Characterization and regulation of adenosine transport in T84 intestinal epithelial cells

EDWARD C. MUN, KEVIN J. TALLY, AND JEFFREY B. MATTHEWS  
Department of Surgery, Beth Israel Deaconess Medical Center,  
Harvard Medical School, Boston, Massachusetts 02215

Mun, Edward C., Kevin J. Tally, and Jeffrey B. Matthews. Characterization and regulation of adenosine transport in T84 intestinal epithelial cells. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G261–G269, 1998.—Adenosine release from mucosal sources during inflammation and ischemia activates intestinal epithelial Cl⁻ secretion. Previous data suggest that A2b receptor-mediated Cl⁻ secretory responses may be dampened by epithelial cell nucleoside scavenging. The present study utilizes isotopic flux analysis and nucleoside analog binding assays to directly characterize the nucleoside transport system of cultured T84 human intestinal epithelial cells and to explore whether adenosine transport is regulated by secretory agonists, metabolic inhibition, or phorbol ester. Uptake of adenosine across the apical membrane displayed characteristics of simple diffusion. Kinetic analysis of basolateral uptake revealed a Na⁺-independent, nitrobenzylthioinosine (NBTI)-sensitive facilitated-diffusion system with low affinity but high capacity for adenosine. NBTI binding studies indicated a single population of high-affinity binding sites basolaterally. Neither forskolin, S₁^r-(N-ethylcarboxamido)-adenosine, nor metabolic inhibition significantly altered adenosine transport. However, phorbol 12-myristate 13-acetate significantly reduced both adenosine transport and the number of specific NBTI binding sites, suggesting that transporter number may be decreased through activation of protein kinase C. This basolateral facilitated adenosine transporter may serve a conventional function in nucleoside salvage and a novel function as a regulator of adenosine-dependent Cl⁻ secretory responses and hence diarrheal disorders.

purinergic receptors; chloride channels; inflammation; ischemia; intestinal secretion

However, in mammalian intestine, adenosine appears to act in novel fashion. We reported that the adenosine released during ischemia in model T84 intestinal epithelial cells, rather than decreasing energy-requiring processes such as active ion transport, paradoxically increases cell work in the form of electrogenic chloride ion (Cl⁻) secretion (25, 42). This unusual secretory response to metabolic stress bears a striking resemblance to the early luminal fluid losses and diarrhea associated with clinical mesenteric ischemia and appears to be mediated through autocrine activation of A2b membrane receptors that are positively coupled to adenosine 3',5'-cyclic monophosphate (cAMP)-dependent Cl⁻ secretory pathways (40, 41). Thus, unlike its usual metabolic feedback role, adenosine released by intestinal epithelia may “feed forward” to activate the cellular machinery for salt and water transport (25).

Adenosine is produced during normal cellular metabolism (7, 42), and adenosine and its precursors are released from damaged cells and discharged from mast cells and platelets (2, 3, 16, 22, 26). Moreover, nucleosides are absorbed luminally from dietary sources. Thus, under a variety of pathological and nonpathological conditions, substantial quantities of adenosine may accumulate in the extracellular space in the vicinity of secretory crypt epithelial cells. The intestine is normally a proabsorptive organ, and secretion is generally a tightly regulated process. This implies that intestinal epithelial cells likely possess a control mechanism that dampens secretory responsiveness to locally released adenosine under normal circumstances. In metabolically intact T84 cells, we showed that constitutive release of adenosine under nonischemic conditions is unmasked by treatment with the conventional inhibitors of nucleoside transport dipyriradomole and nitrobenzylthioinosine (NBTI) and with iodotubercidin, an inhibitor of adenosine kinase (42). Moreover, extracellular adenosine accumulation induced by these agents elicited a Cl⁻ secretory response that was prevented by adenosine receptor blockade. These indirect data suggested the presence of an adenosine transporter, which scavenges extracellular adenosine and limits A2b receptor activation, thereby limiting the potential for autocrine activation of Cl⁻ secretion. Such a transporter could serve as at least one mechanism by which excessive adenosine-elicited secretion is prevented under nonischemic conditions.

