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# Coupling of CFTR-mediated Anion Secretion to Nucleoside Transporters and Adenosine Homeostasis in Calu-3 Cells

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**Abstract.** The purpose of this study was to characterize the role of adenosine-dependent regulation of anion secretion in Calu-3 cells. RT-PCR studies showed that Calu-3 cells expressed mRNA for $A_{2A}$ and $A_{2B}$ but not $A_1$ or $A_3$ receptors, and for hENT1, hENT2 and hCNT3 but not hCNT1 or hCNT2 nucleoside transporters. Short-circuit current measurements indicated that $A_{2B}$ receptors were present in both apical and basolateral membranes, whereas $A_{2A}$ receptors were detected only in basolateral membranes. Uptake studies demonstrated that the majority of adenosine transport was mediated by hENT1, which was localized to both apical and basolateral membranes, with a smaller hENT2-mediated component in basolateral membranes. Whole-cell current measurements showed that application of extracellular nitrobenzylmercaptopurine ribonucleoside (NBMPR), a selective inhibitor of hENT1-mediated transport, had similar effects on whole-cell currents as the application of exogenous adenosine. Inhibitors of adenosine kinase and 5′-nucleotidase increased and decreased, respectively, whole-cell currents, whereas inhibition of adenosine deaminase had no effect. Single-channel studies showed that NBMPR and adenosine kinase inhibitors activated CFTR $Cl^-$ channels. These results suggested that the equilibrative nucleoside transporters (hENT1, hENT2) together with adenosine kinase and 5′-nucleotidase play a crucial role in the regulation of CFTR through an adenosine-dependent pathway in human airway epithelia.

**Key words:** Calu-3 cells — Adenosine metabolism — Nucleoside transport — CFTR

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## Introduction

Adenosine is an endogenous nucleoside that regulates physiological functions through G protein-coupled $P_1$ receptors. Four receptor subtypes, $A_1$, $A_{2A}$, $A_{2B}$, and $A_3$, have been identified based on their molecular structure, pharmacology, and mechanisms of G protein-mediated signaling (Ralevic & Burnstock, 1998; Fredholm et al., 2000). Activation of each of these receptors has been linked to the regulation of ion transport in epithelial tissues (Avila, Stone, & Civan, 2001; Cobb et al., 2002). In airway epithelial cells, stimulation of $A_{2B}$ receptors has been shown to activate anion conduction via a CFTR-dependent pathway (Huang et al., 2001; Cobb et al., 2002), although the underlying mechanisms are not fully understood.

The magnitude of the effects of adenosine on ion transport is related to its concentration in the vicinity of its cell-surface receptors, which depends on the relative rates of its synthesis and metabolism (Deussen, 2000). Extracellular adenosine is produced by hydrolysis of 5′-AMP by membrane-bound ecto-5′-nucleotidase; the 5′-AMP is itself produced by the action of nonspecific (alkaline or acidic) extracellular phosphatases on ADP and ATP. In addition, adenosine may also be produced from S-adenosylhomocysteine by the action of S-adenosylhomocysteine hydrolase. However, this pathway does not seem to play a major role in total adenosine production (Wagner, Bontemps, & van den, 1994). Metabolism of adenosine can occur by the action of either adenosine kinase or adenosine deaminase (ADA), resulting in the conversion of adenosine to AMP or inosine, respectively (Deussen, 2000). The balance in the rates of these activities will determine the amount of physiologically relevant adenosine that is present.

Given that the majority of adenosine production occurs extracellularly, while most of its metabolism

occurs intracellularly, transport of adenosine across the plasma membrane may also be an important determinant of its concentration at cell surface receptors (Baldwin et al., 1999). Since adenosine is a hydrophilic molecule, it requires specialized transporter proteins for permeation of cell membranes. Two distinct families of nucleoside transporters have been characterized in mammalian cells, the equilibrative ($Na^+$-independent) nucleoside transporters (ENTs) and the concentrative ($Na^+$-dependent) nucleoside transporters (CNTs) (Baldwin et al., 1999). The ENTs are facilitative carriers that transport nucleosides down their normally inwardly directed concentration gradients, whereas the CNTs are secondary active symporters that use $Na^+$ gradients to transport nucleosides into cells. Molecular cloning has identified two functional members of the human ENT family. Both are ubiquitously distributed, but only hENT1 is sensitive to inhibition by nitrobenzylmercaptopurine ribonucleoside (NBMPR, $K_i$ 0.1 to 10 nM), whereas hENT2 is unaffected by concentrations of NBMPR ≤1 μM. Furthermore, both transporter types display broad selectivities, accepting a structurally diverse group of pyrimidine and purine nucleosides as permeants. However, only hENT2 also transports nucleobases (Yao et al., 2002). The concentrative transporters have limited tissue distributions and have primarily been described in specialized cells, such as intestinal epithelia, renal epithelia, liver, choroid plexus, splenocytes, macrophages and leukemic cells (Baldwin et al., 1999; Young et al., 2000). Three human members of this family have been identified by molecular cloning and characterized functionally, and while they are insensitive to inhibition by NBMPR, they can be distinguished functionally based on their permeant selectivities (Baldwin et al., 1999). Human CNT1 and CNT2 both transport uridine and certain uridine analogs, but are otherwise selective for either pyrimidine (hCNT1) or purine (hCNT2) nucleosides, except for modest transport of adenosine by hCNT1 (Ritzel et al., 2001). In contrast, hCNT3 transports both purine and pyrimidine nucleosides. All of the human nucleoside transporters described to date can transport adenosine to some extent, and may therefore be relevant to its signaling.

The aim of the present study was to identify potential factors that control adenosine homeostasis, and to determine their impact on ion transport in human airway epithelial cells. Under normal conditions, adenosine is present in the airway surface liquid where it mediates cilia beat frequency and mucus secretion (Taira et al., 2002). For these reasons, adenosine has received significant attention as an endogenous regulator of mucociliary clearance and as a potential therapeutic target for diseases, such as cystic fibrosis, characterized by abnormalities in this process. The results of our study show that equilibrative nucleoside transporters, together with adenosine kinase and 5′-nucleotidase play a major role in the control of adenosine effects in Calu-3 cells.

