhibit cellular and organ work and thus decrease oxygen demand. The stimulatory G protein-linked $A_2$ receptor is typically found in vascular tissue and mediates events such as local vasodilatation which enhance oxygen delivery (28). Adenosine has been found to exert metabolic inhibitory control in this fashion in kidney, heart, brain, and skeletal muscle in response to hypoxia and/or increased work (12, 13, 15–17, 28, 30–34). Recently, endogenous aden-

osine has been identified as an autocrine feedback inhibitor of $Cl^-$ secretion in shark rectal gland (15), an important experimental preparation widely used as a model of secondary active $Cl^-$ transport in the mammalian kidney.

In contrast to this classical adenosine paradigm, the present studies provide direct evidence of adenosine-mediated activation of cellular work (ion transport) during ATP depletion in the human cryptlike intestinal epithelial cell line T84. An early marked stimulation in $I_{sc}$ was observed that correlated with the initial decrease in cellular ATP content; this current persisted until ATP levels fell toward 20% of control values. By modifying the conditions used to induce metabolic inhibition, graded degrees of cellular ATP depletion were achieved that induced more sustained activation of $Cl^-$ secretion. The hypoxic $I_{sc}$ was not affected by a variety of conditions that inhibit $Ca^{2+}$-mediated secretory responses. The extracellular buffer obtained from ATP-depleted monolayers promptly activated secretion when applied to metabolically-intact monolayers, and this pro-secretory activity could be ablated by ADA. The hypoxic secretory current was abolished by 8-PT, a competitive inhibitor of cell surface adenosine receptors, and by prior down-regulation of adenosine-mediated secretory responses by prolonged exposure to NECA. Chromatographic analysis indicated that adenosine indeed accumulated extracellularly in substantial quantities during metabolic inhibition, and the secretagogue bioactivity of the extracellular buffer from ATP depleted cells was confined to the adenosine-containing fraction. The appearance of adenosine was largely unaffected by the 5′-ectonucleotidase inhibitor $\alpha$, $\beta$-methylene-ADP or by an inhibitor of facilitated nuceloside transport, suggesting first that the adenosine derives from intracellular sources and not from extracellular hydrolysis and, second, that its release occurs at least in part by an NBTI-insensitive pathway, possibly by free diffusion.

Adenosine is known to activate $Cl^-$ transport pharmacologically in distal colon and ileum (27, 35–37). It is also a potent agonist in T84 intestinal epithelial cells (27, 36). Thus its action is likely direct and does not require the secondary release of additional substances from adjacent non-epithelial cells. Because adenosine is rapidly degraded by the ubiquitous enzyme ADA, it is thought that in most organ systems adenosine acts as a paracrine or autocrine mediator rather than as a circulating hormone (28). In fact, a substantial (millimolar) concentration of exogenous adenosine is required to elicit $Cl^-$ secretion in native intestinal preparations unless the ADA inhibitor deoxycoformycin is also included (35). Because ADA is abundant in intestine compared to other organs (38), the autocrine and paracrine roles of adenosine may be of particular relevance in the gut. Mast cells, phagocytes, lymphocytes, and other cell types are known to be sources of preformed adenosine, and its local release as a paracrine factor has been postulated to contribute to the secretory diarrhea associated with active intestinal inflammation and certain immune-related diarrheal disorders (27, 36, 37). Activated neutrophils have also been shown to release 5′-AMP, a potent agonist that requires conversion to adenosine at the epithelial cell surface by an ecto-5′-nucleotidase to exert its secretagogue effects (23, 39). Strohmeier et al. recently provided evidence, based on Northern blot analysis and an examination of agonist hierarchy, that the major adenosine receptor subtype present in both the apical and basolateral membrane domains of T84 cells and in native human colon is the recently-cloned stimulatory $A_{2b}$ receptor (40). Thus, unlike most working tissues, in which the predominant adenosine receptor is the inhibitory $A_1$ subtype (28), the human intestinal crypt epithelial cell under normal circumstances is apparently poised to activate its cellular machinery (i.e., electrogenic $Cl^-$ transport) in response to locally released adenosine by virtue of possessing a stimulatory receptor subtype.