Two major classes of nucleoside transport systems have been described in mammalian tissues (31, 32). The first class is the Na⁺-dependent transporters, which have been identified in brush-border membranes.
of absorptive renal tubules and mammalian ileal enterocytes; these are termed concentrative transporters because of their ability to accumulate substrate intracellularly against a concentration gradient (6, 35, 45). Several examples of Na\(^+\)-dependent transporters have been recently cloned (17, 27). These systems characteristically exhibit relative insensitivity to inhibition by the nucleoside analog NBtI. A second and more widely distributed class of nucleoside transporter is present in numerous nonepithelial cell types and is characterized by Na\(^+\) independence. These equilibrative transporters display kinetic properties consistent with facilitated diffusion and display a range of sensitivities to inhibition by NBtI (19, 32).

In nonepithelial systems, the major role of these facilitated transporters is nucleoside salvage to conserve intracellular energy pools. We have speculated that an adenosine transporter in intestinal epithelia would additionally serve to maintain local concentrations of adenosine below the activation threshold for the low affinity A\(_2\) receptor. Thus this transporter may negatively regulate adenosine-dependent epithelial secretion, and changes in the functional expression of this putative “off-switch” could modulate diarrheal responsiveness of the intestinal tract to extracellular adenosine. However, the presence of this transporter has yet to be firmly established, and the possibility of regulated expression of adenosine transport in secretory intestinal epithelia has not been addressed. In this study, we develop direct evidence for the presence of a basolateral membrane nucleoside transporter in model intestinal epithelial cells and define the kinetic properties of this transporter in the context of other known nucleoside transport systems. Additionally we examine whether this adenosine transport can be altered by 1) Cl\(-\) secretory agonists [cAMP and 5′-[(N-ethylcarboxamido) adenosine (NECA)], 2) metabolic inhibitors (oligomycin plus 2-deoxyglucose, iodoacetate), or 3) protein kinase C (PKC) activation [phorbol 12-myristate 13-acetate (PMA)].

**METHODS**

Cell culture and buffers. Human colonic epithelial cells, T84 cells, were maintained in culture as previously described and grown to confluence on collagen-coated permeable filters mounted on supports for 12-well culture dishes from Costar (Cambridge, MA) (13, 24, 25, 42). Cells were fed the day before experiments. Experiments were carried out in a Na\(^+\)-free buffer basolaterally.

Adenosine uptake studies. Monolayers grown on 1.0 cm\(^2\) supports were washed, preincubated in HPBR for 10 min, and then incubated for 1 min in HPBR containing 1.0 µCi/ml \(^3\)H]adenosine either apically or basolaterally as specified. The final concentration of adenosine was 1.0 µM to 1.0 mM. The membrane supports were then removed from incubation medium and dipped 10 times rapidly in three successive beakers of “stop-cold” solution (100 mM MgCl\(_2\), 10 mM tris(hydroxymethyl)aminomethane-HCl, pH 7.4 at 0°C). Each filter was subsequently excised and placed in a vial. Econofluor (3.0 ml; Packard, Meriden, CT) was added before measurement of radioactivity, by a Packard 1600 TR (Packard) liquid scintillation counter. Spectrophotometric determination of protein content of several representative monolayer filters was done using a commercially available bicinchoninic acid assay (Pierce Biochemicals, Rockford, IL) with bovine serum albumin as standard.

For the study of the effect of various agents on adenosine transport, the cells were first preincubated in HPBR containing these compounds for specified durations, and then adenosine transport was measured as previously described by adding \(^3\)H]adenosine. Unless otherwise specified, these agents were present during the transport measurement.

For determination of uptake dependence on Na\(^+\), monolayers were incubated with 1.0 µCi/ml \(^3\)H]adenosine (final adenosine concentration of 10 µM) with or without 2.0 µM NBtI in the above-mentioned Na\(^+\)-free buffer basolaterally. For NBtI inhibition studies, monolayers were incubated in Na\(^+\)-free HPBR containing graded concentrations of NBtI (0.1–0.10 µM) for 20 min. One-minute uptake was then measured using 1.0 µM adenosine in the presence of NBtI. \(^3\)H]NBtI binding assay. We preincubated 1-cm\(^2\) supports containing T84 monolayers in HPBR for 10 min before the initiation of equilibrium binding assays. Supports were then transferred to basolateral solution containing varying concentrations (0.15–10 nM) of \(^3\)H]NBtI in the presence and absence of 10 µM excess nonlabeled NBtI. After incubation at 37°C for 20 min each filter was excised, and radioactivity was determined as previously described.