## Materials and Methods

### Cell Culture

Calu-3 cells were obtained from the American Type Culture Collection (Rockville, MD), and grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 5 μg/ml gentamycin sulfate, 6 μg/ml penicillin-G and 10 μg/ml streptomycin. Cells were maintained in T75 tissue-culture flasks (Costar, Cambridge, MA) at 37°C in a humidified atmosphere of 5% $CO_2$ in air, and typically required 6 to 8 days to reach confluence. Confluent cell layers were passaged using saline solution containing 0.05% trypsin and 0.02% EDTA. Cells were seeded at a density of $5 \times 10^5$ cells/cm$^2$ onto Costar Transwell inserts (0.45 μm pore size, 0.33 cm$^2$ surface area) for adenosine-transport experiments and onto Costar Snapwell inserts (0.45 μm pore size, 1 cm$^2$ surface area) for short-circuit current measurements. For the first six days, cells were grown submerged in culture medium that was changed every two to three days. Subsequently, air interface culturing was used, in which the medium was added only to the basolateral side of the inserts. Inserts were used for experiments 10 to 16 days after the establishment of an air interface. For patch-clamp studies, $1 \times 10^5$ cells were seeded onto 15-mm coverslips (Fisherbrand, Pittsburgh, PA) 24 hours prior to experiments.

### RT-PCR

A portion ($1.5 \times 10^7$) of cells harvested from T75 flasks was used for RNA purification using the Qiagen RNeasy kit (Qiagen), typically yielding 35 μg total RNA. First-strand cDNA was synthesized by reverse transcription of the RNA using Superscript II RNase H Reverse Transcriptase (Invitrogen) and random hexamer primers (200 ng). Thereafter, PCR was performed using the following sets of primers (from 5′ to 3′) and annealing temperatures: $A_1$ (GenBank™ accession number L22214) forward nucleotides 603–623, reverse 847–826, at 55°C; $A_{2A}$ (U40771) forward 546–569, reverse 995–972, at 55°C; $A_{2B}$ (X68487) forward 447–466, reverse 958–938, at 55°C; $A_3$ (L22607) forward 353–376, reverse 793–770, at 55°C; hENT1 (U81375) forward 623–644, reverse 950–930, at 58°C; hENT2 (AF029358) forward 410–430, reverse 751–731, at 58°C; hCNT1 (U62968) forward 1–23, reverse 612–593, at 58°C; hCNT2 (AF036109) forward 1–22, reverse 540–521, at 58°C; hCNT3 (AF305210) forward 864–887, reverse 1319–1295, at 57°C. In addition to the primers designed to amplify sequences of interest, reactions with glyceraldehyde-3-phosphate dehydrogenase-specific primers were run in all rounds of PCR reactions to serve as internal positive controls: GAPDH (accession number M33197) forward 212–235, reverse 806–786, at 60°C. Primers were obtained from Invitrogen (Carlsbad, CA). PCR was performed using the hot-start method, where one tenth of the reverse transcription reaction was combined with 1 μM of each primer, 0.2 mM of each dNTP, 1.5 mM $Mgcl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 U of Taq polymerase, and autoclaved distilled water to a final volume of 20 μl. After 5 min at 94°C, amplifications proceeded under the following conditions: 30 cycles (94°C, 45 s; annealing temperature, 30 s; 72°C, 90 s) with a final elongation period at 72°C for 7 min. PCR products were separated and visualized by electrophoresis on ethidium bromide-stained 1.5% agarose gels. The expected sizes (bp) of the PCR products were: 245 ($A_1$),

450 ($A_{2A}$), 512 ($A_{2B}$), 441 ($A_3$), 327 (hENT1), 341 (hENT2), 613 (hCNT1), 541 (hCNT2), 456 (hCNT3) and 595 (GAPDH). To confirm their identities, all RT-PCR products were sequenced in one (adenosine receptors) or both (nucleoside transporters) directions by Taq dideoxyterminator cycle sequencing using an automated Model 373A DNA sequencer (Applied Biosystems, Foster City, CA). Sequencing was done by the University of Alberta DNA Sequencing Core Facility.

## Nucleoside Transport

Experiments were carried out at 20°C in HEPES-buffered Ringer's solution (HPBR) containing (in mM) 135 NaCl, 5.0 KCl, 3.33 $NaH_2PO_4$, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose and 5.0 HEPES (pH = 7.4 at 20°C) or in $Na^+$-free HPBR containing (in mM) 140 N-methyl-D-glucamine (NMG), 5.0 $KH_2PO_4$, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose and 5.0 HEPES (pH = 7.4 at 20°C). Confluent monolayers of Calu-3 cells, grown on permeable filters, were washed six times with HPBR or $Na^+$-free HPBR and then pre-incubated in the same solution (± 1 μM NBMPR) for 30 min. Uptake was initiated by adding 10 μM $^{14}$C-labelled adenosine (0.5 μCi/ml, Amersham Pharmacia Biotech) in HPBR or $Na^+$-free HPBR (± 1 μM NBMPR) to either the apical or basolateral compartment. Incubation buffer also included 1 μM deoxycoformycin to inhibit ADA activity. Uptake was terminated by ten rapid washes of the cell culture inserts in an ice-cold "stop" solution containing (in mM) 100 $MgCl_2$ and 10 Tris-HCl (pH = 7.4 at 0°C). The monolayers were dissolved in 0.2 ml 5% (w/v) SDS and counted for radioactivity using a Beckman LS 6000IC liquid scintillation counter (Irvine, CA). Non-mediated (passive) uptake was determined in the presence of 1 μM NBMPR and excess (5 mM) unlabeled uridine. The protein content of representative monolayers was measured using the Bio-Rad Protein Standard Assay Procedure. The flux values are expressed in pmol/(mg protein · min) as means ± SEM.

## Transepithelial Measurements

Standard techniques were used in Ussing chamber studies. The tissues were bathed on apical and basolateral sides with 10 ml of Krebs-Henseleit (KH) solution, which was warmed to 37°C and continually circulated with a gas lift using 95% $O_2$-5% $CO_2$. The composition, in mM, was 116 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 $KH_2PO_4$, and 11.1 glucose, pH 7.4. Chemicals were added from concentrated stock solutions and both chambers were continuously and separately perfused to ensure proper oxygenation and stirring of the solutions. The transepithelial potential difference was clamped to zero using a DVC 1000 voltage/current amplifier (WPI, Sarasota, Fl) and the resulting short-circuit current ($I_{SC}$) was recorded through Ag-AgCl electrodes and 3 M KCl agar bridges. The $I_{SC}$ was allowed to stabilize for 30 min prior to the application of adenosine receptor agonists, and all experiments were performed in the presence of 10 μM apical amiloride. Positive currents were defined as anion secretion or movement from basolateral to apical side. The data were collected at 10 Hz and stored using a PowerLab 8SP series (ADInstruments, NSW 2154, Australia). Average $\Delta I_{SC}$ was calculated as the mean current in the first 300 sec after drug addition.

