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Ecto 5′-Nucleotidase and Nonspecific Alkaline Phosphatase

TWO AMP-HYDROLYZING ECTOENZYMES WITH DISTINCT ROLES IN HUMAN AIRWAYS*

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In human airways, extracellular adenosine regulates epithelial functions supporting mucociliary clearance, an important airway defense mechanism against bacterial infection. Thus, defining the mechanisms of adenosine generation is critical for elucidating the role of this nucleoside in airway homeostasis. In this study, we identified the source of adenosine on the mucosal surface of human airway epithelia. Polarized primary cultures of human nasal or bronchial epithelial cells were assayed for transepithelial transport, cytosolic and cell surface adenosine production. Ussing chamber experiments indicated that serosal 1 μM [^{3}H]adenosine was not transported to the mucosal compartment. Messenger RNA for the cytosolic AMP-specific 5′-nucleotidase (CN-I) was not detected in human bronchial epithelial cells, suggesting that mucosal adenosine did not originate from intracellular pools. In contrast, extracellular 0.1 mM ATP was rapidly dephosphorylated into adenosine on the mucosal epithelial surface. We identified two ectonucleotidases that mediated the conversion of AMP to adenosine: ecto 5′-nucleotidase (ecto 5′-NT, CD73) and alkaline phosphatase (AP). Both mucosal and serosal epithelial surfaces displayed ecto 5′-NT activity (K_m = 14 μM, V_{max} = 0.5 nmol·min^{-1}·cm^{-2}), whereas AP activity was restricted to the mucosal surface ($K_{m,high}$ = 36 μM, V_{max} = 1.2 nmol·min^{-1}·cm^{-2}; $K_{m,low}$ = 717 μM, V_{max} = 2.8 nmol·min^{-1}·cm^{-2}). In bronchial cultures and tissues, ecto 5′-NT accounted for >80% of total activity toward 0.01 mM AMP, compared with <15% for 5 mM AMP. The proximal airway AP isoform was identified as nonspecific AP (NS AP) by levamisole sensitivity and mRNA expression. The two ectoenzymes presented opposite airway distributions, ecto 5′-NT and NS AP mRNA dominating in higher and lower airways, respectively. Collectively, these experiments support a major role for extracellular nucleotide catalysis and for ecto 5′-NT and NS AP in the regulation of adenosine concentrations on airway surfaces.

Mucociliary clearance (MCC)[1] constitutes an essential component of airway defense against the development of infectious lung diseases (1). Several epithelial functions involved in MCC are regulated by extracellular nucleotides. For instance, $P2Y_2$ receptor activation by ATP or UTP-stimulated Ca^{2+}-dependent Cl^- channels (I_{CA}) (2, 3), cilia beating frequency (CBF) (4, 5), and mucin secretion from goblet cells and submucosal glands (6, 7). Two members of the P2X receptor subfamily were recently identified in human airway epithelial cultures: $P2X_4$ and $P2X_5$ (8). These ATP-gated cationic channels increased Ca^{2+}-dependent Cl^- secretion (8) and CBF (9) in airway epithelia. Interestingly, extracellular adenosine was found to be responsible for regulating the post-peak sustained phase of increased CBF induced by ATP on human nasal explants (4). In a human bronchial cell line lacking $P2Y_2$ receptors (Calu-3; Ref. 10), the channel activity of the cystic fibrosis transmembrane regulator was inhibited by 8-*p*-sulfophenyltheophylline, a nonspecific blocker of cell surface adenosine receptors (11). Subsequently, adenosine was shown to regulate CBF (4, 5) and ion transport (12–15) through activation of cell surface A_{2B} receptors.