Statistical analysis. Data are expressed as means ± SE. Student’s t-test and analysis of variance (ANOVA) were performed for paired variates and multiple variates, respectively, when appropriate, and P < 0.05 was considered significant. Linear curve fitting was by the least-squares method.

**RESULTS**

Adenosine uptake in T84 monolayers. Adenosine uptake was measured as a function of time (Fig. 2). Both apical and basolateral \(^3\)H]adenosine uptake was linear for 3 min (R = 0.982 and 0.999, respectively). Therefore, all subsequent uptake studies were performed for a 1-min incubation. One-minute uptake was 13-fold higher across the basolateral membrane than the apical membrane (9.96 ± 0.79 vs. 0.75 ± 0.045 pmol·min\(^{-1}\)·mg protein\(^{-1}\), respectively, for extracellular adenosine concentration of 1.0 µM, n = 6, P < 0.001), consistent with our earlier report (42). Adenosine uptake was then measured against graded concentrations of adenosine. Uptake of adenosine across the apical surface of the monolayers displayed linear, nonsaturatable behavior in concentrations up to 1 mM (data not shown), indicating that adenosine movement across this membrane occurs by simple diffusion alone.

In contrast, adenosine uptake across the basolateral membrane (Fig. 2A) yielded data mathematically resolved into two components: 1) a nonsaturable linear component evident at high concentrations of substrate...
and 2) a saturable component that appeared to obey simple Michaelis-Menten kinetics. Figure 2B shows the high linear correlation (R = 0.999, n = 3 for each data point) between 1/v and 1/s of this saturable component. The linear component of basolateral uptake is characteristic of simple diffusion. Diffusive uptake across the apical membrane appeared to be slightly higher than across the basolateral membrane (7.60 ± 0.70 vs. 4.15 ± 0.32 pmol·min⁻¹·mg protein⁻¹, respectively, at extracellular adenosine concentration of 10 µM, n = 3 each, P < 0.01), an observation that may reflect differences in membrane surface area and/or composition. Lineweaver-Burke transformation of the saturable component of basolateral adenosine uptake yielded an apparent Michaelis constant (Kₘ) value of 114 ± 7.3 µM and maximal velocity (Vₘₐₓ) of 491 ± 27.4 pmol·min⁻¹·mg protein⁻¹ (n = 72 monolayers, representing the results of 4 separate experiments performed in triplicate).

NBTI inhibition studies. To study the sensitivity of adenosine transport to NBTI, adenosine uptake was measured in the presence of different concentrations of NBTI at a constant 1.0 µM extracellular adenosine concentration. NBTI inhibited basolateral adenosine uptake in a dose-dependent manner, as shown in Fig. 3. Inhibition was observed with a 50% inhibitory concentration (IC₅₀) of 1.55 ± 0.73 nM (result of 3 separate experiments performed in triplicate). Approximately 20% of the total uptake probably represents the simple diffusion component of adenosine movement across the basolateral membrane.

Na⁺ independence of adenosine transport in T84 cells. To explore the Na⁺ dependence of basolateral adenosine transport, extracellular Na⁺ was replaced with NMG (Fig. 4). There was minor reduction of adenosine uptake in the absence of Na⁺, and this was statistically insignificant (49.5 ± 1.7 vs. 46.3 ± 0.9 pmol·min⁻¹·mg protein⁻¹ for control vs. Na⁺ free, respectively, at 10 µM adenosine, n = 15, P = 0.097). The residual adenosine transport insensitive to 2.0 µM NBTI inhibition in the absence of Na⁺ was the same as that observed in the presence of Na⁺ (5.84 ± 0.30 vs. 4.96 ± 0.40 pmol·min⁻¹·mg protein⁻¹ for control vs. Na⁺ free, respectively, at 10 µM adenosine and 2.0 µM NBTI, n = 15, P = 0.090). Approximately 12% of the total uptake in the presence of Na⁺ was insensitive to NBTI inhibition (5.84 ± 0.30 vs. 49.5 ± 1.7 pmol·min⁻¹·mg protein⁻¹ at 2.0 µM NBTI, n = 15, P < 0.0001) and may represent the nonsaturable portion of the total uptake (estimated nonsaturable uptake from Fig. 2A is ~4.15 pmol·min⁻¹·mg protein⁻¹ at 10 µM adenosine). Thus the adenosine transport system in T84 cells appears to consist predominantly of Na⁺-independent transporters.