Ischemia—cessation of blood flow—results in both hypoxia and substrate deprivation to the affected tissue. Occlusion of the superior mesenteric artery of rats has been shown to reduce intestinal tissue ATP content by 60–80% within 20 min (41, 42). Using a variety of pharmacologic agents to achieve similar degrees of ATP depletion in the T84 cell model, we demonstrate that substantial quantities of adenosine are released and that this adenosine retains secretagogue activity until cellular ATP content falls below ~ 20% of control values. However, when metabolic inhibition was achieved by IA, in contrast to DOG or substrate deprivation, adenosine did not accumulate extracellularly and $Cl^-$ secretion was not stimulated despite comparable cellular ATP depletion. This disparity can perhaps be explained by the two competing pathways that exist for the intracellular catabolism of ATP (29, 31, 43, 44). ATP may be degraded via sequential hydrolysis (i.e., ATP → ADP → AMP → adenosine), followed by ring deamination to inosine or, alternatively, ring deamination may occur at the level of AMP rather than adenosine (i.e., ATP → ADP → AMP → IMP → inosine). These two pathways for ATP catabolism, which diverge at the level of AMP, depend in large part upon the relative activities of intracellular 5′-nucleotidase and AMP deaminase. In human erythrocytes, IA has been shown to induce ATP degradation almost exclusively via IMP and not through adenosine, suggesting that IA selectively enhances AMP deaminase (29). Because AMP deaminase is strongly inhibited by 2,3-diphosphoglycerate (DPG) (45), it is postulated that IA (which inhibits glyceraldehyde-3-phosphate dehydrogenase in the glycolytic pathway) causes a rapid loss of 2,3-DPG and secondary activation of AMP deaminase. In the absence of inhibitors of glycolytic enzymes, the level of 2,3-DPG is thought to be more stable, and thus AMP may be preferentially degraded via hydrolysis to adenosine (29). Thus, the absence of both the extracellular adenosine accumulation and the $Cl^-$ secretory response in IA-treated monolayers provides further indirect support for a causal relationship between these two events in DOG-treated or substrate-deprived cells.

Active electrogenic $Cl^-$ transport by T84 cells bears a striking resemblance to that of native intestine, both in terms of the specific membrane ion transport pathways involved and the cellular mechanisms of regulation of this event. Thus, this cultured human intestinal epithelial cell line is widely used as a model of intestinal $Cl^-$ secretion, the process which underlies secretory diarrhea (18, 19, 46–49). Our findings may therefore have important physiologic and pathophysiologic implications. Endogenous adenosine may play a role in the conversion of the normally proabsorptive gut to its observed prosecretory status during short-term ischemic stress. During both chemical and true hypoxia, adenosine has been shown to accumulate in the extracellular space and to exert local regulatory effects in several non-intestinal tissues and cell types, including kidney and isolated renal tubules, hepatocytes, cardiac muscle, brain, and erythrocytes (12, 16, 17, 30, 33, 34, 43, 44, 50). The release of adenosine from T84 cells observed during chemical hypoxia in the present study is thus consistent with findings in nonintestinal systems. Nevertheless, one should remain cautious when extrapolating these results to the in vivo situation. For example,

transformed cells may be more resistant than normal cells to the injurious effects of hypoxia not only because of their ability to switch to anaerobic glycolytic ATP synthetic pathways but also due to differences in membrane phospholipase activity and free fatty acid accumulation during hypoxia (51). There may also be subtle differences between chemical and true hypoxia. Additionally, the reductionist nature of the cell culture model does not account for possible interactions between epithelial cells and subepithelial neural and stromal elements or lumenal bacteria. These and other considerations limit the potential usefulness of T84 cells as a general model of bowel ischemia. A direct examination of the role of local adenosine release during mesenteric ischemia in native intestine will be useful, but such studies may be hampered by the constraints imposed by the high levels of ADA normally present (38).

Epithelial $Cl^-$ secretion is thought to represent a primitive defense mechanism whereby mucosal surfaces can be "flushed" in response to potentially injurious stimuli (39, 49). The seemingly paradoxical activation of salt and water secretion observed clinically during intestinal ischemia or, here, during experimentally induced "chemical hypoxia" may, in fact, reflect an adaptive or protective response that limits access of noxious lumenal factors to the internal milieu during a period of potentially increased mucosal vulnerability. Alternatively, because regional mucosal ischemia may be exacerbated by excessive fluid losses into the intestine, leading to further intravascular volume depletion, this secretory response may be inappropriate and systemically deleterious. Moreover, as $Cl^-$ secretion requires energy, activation of this cellular machinery during intestinal ischemia may confer an additional metabolic stress on the already-threatened enterocyte. In view of these speculative considerations, it is clear that an improved understanding of the pathways of purine nucleotide degradation and the paracrine and autocrine roles of adenosine may yield new approaches to the treatment of ischemic diseases of the intestine.

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