## Patch-Clamp Recordings

Pipette electrodes were made from thin-walled borosilicate glass (A-M Systems, Everett, WA) using a two-stage vertical puller (Narishige, Japan). Electrode tips were fire-polished to a final resistance of 3 to 6 MΩ for whole-cell recordings and 8 to 10 MΩ for cell-attached recordings. Cells, cultured on 15-mm round coverslips, were rinsed three times in bath solution immediately before being mounted into an open bath chamber (Warner Instruments, Hamden, CT), maintained at 37°C and fixed to the stage of an Olympus IMT-2 Inverted Research Microscope (Lake Success, NY). After the pipette had been immersed in bath solution, offset potentials were compensated before forming a GΩ seal. Currents were recorded, using an Axopatch 200A amplifier and Clampex 8.0 software, both from Axon Instruments (Foster City, CA). All currents were reported with reference to zero in the bath and data were analyzed by pClamp 8.0 (Axon Instruments) and Microcal Origin 6.0 (Northampton, MA) software.

### *Whole-cell Recordings*

The pipette solution contained (in mM): 5 NaCl, 135 KCl, 1.8 $MgCl_2$, 0.2 $CaCl_2$, 1 MgATP, 0.2 LiGTP, 10 glucose, 0.5 EGTA and 10 HEPES (pH 7.4). Bath solution contained (in mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose and 10 HEPES (pH 7.4). Once the whole-cell configuration was obtained, the cell was clamped to −40 mV. The cell capacitance and the access resistance of the patch were determined directly by the compensation circuitry of the patch-clamp amplifier, as well as by Clampex 8.0 software. The whole-cell capacitance, expressed as a mean ± SD, was 49 ± 18 pF ($n$ = 105) and only seals with a series resistance of less than 20 MΩ were analyzed. Current recordings were obtained at one-minute intervals in response to voltage steps, each lasting 200 ms, from −80 mV to 80 mV in 20 mV increments. Traces were normalized to 1 pF, in order to remove variability due to differences in cell size, and the current-voltage relationship was obtained from the mean current during the central 140 ms of each recording.

### *Cell-attached Recordings*

Both the bath and pipette solution contained (in mM): 160 TrisCl, 30 sucrose (pH 7.0). Once the seal was obtained, the pipette potential was clamped to −40 mV. Continuous recordings of channel activity, sampled at 5 kHz, were made. Voltage was briefly stepped from −80 to 80 mV in 20 mV increments to obtain a current-voltage relationship. All kinetic data were obtained at −80 mV, where CFTR $Cl^-$ channel activity was most apparent. Recordings were filtered at 100 Hz, using an 8-pole Bessel Filter; current-amplitude histograms were made and fit with Gaussian functions. The closed-probability ($P_C$) was calculated as the proportion of the area under the curve that corresponded to the state in which all channels were closed. The open-probability ($P_O$) was then calculated, for patches that contained one or more CFTR channels, as the total area under the curve minus the closed probability ($1-P_C$).

## Chemicals

Stock solutions were prepared in $H_2O$ for adenosine (10 mM), erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA, 1 mM), deoxycoformycin (1 mM), 2-p-(2-carboxyethyl)phenethylamino-5′-(N-ethylcarboxamido)adenosine (CGS-21680, 1 mM), α,β-methylene-ADP (100 mM), 8-(p-sulfophenyl)theophylline (8-SPT, 10 mM), and amiloride (10 mM). Stock solutions of NBMPR were in methanol (3 mM), of 5′-(N-ethylcarboxamido)adenosine (NECA) were in 0.1 N HCl (10 mM), and of 5′-amino-5′-deoxyadenosine were in 1:1 (v:v) glacial acetic acid:water (20 mM). All the above drugs were obtained from Sigma (St. Louis, MO).

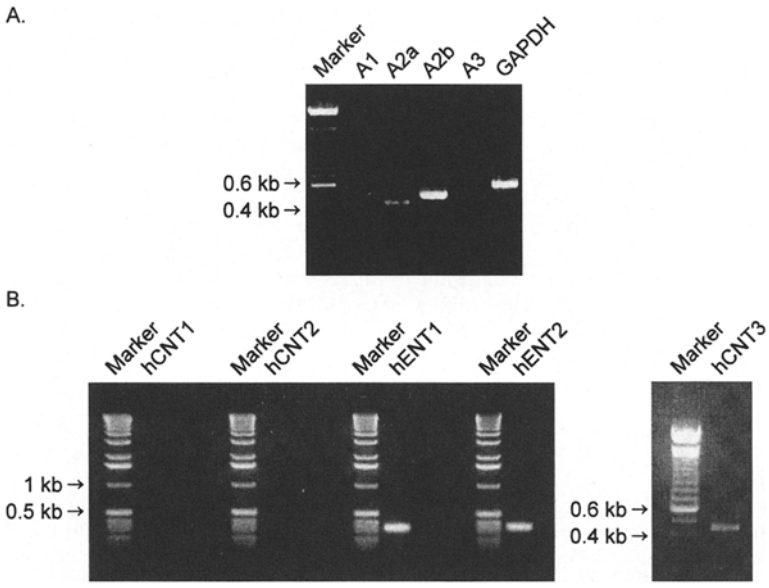


**Fig. 1.** RT-PCR analysis of adenosine receptors and transporters. Arrows indicate the positions of the marker fragments. (*A*) Calu-3 cells express transcripts for $A_{2A}$ and $A_{2B}$ but not $A_1$ and $A_3$ receptors. A representative positive control, using primers specific for GAPDH mRNA, is also included. (*B*) Transcripts for hENT1, hENT2, and hCNT3, but not hCNT1 and hCNT2 were detected. Equal amounts of cDNA were used for the PCR reactions.

### Data Analysis

Data are presented as means ± SEM, unless otherwise indicated; $n$ refers to the number of experiments. The paired Student's $t$-test was used to compare the means of two groups. Statistically significant differences among the means of multiple groups were determined by one-way analysis of variance (ANOVA) with the Tukey-Kramer posttest using Graphpad Instat 3.05 software (San Diego, CA). A value of $P < 0.05$ was considered statistically significant.