**Fig. 1.** Time course of adenosine transport in T84 intestinal epithelial cells. Adenosine uptake was measured as a function of time at 1.0 µM adenosine (1.0 µCi/ml) at 37°C as described in METHODS. •, Uptake via basolateral membrane. ○, Apical transport. Values are means ± SE of 2 experiments performed in triplicate.

**Fig. 2.** Kinetic analysis of adenosine transport in T84 intestinal epithelial cells. Adenosine uptake was measured at 1 min at 37°C for varying concentrations of extracellular adenosine (1.0–1,000 µM) as described in METHODS. A: total transport (○) exhibits linear behavior at higher concentrations, and this linear component (−−) was mathematically subtracted from total uptake to yield saturable component (●). B: Lineweaver-Burke transformation was applied on saturable component to yield a highly linear relationship. Michaelis constant (Kₘ) and maximum velocity (Vₘₐₓ) were then calculated from this plot using nonlinear regression of Michaelis-Menten enzyme kinetics. Data represent typical experiment performed in triplicate. [ADO], adenosine concentration.
Equilibrium binding study of [3H]NBTI to T84 cells. Figure 5 shows the concentration dependence of specific basolateral membrane [3H]NBTI binding, where membrane-associated binding is plotted against the equilibrium free concentration of the inhibitor. There was saturable specific [3H]NBTI binding and when transformed for Scatchard analysis, a highly linear relationship (R = 0.995) was seen, indicating a single population of high-affinity NBTI binding sites with an apparent dissociation constant (Kd) value of 3.11 ± 1.09 nM and a maximal binding (Bmax) of 0.311 ± 0.021 pmol/mg protein (result of 2 experiments performed in triplicate). The calculated turnover number for the transport system operating at Vmax (491 pmol·min⁻¹·mg protein⁻¹) is ~26 molecules·transporter⁻¹·s⁻¹ and is consistent with several reported values for the turnover rate in other transporters (31, 38, 39). Taken together with the results presented above, the basolateral adenosine transporter in T84 cells appears to be Na⁺ independent and NBTI sensitive, consistent with so-called “equilibrative” facilitated nucleoside transporters identified in other systems.

Equilibrium binding study of [3H]NBTI to T84 cells. Figure 5 shows the concentration dependence of specific basolateral membrane [3H]NBTI binding, where membrane-associated binding is plotted against the equilibrium free concentration of the inhibitor. There was saturable specific [3H]NBTI binding and when transformed for Scatchard analysis, a highly linear relationship (R = 0.995) was seen, indicating a single population of high-affinity NBTI binding sites with an apparent dissociation constant (Kd) value of 3.11 ± 1.09 nM and a maximal binding (Bmax) of 0.311 ± 0.021 pmol/mg protein (result of 2 experiments performed in triplicate). The calculated turnover number for the transport system operating at Vmax (491 pmol·min⁻¹·mg protein⁻¹) is ~26 molecules·transporter⁻¹·s⁻¹ and is consistent with several reported values for the turnover rate in other transporters (31, 38, 39). Taken together with the results presented above, the basolateral adenosine transporter in T84 cells appears to be Na⁺ independent and NBTI sensitive, consistent with so-called “equilibrative” facilitated nucleoside transporters identified in other systems.

Nonregulation of adenosine transport in T84 intestinal epithelial cells by secretory agonists and chemical hypoxia. We next examined the possibility that the facilitated adenosine transporter itself displays the capacity for regulation. We found that neither the cAMP receptor agonist forskolin (10 µM) nor the nontransported adenosine A2b receptor agonist NECA (1.0 µM) affected adenosine transport at two relevant concentrations of [3H]NBTI (0.15–10 nM) in presence and absence of nonlabeled NBTI (10 µM) as described in METHODS. A: specific binding was obtained by subtracting nonspecific binding measured in presence of 10 µM nonlabeled NBTI from total binding measured in absence of nonlabeled NBTI; B: after conversion of specific binding for Scatchard analysis, binding parameters (dissociation constant (Kd) and maximal binding (Bmax)) were calculated. This represents typical experiment performed in triplicate.