The importance of adenosine receptor-mediated regulation of MCC remains unclear because of the lack of information on endogenous sources of extracellular adenosine on the mucosal surface of airway epithelia. Adenosine could originate from the interstitial compartment and penetrate airway epithelial tight junctions to reach the lumen. The nucleoside could also be generated intracellularly by the cytosolic AMP-specific 5′-nucleotidase (CN-I) (16, 17) and reach the mucosal surface through nucleoside transporters (for review see Ref. 18). Alternatively, ATP release and cell surface conversion into adenosine has been reported in numerous mammalian cells (19). Human airway epithelial cells release ATP under basal conditions (20, 21) and by mechanical stimulations such as membrane stretch (22, 23), shear stress (24, 25), or hypotonicity-induced swelling (26–28). All adenine nucleotides and nucleosides have been detected in the airway surface liquid under basal conditions (29). Lazarowski *et al.* (30) established that cell surface adenine nucleotide and nucleoside concentrations are maintained by a balance between ATP release and cell surface metabolism.

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[1] The abbreviations used are: MCC, mucociliary clearance; α,β-met-ADP, α,β-methylene adenosine diphosphate; AP, alkaline phosphatase; Cat_{eff}, catalytic efficiency; ecto 5′-NT, ecto 5′-nucleotidase (CD73); G AP, germ-cell alkaline phosphatase; HPLC, high-pressure liquid chromatography; I AP, intestinal alkaline phosphatase; KRB, Krebs buffer; NS AP, nonspecific alkaline phosphatase; PLA AP, placental alkaline phosphatase; RPA, RNase protection assay; RT, reverse transcriptase; TBASH, tetrabutyl ammonium hydrogen sulfate; CBF, cilia beating frequency; IL, interleukin; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; CHES, 2-(cyclohexylamino)ethanesulfonic acid.

We recently demonstrated that exogenous ATP is dephosphorylated into ADP, AMP, and adenosine at the surface of human nasal and bronchial epithelial cells (31). Two ectoenzymes have been reported to dephosphorylate AMP into adenosine on mammalian cells: ecto 5′-nucleotidase (ecto 5′-NT, CD73, eN; EC 3.1.3.5; Ref. 32) and alkaline phosphatases (APs, EC 3.1.3.1; Ref. 33). Whereas ecto 5′-NT specifically dephosphorylates nucleoside monophosphates (AMP → adenosine (32)), APs will metabolize a spectrum of substrates, including 5′-nucleotides (ATP → ADP → AMP → adenosine), pyrophosphate, and *p*-nitrophenyl phosphate (33). The AP family contains four ectoenzymes: intestinal AP (I AP), tissue nonspecific AP detected in several organs including liver, bone, and kidney (NS AP), placental AP (PLA AP), and germ-cell AP (G AP) reported in testis and malignant tumors (34, 35). Two AP isoforms have been localized in human airways: NS AP and PLA AP. NS AP activity was detected by histochemistry on the epithelial surface lining the entire respiratory system, except for Type I pneumocytes (36), which express PLA AP (37). Bronchoalveolar fluid AP activity has been used for decades to diagnose acute lung injury and chronic disorders like idiopathic pulmonary fibrosis (38, 39). Because bronchoalveolar fluid and lung tissue AP activities are classically assayed with *p*-nitrophenyl phosphate as substrate (38–40), the possible role of human airway AP in the production of extracellular adenosine has not been investigated. Furthermore, ecto 5′-NT activity has not been investigated in human airways. Thus, the pharmacological manipulation of endogenous adenosine levels by targeting these ectoenzymes remains an unexplored area.

The purpose of this study was to identify the source(s) of extracellular adenosine on the mucosal surface of human airway epithelia, distinguishing between permeation from the interstitium, synthesis and secretion from the epithelial cells, and/or cell surface metabolism of released nucleotides. All experiments were performed on primary epithelial cultures, freshly excised airway epithelia or bronchial sections. Serosal to mucosal permeability was measured by Ussing chamber experiments with [^{3}H]adenosine, and the transported species were identified by high-performance liquid chromatography (HPLC). The expression of cytosolic CN-I was investigated by reverse transcriptase-polymerase chain reaction (RT-PCR). Based on our data suggesting that adenosine resulted from cell surface conversion of ATP → ADP → AMP → adenosine (31), we investigated in detail the biochemical properties and relative contribution of ecto 5′-NT and APs. Enzyme expression and tissue distribution were determined by RNase protection assays (RPA). Because of the potential importance of adenosine in diseases associated with airway inflammation (41), we investigated the impact of a prototypic inflammatory cytokine, interleukin-1β (IL-1β), on ecto 5′-NT and APs expression.