## Results

### Identification of Adenosine Receptors and Transporters using RT-PCR

The regulatory actions of adenosine are mediated via four subtypes of G-protein coupled receptors known as $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$. Using RT-PCR, we found that $A_{2A}$ and $A_{2B}$ mRNAs were present in Calu-3 cells at relatively high concentrations, with the $A_{2B}$ signal being the strongest (Fig. 1*A*). The mRNAs for the $A_1$ and $A_3$ receptors were not detected. Figure 1*B* shows RT-PCR amplification of nucleoside transporter transcripts in Calu-3 cells. The cells expressed mRNA for the equilibrative nucleoside transporters, hENT1 and hENT2, and the concentrative nucleoside transporter hCNT3, whereas mRNA transcripts for hCNT1 and hCNT2 were not detected. All PCR products were sequenced and found to be identical to the corresponding GeneBank™ sequences. Control amplifications in which mRNA was used as a template for PCR, or in which no template was used, were negative for reactions involving adenosine receptors ($A_{2A}$ and $A_{2B}$), and nucleoside transporters (hENT1, hENT2, and hCNT3). Control amplifications with GAPDH primers were always positive.

### Polarized Distribution of Adenosine Receptors

We used short-circuit current ($I_{SC}$) measurements as a functional assay to determine the distribution of adenosine receptors in apical and/or basolateral membranes. The experiments were performed using CGS-21680, a specific $A_{2A}$ receptor agonist, and NECA, a nonspecific adenosine receptor agonist. Representative recordings that demonstrated the effects of sequential addition of CGS-21680 (1 μM) and NECA (10 μM) on $I_{SC}$ are shown in Fig. 2. Baseline $I_{SC}$ was 17 ± 4 μA/cm$^2$ ($n$ = 10), expressed as a mean ± SD. CGS-21680 consistently activated $I_{SC}$ when added to the basolateral aspect of the monolayer regardless of the presence or absence of apical CGS-21680. In contrast, application of CGS-21680 to the apical membrane induced only a small $I_{SC}$ increase (0.9 ± 0.3 μA/cm$^2$), which was not statistically significant ($P > 0.05$, $n$ = 5). Furthermore, when CGS-21680 was added to the apical membrane in the presence of basolateral CGS-21680, there was never any effect on $I_{SC}$ ($n$ = 5). Thus, $A_{2A}$ receptors play a significant role in regulating anion secretion, but only from the basolateral side.

The role of $A_{2B}$ receptors was examined by applying NECA to each side of the monolayer in the presence of bilateral CGS-21680. Because RT-PCR experiments demonstrated the expression of only $A_{2A}$ and $A_{2B}$ receptor subtypes in Calu-3, this protocol allowed for the assessment of $A_{2B}$ function despite the lack of specific agonists and antagonists. Under these conditions NECA stimulated an increase in $I_{SC}$ when applied to either the apical or the basolateral surface (Fig. 2). However, the average $\Delta I_{SC}$ was greater when NECA was applied to the apical membrane first rather than to the basolateral mem-

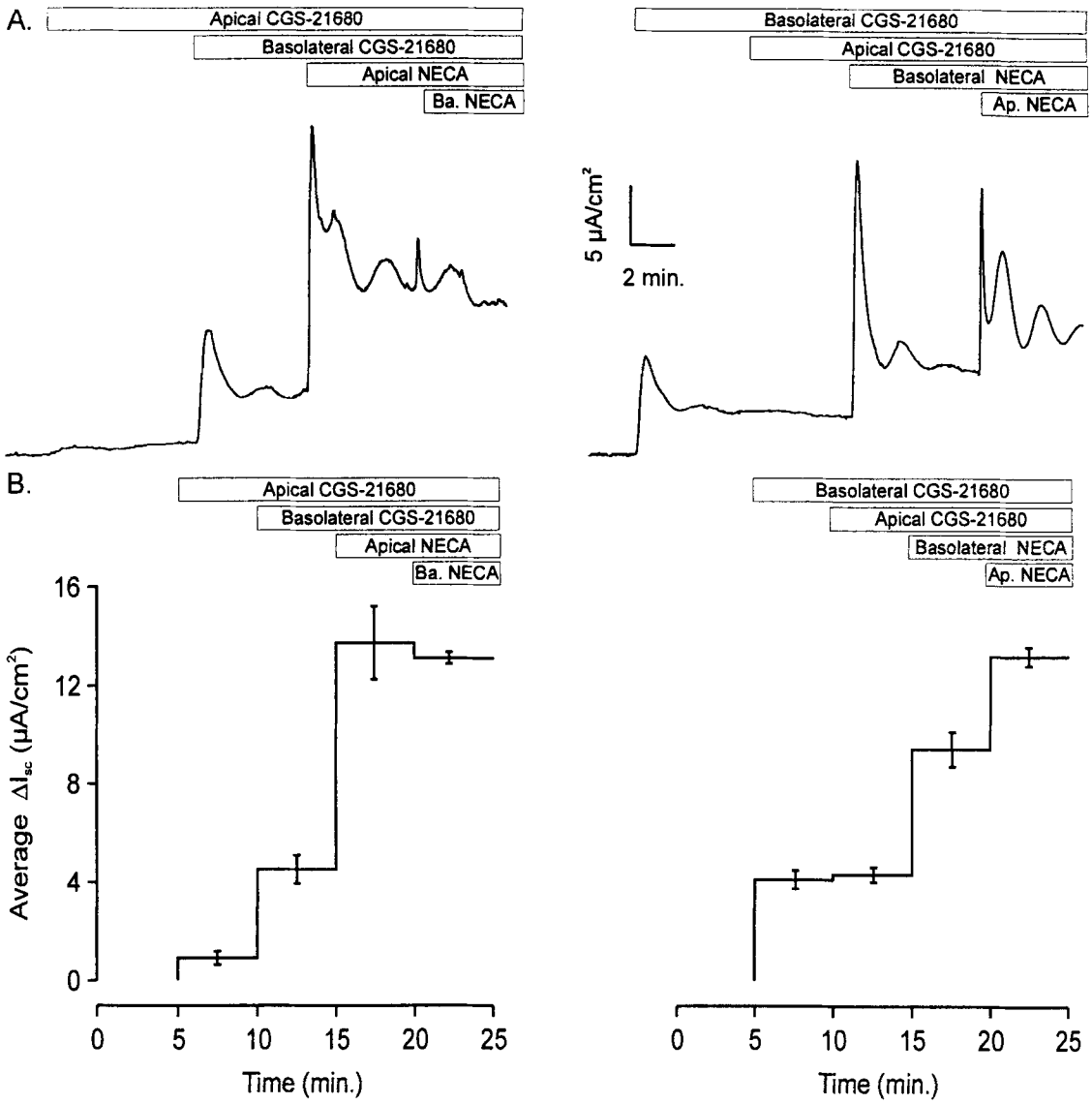


**Fig. 2.** Functional characterization of polarized adenosine receptor distribution. (*A*) Representative tracings showing the effects of 1 μM CGS-21680, a specific $A_{2A}$ agonist, and 10 μM NECA, a nonspecific adenosine receptor agonist, on transepithelial short-circuit current ($I_{SC}$). (*B*) A summary of experiments done with CGS-21680 in combination with NECA. All values are expressed as means ± SEM.