G264 ADENOSINE TRANSPORT IN INTESTINAL EPITHELIA

Fig. 3. Nitrobenzylthioinosine (NBTI) inhibition of adenosine transport in T84 intestinal epithelial cells. After preincubation for 20 min at 37°C with graded concentrations of NBTI (0.1 pM-1.0 µM), 1.0 µM adenosine ([3H]adenosine = 1.0 µCi/ml) was added to cells. Adenosine transport was measured at 1 min as described in METHODS. Data are expressed as percentages of adenosine uptake after 20 min NBTI inhibition with respect to control transport measured in absence of NBTI. Data represent typical experiment from 3 separate experiments performed in triplicate.

Fig. 4. Na⁺ independence of adenosine transport in T84 intestinal epithelial cells. Na⁺ was replaced (crosshatched bar) from control HEPES-buffered Ringer solution (HPBR) buffer (open bar) with N-methyl-D-glucamine as described in METHODS, and adenosine transport was measured at 37°C at 10 µM adenosine. Solid and hatched bars represent adenosine transport measured in presence and absence of Na⁺, respectively, but in presence of 2.0 µM NBTI. Values are means ± SE of 5 experiments performed in triplicate.

Fig. 5. [3H]NBTI equilibrium binding to T84 intestinal epithelial cells. Cells were incubated for 20 min at 37°C with varying concentrations of [3H]NBTI (0.15–10 nM) in presence and absence of nonlabeled NBTI (10 µM) as described in METHODS. A: specific binding was obtained by subtracting nonspecific binding measured in presence of 10 µM nonlabeled NBTI from total binding measured in absence of nonlabeled NBTI; B: after conversion of specific binding for Scatchard analysis, binding parameters (dissociation constant (Kd) and maximal binding (Bmax)) were calculated. This represents typical experiment performed in triplicate.

Fig. 6. A: Nitrobenzylthioinosine (NBTI) inhibition of adenosine transport in T84 intestinal epithelial cells. After preincubation for 20 min at 37°C with graded concentrations of NBTI (0.1 pM-1.0 µM), 1.0 µM adenosine ([3H]adenosine = 1.0 µCi/ml) was added to cells. Adenosine transport was measured at 1 min as described in METHODS. Data are expressed as percentages of adenosine uptake after 20 min NBTI inhibition with respect to control transport measured in absence of NBTI. Data represent typical experiment from 3 separate experiments performed in triplicate.
are means extracellular adenosine concentrations of 10, 50, and 100 µM. Values µM) plus 2-deoxyglucose (2-DG, 10 mM) or iodoacetate (IA, 1.0 mM) preincubated in absence (control) or presence of oligomycin (oli, 1.0 

on adenosine transport in T84 intestinal epithelial cells. Cells were experiments performed in triplicate.

concentration of 0.05, 0.1, and 0.5 mM, respectively, 

33, and 37% inhibition for extracellular adenosine 

duced by PMA (100 nM). As seen in Fig. 7 

significant decrease in adenosine transport was in-

by phorbol ester.

In contrast to the above results, a 

not affect adenosine uptake (data not shown).

formation of cellular adenosine may be high, also did 

Shorter incubation periods with above ischemia-

were preincubated in absence (control) or presence of forskolin (10 µM) or NECA (1.0 

in absence (control) or presence of forskolin (10 µM) or NECA (1.0 

m values for adenosine of 100–1,000 µM. The observed 

K_m values for adenosine of 100–1,000 µM. The observed 

V_max, after PMA treatment (0.332 ± 0.013 vs. 0.217 ± 0.022 

pmol·min⁻¹·mg protein⁻¹ for control vs. PMA, respectively, result of 2 experiments done in triplicate, P < 0.005). However, there was no significant change in K_d 

(2.02 ± 0.52 vs. 1.91 ± 0.37 nM for control vs. PMA, respectively, result of 2 separate experiments performed in triplicate). Therefore, PMA inhibition on adenosine transport appears primarily to affect the number of functional adenosine transport sites.