EXPERIMENTAL PROCEDURES

Cell Culture—Well differentiated primary cultures of human nasal and bronchial epithelial cells were grown as previously described (42). In brief, cells were isolated from freshly excised nasal turbinate and mainstem bronchi by protein digestion (43), and plated on porous Transwell Col filters (well diameter, 12 mm; pore size, 0.45 μM; Costar) in air-liquid interface medium (50:50 mixture of LHC basal and Dulbecco's modified Eagle's medium-H, 0.5 ng/ml epidermal growth factor, 50 nM retinoic acid, 0.5 mg/ml bovine serum albumin, 0.8% bovine pituitary extract, 50 units/ml penicillin, and 50 μg 47 μl streptomycin) (44). Once they reached confluence, the cultures were maintained at an air-liquid interface with air-liquid interface medium added only to the serosal compartment. After 4 weeks, the cultures were composed of columnar ciliated cells (>90%) and secretory cells, covering a layer of basal-like cells (45). Enzyme assays were conducted on cultures of transepithelial electrical resistance ≥300 Ω/cm^2. Lactate dehydrogenase activity was employed as a test of cellular integrity.

Enzyme Assays on Epithelial Cultures—The epithelial cultures were rinsed three times with Krebs buffer (KRB (in mM)), 140 Na^+, 120 Cl^-, 5.2 K^+, 25 HCO_3^-, 2.4 HPO_4^-, 1.6 Ca^{2+}, 1.6 Mg^{2+}, 5.2 glucose, and 25 HEPES (pH 7.4), and preincubated in KRB (0.35 ml mucosal/serosal) for 30 min at 37 °C (5% CO_2, 95% O_2). Reactions were initiated with the substrate (AMP, UMP, CMP, GMP, or IMP) dissolved in 35 μl of KRB. Aliquots (10 μl) were collected over 10 to 60 min, boiled 3 min, filtered, and analyzed by HPLC. For the determination of pH sensitivity, HEPES was used to buffer solutions at pH 6.5 to 8.0, Tricine for solutions at pH 8.0 and 8.5, and CHES for solutions at pH 8.5 and 9.0. In these buffers, bicarbonate was omitted for better control of pH.

Ussing Chamber Experiments—Primary nasal epithelial cultures grown on Snapwells™ (Costar; Cambridge, MA) were mounted in modified Ussing chambers (Physiologic Instruments, San Diego, CA) with an aperture of 1.0 cm^2. The epithelium was bathed on each side with 5 ml of HEPES-free KBR (pH 7.4, 37 °C), circulated by gas lift with 95% O_2, 5% CO_2. The voltage was clamped to 0 mV, except for 3-s pulses (±10 mV) every 60 s. Short-circuit current (I_{sc}) and transepithelial resistance were digitally recorded from the output of voltage clamp. The permeability coefficients (P_{coeff}) for adenosine and mannitol were measured. Mannitol P_{coeff} was used as an index of paracellular permeability. Following a 45-min preincubation period with [^{3}H]adenosine (0.01 μCi/μl; 1 μM) and [^{14}C]mannitol (0.2 μCi/μl) added to the serosal bath, aliquots (500 μl) were collected from the mucosal side (sink) every 30 min and replaced with 500 μl of HEPES-free KBR. Every 60-min interval, 50 μl was collected from the serosal (source) side. [^{14}C]Mannitol and [^{3}H]adenosine in the source and sink buffer samples were measured using a liquid scintillation counter (Beckman LS 6500 counter) and P_{coeffs} calculations were calculated with the established equation: $P_{coeff} = (\Delta Q/\Delta t/S{\cdot}A)$, where $\Delta Q/\Delta t$ is the steady-state rate of appearance of the tracer in the sink (cpm/s), S is the source radioactivity (cpm/cm^3), and A is the surface area (cm^2) (46). The units of P_{coeff} are cm/s. Mucosal and serosal buffer samples were also analyzed for nucleoside and nucleobase composition by HPLC.