brane. Furthermore, the effects of apical and basolateral NECA were not additive. Apical NECA stimulated the $I_{SC}$ by 9.3 ± 1.5 μA/cm$^2$ ($n$ = 5), which could not be further increased by subsequent addition of basolateral NECA. Basolateral NECA stimulated the $I_{SC}$ by 5.2 ± 0.7 μA/cm$^2$ ($n$ = 5), which was further increased by an additional 3.8 ± 0.9 μA/cm$^2$ ($n$ = 5) by apical NECA.

### Polarized Distribution of Nucleoside Transporters

Uptake of $^{14}$C-adenosine (10 μM) was measured as a function of time across both the apical and basolateral membranes. The uptake was linear for at least 3 min on both sides (apical < basolateral, $R$ = 0.989 and 0.975, respectively), therefore all subsequent initial rate measurements were carried out using 2-min incubations.

Adenosine uptake activities were further examined in detail to determine which transporter types were functional in each membrane (Fig. 3). The transport of adenosine was not significantly reduced by removal of extracellular $Na^+$ in either the apical or the basolateral membrane. However, addition of 1 μM NBMPR, a concentration sufficient to block all hENT1-mediated transport activity, substantially inhibited adenosine uptake across both membranes. Furthermore, when excess unlabelled uridine (5 mM), which saturates nucleoside transporters and effectively blocks transporter-mediated adenosine uptake, was added to the basolateral side, an NBMPR-insensitive $Na^+$-independent process was evident. Any remaining

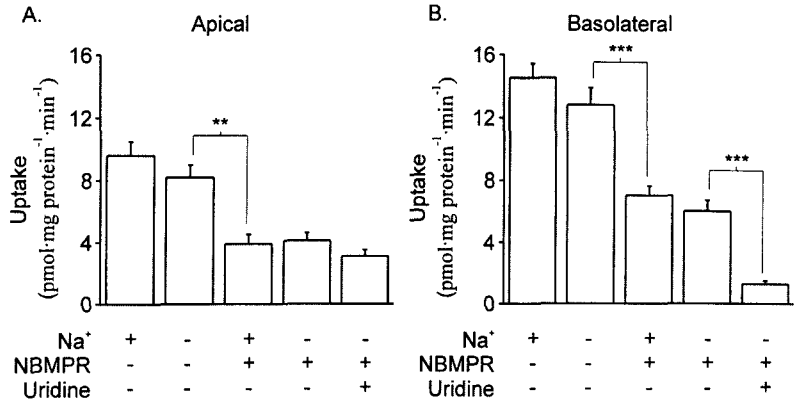


**Fig. 3.** Functional characterization of polarized nucleoside transporter distribution. Initial rates of 10 μM $^{14}$C-adenosine uptake [pmol/(mg protein·min)] across the apical (*A*) and basolateral (*B*) membranes were measured over a 2 min time course. In all experiments, nucleoside uptake was not significantly affected by the removal of $Na^+$, demonstrating the lack of concentrative $Na^+$/nucleoside cotransport activity. In contrast, adenosine uptake across both apical and basolateral membranes was sensitive to inhibition by 1 μM NBMPR. In addition, NBMPR-insensitive transporter activity was detected on the basolateral membrane that could be blocked by saturating with excess unlabeled uridine (5 mM). The remaining nucleoside uptake was via passive (non-transporter mediated) mechanisms. Data are presented as means ± SEM of 20–62 inserts; $^{**}P < 0.01$, $^{***}P < 0.001$.

fluxes in the apical and basolateral membranes were attributed to passive (non-transporter mediated) processes. These experiments identified hENT1 activity as the dominant transport process in the apical membrane, while both hENT1 and hENT2 were functional in the basolateral membrane. When corrected for passive uptake, the ratio of hENT1 to hENT2 activity at the basolateral surface was 3:2.

### The Effect of Exogenous and Autocrine Adenosine on Whole-Cell Current

Figure 4 shows typical recordings of the whole-cell current in Calu-3 cells. Under our experimental conditions the reversal potentials for $K^+$, $Na^+$ and $Cl^-$ currents were −84.6 mV, 84.6 mV, and 0 mV, respectively. Measured baseline reversal potential, was −11 ± 0.5 mV ($n$ = 105), indicating that the majority of the whole-cell current was carried by $Cl^-$ ions with some contribution by $K^+$ ions, while measured baseline whole-cell conductance was 127 ± 4 pS/pF ($n$ = 105). Addition of adenosine (100 μM) to the bath solution significantly activated whole-cell conductance (to 320 ± 36 pS/pF) and shifted the reversal potential to 1 ± 2 mV ($n$ = 8, Fig. 4*B*). This indicates that although both $K^+$ and $Cl^-$ channels contribute to the baseline whole-cell current, adenosine predominantly activates chloride current.

The effect of autocrine adenosine on the whole-cell current was studied using NBMPR (1 μM) to inhibit uptake of extracellularly produced adenosine. We reasoned that this would increase extracellular adenosine, thereby leading to activation of adenosine receptors. Figure 4*C* shows that addition of NBMPR to the bath solution increased the whole-cell conductance (to 246 ± 28 pS/pF, $n$ = 6) in a manner that was similar to that observed with the addition of exogenous adenosine. In particular, NBMPR shifted the reversal potential to −1 ± 2 mV, consistent with $Cl^-$ current activation. The requirement for adenosine receptors was confirmed by demonstrating that addition of NBMPR in the presence of the nonselective adenosine receptor antagonist, 8-SPT (1 μM), had no effect on the whole-cell conductance when compared to the baseline (107 ± 36 pS/pF, $n$ = 4, paired Student's $t$-test). Interestingly, 8-SPT alone inhibited basal whole-cell conductance (to 42 ± 8 pS/pF, $n$ = 4, $P < 0.001$). This indicates that endogenous adenosine is formed extracellularly under baseline conditions.