DISCUSSION

These data demonstrate the presence of a Na⁺-independent facilitated adenosine transport system within the basolateral membrane domain of model intestinal crypt epithelial cells. Equilibrative adeno-
sine transporters in neural systems display higher affinity transporters with typical K_m values in the range of 1–10 µM, whereas peripheral systems tend to be characterized by broader substrate specificity, with K_m values for adenosine of 100–1,000 µM. The observed K_m value of 114 µM for adenosine transport in T84 intestinal epithelial cells is comparable to nucleoside transporters in peripheral tissues and is thus of low affinity (10, 15). Other investigators have identified high affinity (K_m ~17 µM), Na⁺-dependent nucleoside transport in the apical membrane of mammalian absorptive enterocytes (6), but this process does not appear to be a major component of the adenosine transport in the Cl⁻-secreting T84 cell line. This sugges
ts the possibility that expression of Na⁺-coupled adenosine transporter may be regulated along the crypt-villus axis, similar to a number of other Na⁺-coupled transporters. However, Na⁺-dependent transport has been reported to be present in monolayers of undifferentiated, cryptlike IEC-6 cells (18). Betcher and co-workers (6) described Na⁺-independent adeno-
sine uptake in rabbit ileal basolateral membrane vesicles and suspected the presence of facilitated transport in absorptive enterocytes but postulated that facilitated trans-

Fig. 6. A: effects of secretory agonists on adenosine transport in T84 intestinal epithelial cells. Cells were preincubated at 37°C for 20 min in absence (control) or presence of forskolin (10 µM) or NECA (1.0 

B: effects of metabolic inhibition on adenosine transport in T84 intestinal epithelial cells. Cells were preincubated in absence (control) or presence of oligomycin (oli, 1.0 

2-deoxyglucose (2-DG, 10 mM) or iodoacetate (IA, 1.0 mM) for 30 min at 37°C. Adenosine transport was then measured at extracellular adenosine concentrations of 10, 50, and 100 µM. Values are means ± SE of 4 experiments performed in triplicate.

sine surface receptor responses. Similarly, adenosine uptake was not affected by metabolic inhibition using oligomycin A plus 2-deoxyglucose or iodoacetate, as seen in Fig. 6B. This treatment decreased cellular ATP levels to ~20% of initial levels within 30 min, as determined by chemiluminescence (data not shown). Shorter incubation periods with above ischemia-

inducing agents (10 and 15 min), during which time formation of cellular adenosine may be high, also did not affect adenosine uptake (data not shown).

Downregulation of basolateral adenosine transport by phorbo1 ester. In contrast to the above results, a significant decrease in adenosine transport was induced by PMA (100 nM). As seen in Fig. 7A, after 4 h PMA inhibited ~37% of total adenosine uptake at varying concentrations of extracellular adenosine (42, 33, and 37% inhibition for extracellular adenosine concentration of 0.05, 0.1, and 0.5 mM, respectively, n = 4 for each group, P < 0.0001 for all 3 concentrations). Shorter incubation periods (30 min) resulted in similar reductions in adenosine transport (data not shown), suggesting that these effects may be due to activation rather than downregulation of PKC. Detailed kinetic analysis of adenosine transport (Fig. 7, B and C) revealed that 100 nM PMA significantly increased K_m (219 ± 19 vs. 332 ± 5.9 µM for control vs. PMA, respectively, n = 3, P < 0.005) and reduced V_max (410 ± 46 vs. 254 ± 15 pmol·min⁻¹·mg protein⁻¹ for control vs. PMA, respectively, n = 3, P < 0.05), suggesting that activation of PKC decreases adenosine transport by reducing the number of basolateral adenosine transporters and reducing transporter affinity for its substrate.

To test whether the decrease in adenosine transport was due to a reduction in the number of adenosine transport sites, equilibrium binding studies were performed after the preincubation in medium containing either 100 nM PMA or an equivalent concentration of dimethyl sulfoxide (DMSO) as control for 4 h at 37°C. Both groups displayed high linearity as seen in Fig. 8 (R = 0.957 vs. 0.976 for control vs. PMA, respectively). There was a significant reduction (35%) in B_max after PMA treatment (0.332 ± 0.013 vs. 0.217 ± 0.022 pmol·min⁻¹·mg protein⁻¹ for control vs. PMA, respectively, result of 2 experiments done in triplicate, P < 0.005). However, there was no significant change in K_d 

(2.02 ± 0.52 vs. 1.91 ± 0.37 nM for control vs. PMA, respectively, result of 2 separate experiments performed in triplicate). Therefore, PMA inhibition on adenosine transport appears primarily to affect the number of functional adenosine transport sites.
port served as the exit rather than the entry pathway for transcellular adenosine movement, in analogy to the absorption of glucose and various amino acids.