Identification of the AMP-hydrolyzing Enzymes—Three enzymes could be responsible for the conversion of extracellular AMP into adenosine on human airway epithelial surfaces: cytosolic 5′-NT, ecto 5′-NT, and AP. The possible contribution of each enzyme was evaluated with specific substrates and inhibitors. The non-hydrolyzable analog of ADP, α,β-methylene ADP (α,β-met-ADP), inhibits ecto 5′-NT and AP, but not cytosolic 5′-NT (32). Hence, complete inhibition of AMP hydrolysis by α,β-met-ADP would rule out the contribution of released cytosolic 5′-NT. Ecto 5′-NT activity was identified with concanavalin A, a plant lectin that binds specifically to α-D-glucosyl and α-D-mannosyl residues of glycoproteins (47). Concanavalin A has been reported to completely inhibit the activity of ecto 5′-NT, without affecting AP (48). The AP activity was measured with a specific substrate (β-glycerophosphate) and inhibitor (levamisole). The substrate reports the biochemical activity of all AP isoforms (49), whereas levamisole sensitivity discriminates between NS AP, PLA AP, and I AP (50). The identity of the AP isoform(s) was further addressed with L-phenylalanine (I AP and PLA AP) and L-leucine (G AP) (34, 35). All experiments were conducted as described above for enzyme assays. Reactions were initiated with β-glycerophosphate mixed with AMP. Alternatively, the cultures were preincubated 20 min with an inhibitor (α,β-met-ADP, levamisole, amino acids, or concanavalin A) before the onset of the reaction.

Kinetic Analysis of AMP Hydrolysis—All assays were conducted on the mucosal surface of human bronchial epithelial cultures with [^{3}H]AMP (0.1 mCi; 1–3000 μM), as described above for enzyme assays. Samples were collected after incubation periods that limited substrate hydrolysis to ≤10%, and were analyzed by HPLC. Kinetic parameters for ecto 5′-NT and AP were determined in the presence 10 mM levamisole and 5 mM concanavalin A, respectively. Michaelis constants (K_m) and maximal velocities (V_{max}) were obtained from the slope and the ordinate of Woolf-Augustinson Hoftsee transformations, respectively. Catalytic efficiency (Cat_{eff}) was calculated from the velocity (V_o) at K_m divided by K_m (51).

Competition studies were conducted to determine whether ADP and/or ATP inhibited the hydrolysis of nucleoside monophosphates. Because ecto 5′-NT (48) and AP (33) hydrolyze AMP and UMP at similar rates, UMP was chosen over AMP as substrate for two reasons: 1) to rule out interference from the adenylate kinase activity (ATP + AMP ↔ 2ADP) we recently detected on these cells (52) and 2) to distinguish substrates from inhibitors and their metabolites on the HPLC chromatograms. Because we recently demonstrated that ATP and ADP are hydrolyzed into AMP on these cells (31), the protocol was designed to prevent interference of UMP hydrolysis by AMP produced from ATP and ADP hydrolysis. Reactions were started with previously mixed UMP (10, 30, or 100 μM) and ATP or ADP (10, 30, 60, or 100 μM).

TABLE I
Specific primers for the human ecto 5′-NT alkaline phosphatases, and CN-1

Gene	Accession number	Strand[a]	Sequence	Position	Fragment size
					bp
5′-NT	NM 002526	S	GGC GCC GAG GTG GCG CAC TTC ATG AAC GCC	341	587
		AS1	GGA GTG TCC TCC CAC CAC GAC GTC CAC ACC	781	
		AS2	CTC GAT CTT CAG ATA GCC TAG GTA TTT GCC	928	
NS AP	NM 000478	S	TGG AAC ATG AGT TAA CAT CTG ACC ACT GCC	300	640
		AS1	TAC CAG TCC CGG TCA GCC GAG TGG GCG TAG	960	
		AS2	CTG ATG TTA TGC ATG AGC TGG TAG GCG ATG	1038	
PLA AP	NM 001632	S	CTC CAG ACA TGC TGG GGC CCT GCA TGC TGC	56	691
		AS1	TCT GAG CTC TTC TCT GGG GCA GAC ACG GAG	747	
		AS2	CTT GGC CCG ATT CAC CAC GGA GAT GAC CTC	1314	
I AP[b]	NM 001631	S	CCT CCC TGA TGG AGA TGA CAG AGG CTG CCC	2151	582
		AS1	CGG GAG GCG CCA GGT CGC AGG CCG TGT AGG	3150	
		AS2	CAA CTG GGT GCG CGG CGT CGG TGG TGC AGG	3180	
G AP	J03252	S	GAC TGC TTC CAG ACA TGC AGG GGC CCT GGG	436	693
		AS1	CTT GGC CCG ATT CAC CAC GGA GAT GAC CTC	930	
		AS2	CTG AGC TCT TCT CTG GGG CAG ACA AAC AAG	1129	
CN-I	NM 032526	S	TGG TTG TGT CCC AGA GTC AG	596	183
		AS1	CTA CCC AGT GCC TCC AGA AA	757	