### Enzymes Involved in Adenosine Production and Metabolism Control Whole-Cell Current

Figure 5*A* shows the effect of the adenosine kinase inhibitor, 5′-amino-5′-deoxyadenosine (20 μM, $n$ = 4) on the whole-cell current. The activated conductance (350 ± 29 pS/pF, $n$ = 4) was similar to that seen with the application of exogenous adenosine. This fact suggests that under baseline conditions adenosine kinase converts adenosine to AMP, thereby lowering its effective concentration in the vicinity of receptors. Like the effect of NBMPR, the effect of 5′-amino-5′-deoxyadenosine could be prevented by pretreatment of the cells with 8-SPT (whole-cell conductance was 90 ± 17 pS/pF, $n$ = 3). Figure 5*C* shows the effect of the 5′-nucleotidase inhibitor, α,β-methylene-ADP (300 μM) on the whole-cell current. Inhibition of 5′-nucleotidase significantly reduced whole-cell conductance (to 29 ± 16 pS/pF) and shifted the reversal potential to 21 ± 2 mV ($n$ = 3), consistent with inhibition of chloride channel activity. This suggests that en-

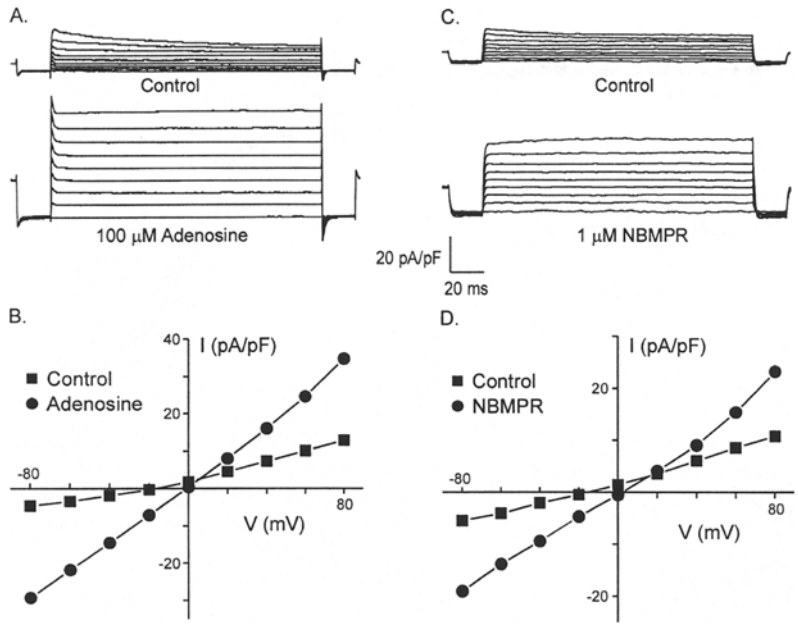


**Fig. 4.** The effect of exogenous and autocrine adenosine on whole-cell current. (*A*) Representative traces showing the activation of whole-cell current by 100 μM adenosine ($n$ = 8), and the corresponding current-voltage plot (*B*). (*C*) Representative traces showing the activation of whole-cell current by 1 μM NBMPR ($n$ = 6), and the corresponding current-voltage plot (*D*).

dogenous adenosine activates $Cl^-$ channels under baseline conditions, which is in agreement with the results of experiments using 8-SPT.

In other studies, we used an inhibitor of ADA, EHNA (10 μM), to evaluate the role of this enzyme in adenosine homeostasis. There was no significant effect of EHNA on the whole-cell conductance over a period of 5 min, when added to either the pipette (180 ± 56 pS/pF, $n$ = 5) or bath (184 ± 73 pS/pF, $n$ = 3) solutions ($P > 0.05$, compared with baseline). This suggests that adenosine deaminase does not play a significant role in adenosine metabolism in Calu-3 cells under these conditions.

### Inhibition of Adenosine Transporters or Adenosine Kinase Activates CFTR Chloride Channels

We used the single-channel patch-clamp technique in the cell-attached mode, to identify ion channels activated by inhibition of adenosine transport or enzymes involved in adenosine metabolism. Figure 6 shows a representative recording of an ion channel under control conditions and after treatment with NBMPR. Since chloride was the only permeant ion present, and the channel conductance is 8 ± 3 pS (mean ± SD, $n$ = 3), this strongly suggests that inhibition of nucleoside transport by NBMPR activates CFTR $Cl^-$ channels. NBMPR had no effect on the channel conductance (Fig. 6*B*), but significantly increased its open probability (Fig. 6*C*). Similarly, 5′-amino-5′-deoxyadenosine increased ion channel $P_O$ from 0.04 ± 0.02 to 0.54 ± 0.10 ($n$ = 3, $P < 0.05$), without changing its conductance.

## Discussion

Extracellular adenosine has been previously shown to modulate the function of both cation (Szkotak et al., 2001) and anion (Huang et al ., 2001; Cobb et al., 2000) channels in human airway epithelial cells. There are also reports suggesting that the presence of nucleoside transporters and enzymes involved in adenosine metabolism may regulate these effects by controlling the effective concentration of adenosine in the vicinity of its receptors (Musante et al., 1999; Szkotak et al., 2001). The results presented in this paper, and summarized in Fig. 7, confirm and extend these observations by identifying enzymes that determine adenosine concentrations, and by characterizing adenosine receptors and nucleoside transporters involved in the regulation of ion channels in Calu-3 cells.

The regulatory actions of adenosine are mediated via four subtypes of G protein-coupled receptors, $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ (Ralevic & Burnstock, 1998). Activation of each of these receptors has been linked to the regulation of ion transport in epithelial tissues (Musante et al., 1999; Avila, Stone, & Civan, 2001; Cobb et al., 2002). The results of our RT-PCR experiments show that Calu-3 cells express $A_{2A}$ and $A_{2B}$, but not $A_1$ or $A_3$ receptors. The presence of $A_{2B}$ receptors in Calu-3 cells has been reported previously (Cobb et al., 2002). These receptors were localized predominantly to the apical side, with some presence at the basolateral membrane. Our data confirm that report and show, in addition, that $A_{2A}$ receptors are present at the basolateral membrane.