Facilitated adenosine transporters are an integral component of the nucleoside salvage pathways in mammalian cells; this is a critical biochemical process in intestinal epithelial cells, which are particularly deficient in the ability to synthesize nucleosides de novo (20, 22). Brush-border Na\(^{+}\)-dependent transporters allow scavenging of luminal nucleosides from dietary sources, and recent molecular evidence from Patil and Unadkat (29) indicates the presence of two cloned isoforms (N1 and N2) and the absence of Na\(^{+}\)-independent transport in brush-border membrane vesicles from human ileum (29). Nucleoside scavenging from the subepithelial compartment is likely to occur through the facilitated transport system we have characterized in the present report. The uptake capacity of this transporter appears large as evidenced by its substantial \(V_{\text{max}}\) of 491 pmol·min\(^{-1}\)·mg protein\(^{-1}\).

Human erythrocytes, rat cardiac myocytes, and bovine adrenal endothelial cells have adenosine transport systems that are inhibited by low concentrations (IC\(_{50}\), 10 nM) of NBTI (10, 19, 38), whereas other cells (for example, Walker 256 carcinosarcoma and Bovikoff hepatoma N1S1–67) possess systems that require higher concentrations of NBTI (IC\(_{50}\). 1.0 µM) for inhibition (28). High-affinity binding of NBTI to the specific membrane sites seems to correlate with inhibition of nucleoside transport (9). We observed an IC\(_{50}\) of 2 nM in T84 cells, consistent with the measured high-affinity binding of NBTI (\(K_{d}\), 3 nM). This high sensitivity of the adenosine transporter to NBTI inhibition in T84 cells additionally indicates that this is predominantly a facilitated-diffusion process.

The presence of this basolaterally restricted low-affinity but high-capacity NBTI-sensitive facilitated nucleoside transporter in T84 cells appears to account for effective scavenging of physiologically relevant amounts of extracellular adenosine released during...
normal cellular metabolism (42). According to the data of Strohmeier et al. (41), A_{2b} receptor activation in T84 cells requires an extracellular concentration of adenosine >10^{-6} M for either cAMP generation or short-circuit current (I_{sc}) response with a 50% effective dose (D_{50}) of 7.78 µM. These values, although slightly lower than the observed K_{m} (114 µM) of our transport system, appear to fall within physiologically operative concentrations of the transporter. The data of Strohmeier et al. (41) further support this hypothesis that the transporter may keep the extracellular adenosine concentration below the threshold of A_{2b} receptor activation. In that study, Cl^- secretory (I_{sc}) responses elicited by basolateral extracellular adenosine were enhanced 10- to 15-fold by adenosine transport inhibitors, suggesting that effective removal of local extracellular adenosine is mediated by this nucleoside transport system. The work of Dobbins et al. (14), also attests to the potential biological relevance of this transporter; these investigators demonstrated that diprydiamole enhanced the Cl^- secretory response to exogenous adenosine in mammalian ileum. The adenosine scavenging system thus may represent a mechanism for limiting adenosine-dependent activation of Cl^- secretion, and hence diarrhea. However, in acute ischemic conditions, where relatively large quantities of adenosine are released into the extracellular space, the capacity of this transport system may be overwhelmed and thus insufficient to prevent stimulation of the A_{2b} receptors.

Could this nucleoside transporter represent an endogenous regulatory site for adenosine-dependent intestinal secretory responses? The second part of this study attempts to address this question. Whether regulation can occur at the level of a nucleoside transporter has to date been studied most thoroughly in cultured chromaffin cells of bovine adrenal gland (11, 12, 36, 37, 43). Sen et al. (37) observed that effectors of secretion in these cells such as forskolin or other direct activators of protein kinase A (PKA) inhibit adenosine transport by decreasing the number of membrane transporters without affecting affinity (37). Subsequently, these investigators observed downregulation of transport sites mediated by various agonists for purinergic P_{2Y} receptor (36). Conversely, the nontransported adenosine receptor agonist NECA, which does not induce granule secretion in these cells, increases adenosine transport capacity by upregulating the transport sites without altering affinity (11). In the present experiments, neither forskolin nor NECA (short- and long-term incubation) exerted a significant effect on T84 cell adenosine transport. Therefore, these regulatory mechanisms are likely to be cell tissue specific; in bovine adrenal endothelial cells adenosine transport is not regulated by PKA (38), and indeed in most systems evidence for regulation of facilitated adenosine transport has been difficult to demonstrate. Adenosine transport was also not altered by cellular energy depletion in these experiments, suggesting that the adenosine-dependent activation of secretion observed in metabolically stressed T84 cells is probably not associated with a decrease in adenosine reuptake capacity. We cannot entirely exclude the possibility that early in the course of the chemical hypoxia there could have been a period during which adenosine uptake was decreased. However, these data displayed enough scatter to render firm conclusions impossible, likely confounded by the combination of nonequilibrium conditions and trans-inhibition of facilitated adenosine uptake during intracellular adenosine formation.