Epithelial nucleoside transport has been most extensively studied in intestine, kidney, liver and choroid plexus (Baldwin et al., 1999). Enterocytes of

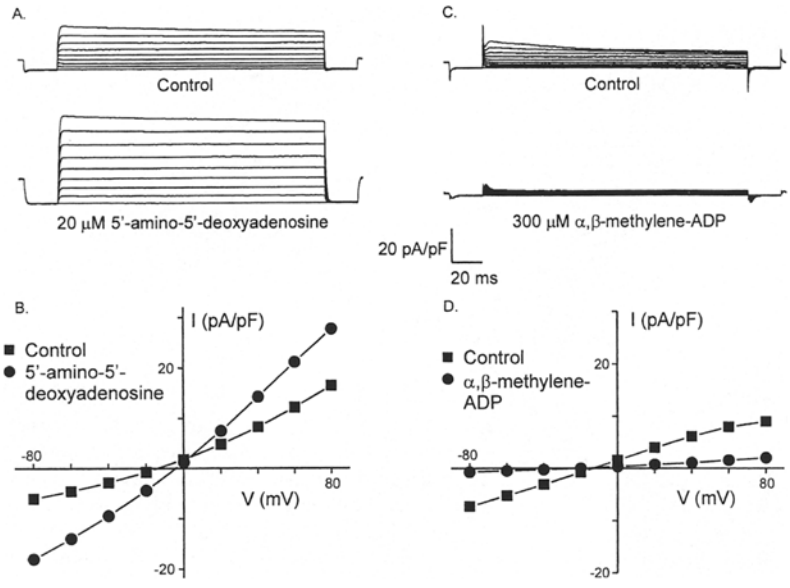


**Fig. 5.** Enzymes involved in adenosine production and metabolism control whole-cell current. (*A*) Representative traces showing the activation of whole-cell current by 20 μM 5′-amino-5′-deoxyadenosine ($n$ = 4). and the corresponding current-voltage plot (*B*). (*C*) Representative traces showing the inhibition of whole-cell current by 300 μM α,β-methylene-ADP ($n$ = 3) and the corresponding current-voltage plot (*D*).

the small intestine, for example, contain transcripts for all five of the CNT and ENT transporter isoforms and exhibit CNT1 and CNT2 functional activities in their apical membranes and ENT1 and/or ENT2 functional activities in their basolateral membranes (reviewed in Young et al., 2000). Cultured T84 cells, a model of intestinal crypt cells, exhibit basolaterally-restricted ENT1 and ENT2 functional activities, while nucleoside uptake across the apical membrane has the characteristics of passive diffusion (Mun, Tally, & Matthews, 1998; Ward & Tse, 1999). Immunocytochemical analyses have demonstrated the presence of CNT1 protein in the apical membrane of rat small intestine, but not at the basolateral membrane, and a similar apical localization was identified for kidney proximal tubule (Hamilton et al., 2001). In rat liver parenchymal cells, CNT1 was abundant in bile canalicular membranes, but largely excluded from sinusoidal membranes (Hamilton et al., 2001) which, instead, are enriched in CNT2 immunoreactivity (Felipe et al., 1998). Choroid plexus exhibits CNT3-type functional activity (Wu et al., 1992). Much less is known about nucleoside transport in other epithelia, although pharmacological and RT-PCR studies suggest the presence of CNT2 but not CNTl in rat epididymal epithelium (Leung et al., 2001).

In the present study, we have used complementary molecular and functional approaches to investigate the nucleoside transport capabilities of Calu-3 cells. In the first series of experiments, RT-PCR was used in conjunction with isoform-specific oligonucleotide primers to test for the presence of hCNT1, hCNT2, hCNT3, hENT1 and hENT2 mRNA. In the second, transport studies were used to confirm the identity of the expressed nucleoside transporters, and to investigate their vectorial distribution (apical versus basolateral membrane). RT-PCR results show that Calu-3 cells express hENT1, hENT2 and hCNT3 mRNAs, but lack transcripts for hCNT1 and hCNT2. However, despite the presence of hCNT3 mRNA we failed to detect any $Na^+$-dependent adenosine transport activity in functional studies, indicating a minor role, if any, for hCNT3 in adenosine transport in Calu-3 cells. hENT1 and hENT2 transport activities were detected in basolateral membranes (hENT1 > hENT2), but only hENT1 activity was found in apical membranes. While hENT1 and hENT2 both transport adenosine and uridine and are broadly selective for other purine and pyrimidine nucleosides, the two transporters are not functionally equivalent. For example, hENT1 has generally higher apparent permeant affinities than hENT2, while hENT2 is capable of interacting with nucleobases and hENT1 is not (Griffiths et al., 1997a, 1997b; Crawford et al., 1998). The two transporters may therefore fulfill complementary, but distinct physiological functions.

The results presented in this study show that application of NBMPR, a selective inhibitor of hENT1-mediated adenosine transport, had similar effects on whole-cell current as the application of exogenous adenosine. Furthermore, the effect of NBMPR was inhibitable by the adenosine receptor antagonist, 8-SPT, indicating that this effect was mediated through the activation of adenosine receptors. Therefore, adenosine transporters may regulate epithelial electrolyte secretion by controlling adenosine concentration in the vicinity of its receptors.

Enzymes involved in adenosine homeostasis exist in both intracellular and extracellular compartments. Adenosine is produced mainly by the

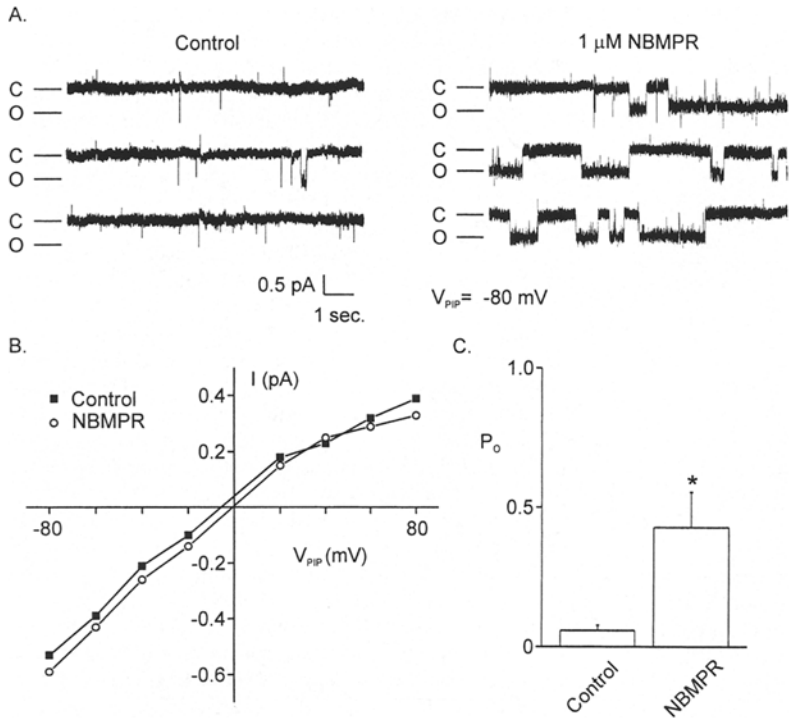


**Fig. 6.** Nucleoside transport inhibition leads to activation of CFTR $Cl^-$ channels. (*A*) Representative recordings of an ion channel under control conditions and after stimulation with 1 μM NBMPR. The recordings were obtained in the cell-attached patch-clamp configuration, when pipette potential was clamped to −80 mV; the channel-closed and-open states are designated by *C* and *O*, respectively. (*B*) The current-voltage plot derived from the CFTR channel recording at various voltages, before and after NBMPR addition, showing that this drug does not influence channel conductance. (*C*) The graph shows the increase in $P_O$ after NBMPR addition ($n$ = 4). *$P$ < 0.05.

action of 5′-nucleotidase, whereas its metabolism is controlled by adenosine kinase and ADA. The results presented in this study show that inhibition of 5′-nucleotidase by α,β-methylene-ADP significantly reduces whole-cell current. This is corroborated in experiments using the nonselective adenosine receptor antagonist 8-SPT, which also reduces basal whole-cell current. These observations suggest that adenosine is an endogenous regulator of Calu-3 chloride channel activity. Interestingly, similar manipulations to change endogenous adenosine levels had no effect on the whole-cell current in A549 cells, another airway epithelial cell line (Szkotak et al., 2001). However, nucleoside transport activity in A549 cells is an order of magnitude greater than in Calu-3 cells. Therefore, nucleoside transporter activity may control the effectiveness of endogenous adenosine on epithelial ion transport.

Under physiological conditions, adenosine kinase is the main contributor to adenosine metabolism (Deussen, 2000). Its $K_m$ of 40 nM (Spychala et al., 1996) is strategically positioned between the high-affinity binding of adenosine to $A_1$ (3 to 30 nM), intermediate-affinity binding to $A_{2A}$ (20 to 200 nM) and low-affinity binding to $A_{2B}$ (5 μM) adenosine receptors (Poulsen & Quinn, 1998). Inhibition of adenosine kinase would be expected to increase intracellular adenosine concentration, thereby leading to reversal of its normally inwardly directed concentration gradient, with release of adenosine and receptor activation. Our study supports this hypothesis since the inhibition of adenosine kinase by 5′-amino-5′-deoxyadenosine had similar effects on the whole-cell current as application of exogenous adenosine.

ADA is responsible for the hydrolytic deamination of adenosine to inosine. It has been shown recently that ADA-deficient mice exhibit a lung phenotype with features of asthma, including increased mucus secretion (Blackburn et al., 2000). These studies also showed that restoring ADA enzymatic activity to ADA-deficient mice attenuated mucus production, suggesting that, by regulating effective adenosine concentration, ADA plays an important role in the control of airway secretions. Interestingly, inhibition of ADA by EHNA had no significant effect on the whole-cell current. Therefore, ADA was probably not involved in the regulation of adenosine effects in Calu-3 cells under our experimental conditions. This may represent an attempt by the cells to conserve adenosine, by converting it to AMP and trapping it intracellularly, rather than deaminating it to produce inosine, which is essentially a waste product.

Activation of CFTR $Cl^-$ channels by adenosine has been shown in several studies (Cobb et al., 2002; Huang et al., 2001). In particular, $A_{2B}$ receptors in Calu-3 were shown to stimulate CFTR through a pathway involving activation of protein kinase A and phospholipase $A_2$ (Cobb et al., 2002), Other studies suggested that $A_{2B}$ receptors were coupled to CFTR by means of $G_S$ proteins, adenylyl cyclase and protein kinase A (Huang et al., 2001). Our re-

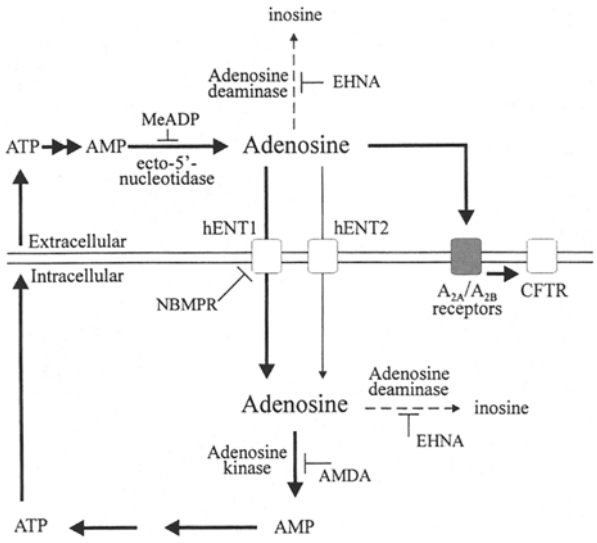


**Fig. 7.** Schematic diagram showing coupling of adenosine homeostasis to adenosine signaling in Calu-3 cells. Intracellular adenosine is rapidly metabolized to AMP by adenosine kinase such that its concentration is normally very low. Intracellular AMP can be converted to ATP, which can exit cells through mechanisms that are not completely understood. Extracellular ATP is converted to AMP by phosphatases, which is then metabolized to adenosine by ecto-5′-nucleotidase. This adenosine is available for either cellular uptake or receptor stimulation. Uptake occurs through hENT1 and, to a lesser extent, hENT2. Adenosine that is not taken up can stimulate $A_{2A}$ or $A_{2B}$ receptors, leading to CFTR activation. The most important pathways are indicated with thick arrows, those of lesser importance are indicated with thin arrows, and those that appear to be unimportant in Calu-3 cells are indicated with dashed arrows. Abbreviations: 5′-amino-5′-deoxyadenosine (AMDA), α,β-methylene-ADP (MeADP).

sults are consistent with these studies but also show, for the first time, that inhibition of nucleoside transporters and enzymes involved in adenosine homeostasis activates CFTR $Cl^-$ channels. Thus, modulators of equilibrative nucleoside transporters or enzymes such as adenosine kinase or 5′-nucleotidase could play an important role in the regulation of CFTR-mediated anion secretion by adenosine receptors.

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