PKC-dependent regulation of adenosine transport has been observed in adrenal chromaffin cells (12), and we also observed that short-term incubation with a PKC-activating phorbol ester significantly altered adenosine transport. This effect appeared to reflect a decrease in affinity for substrate and V_{max} of transport. The NBTI binding experiments suggested that a reduction in transporter number rather than a change in binding affinity for nucleoside analog primarily accounted for the observed transport decrease. There was a rather consistent quantitative relationship between adenosine transport reduction (~37%) with the decrease in transporter number (~35%). Whether the PMA-induced reduction in B_{max} is the result of internalization of functional transporters from the plasma membrane or is a reflection of PKC-dependent deactivation of functional transport sites without a change in total number of surface transporters cannot be distinguished from the present experiments. Both types of PKC-dependent regulatory mechanisms have been described in adrenal chromaffin cells (12). Whereas the above data suggest that the prosecretory effects of extracellular adenosine could be enhanced by PMA due to reduced adenosine uptake from the extracellular space, we could not demonstrate this in monolayers subjected to short-term (30 to 60 min) incubation with PMA. We previously showed that PMA alone exerts an inhibitory effect on cAMP-elicited Cl^- secretion in T84 cells by decreasing the basolateral Na^-K^-2Cl^- cotransporter (23). Additionally, others have found negative interactions of PMA with apical cystic fibrosis transmembrane conductance regulator and basolateral K^-channels (33, 44). Therefore PMA appears to have multiple, primarily negative, effects on the secretory apparatus, thus making it difficult to show an isolated stimulatory effect on Cl^- secretion by downregulation of the nucleoside transport.

In summary, we have characterized the kinetics of adenosine transport across the apical and basolateral surfaces of a polarized Cl^-secreting human intestinal epithelial cell line. Uptake of adenosine across the apical membrane was nonsaturable and consistent with simple diffusion. Basolateral uptake studies revealed the presence of a carrier system that obeyed Michaelis-Menten kinetics in addition to a diffusional component. The results indicate the presence of a basolaterally restricted Na^-independent NBTI-sensitive facilitated adenosine transporter. This transporter may, as elsewhere, be important in nucleoside salvage; however, in this model system and likely in native intestinal crypts, this transporter may serve a novel function in limiting adenosine-dependent secretory responses. The transport capacity of this system appears...
to be modulated by agents affecting PKC but not by cAMP-dependent secretory agonists or metabolic inhibition, although whether such regulation is meaningful in terms of integrated Cl⁻ secretory function and its metabolic regulation remains to be determined. A deeper understanding of the characteristics of this adenosine scavenging system could lead to the development of novel antidiarrheal agents. Because adenosine transporters in nongastrointestinal (e.g., cardiac) systems already represent an attractive target for new pharmacotherapy in ischemic disorders and because some agents already in use (e.g., dipyridamole) are directed already represent an attractive target for new transporters in nongastrointestinal (e.g., cardiac) systems.

Novel antidiarrheal agents. Because adenosine transporters in nongastrointestinal (e.g., cardiac) systems already represent an attractive target for new pharmacotherapy in ischemic disorders and because some agents already in use (e.g., dipyridamole) are directed already represent an attractive target for new transporters in nongastrointestinal (e.g., cardiac) systems.

This work was supported by National Institutes of Health Training Grant GMO-780617 to E. C. Mun, National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-48010 and DK-51630, and the George H. A. Clowes Memorial Career Development Award from the American College of Surgeons to J. B. Matthews.

Address for reprint requests: J. B. Matthews, Dept. of Surgery, Beth Israel Deaconess Hospital, 330 Brookline Ave, Boston, MA 02215.

Received 23 July 1997; accepted in final form 30 October 1997.

REFERENCES