[a] S, sense strand; AS1, inner antisense strand; AS2, outer antisense strand.
[b] Genomic DNA, fragment size without intron: 582 bp.

Samples were collected after incubation periods that limited AMP production from ATP and ADP to <0.1 μM. These conditions also limited ATP conversion into ADP to ≤10%. Inhibition patterns and constants (K_i) were derived from Dixon plots.

Tissue Measurements of Enzyme Activities—The epithelial surface of human bronchial sections was assayed for AMP hydrolysis by ecto 5′-NT and NS AP. From each tissue sample, three circular pieces (diameter, 12 mm) were assayed in parallel with 5 mM AMP. Each piece was inserted into a 12-mm porous Transwell and sealed along the edges with silicone. Epithelial surfaces were rinsed three times with KRB and preincubated in KRB (0.35 ml of mucosal/serosal) for 30 min at 37 °C (5% CO_2, 95% O_2). Reactions were initiated with the substrate dissolved in 35 μl of KRB. Five aliquots (10 μl) were collected over 10–30 min, boiled 3 min, filtered, and analyzed by HPLC. All three pieces were submitted to a second set of experiments. Surfaces were rinsed and tissues were preincubated 30 min at 37 °C (5% CO_2, 95% O_2) in KRB (0.35 ml of mucosal/serosal) containing 10 mM levamisole, 5 mM concanavalin A or vehicle. Reactions were conducted as in the first set of experiments. Ecto 5′-NT and NS AP activities were calculated from the differences in the rate of AMP hydrolysis between control and treated experiments conducted on the same piece of tissue. The piece assayed twice with 5 mM AMP in the absence of enzyme inhibitor provided a control for tissue integrity. This protocol was repeated on different sets of tissues with 0.01 mM AMP and 0.1 mM AMP. At the end of the experiments, all sections were fixed and counterstained with hematoxylin and eosin (H&E) for light microscopic examination of the epithelium. A Nikon microphot SA microscope connected to a 3CC-Chilled Camera (Sony, Marietta, GA) and interfaced to a powerMac 8100 were used to capture the images via Adobe Photoshop.

HPLC Analysis—All samples were analyzed by reversed-phase paired-ion HPLC. The separation system consisted of a Dinamax C-18 column and a mobile phase developed with buffer A (10 mM KH_2PO_4 and 8 mM TBASH, pH 5.3) from 0 to 10 min, buffer B (100 mM KH_2PO_4, 8 mM TBASH, and 15% MeOH, pH 5.3) from 10 to 20 min, and buffer A from 20 to 30 min. Absorbance was monitored at 254 nm with an on-line model 490 multiwavelength detector (Shimadzu Science Instruments Inc., Kyoto, Japan), and radioactivity was determined on-line with a Flo-One Radiomatic β detector (Packard Instrument Co.).

Exposure of Human Airway Epithelia to IL-1β—Primary cultures of human bronchial epithelial cells were incubated during 24 h at 37 °C (5% CO_2, 95% O_2) with a range of IL-1β concentrations reported to induce the cyclooxygenase pathway (0.0–1.0 ng/ml) of human airway epithelial cells (53). Because polarized airway cultures respond to prolonged (>12 h) mucosal flooding by an increased acid production (54), the cytokine was added to air-liquid interface medium in the serosal bath. At the end of the challenges, the cultures were processed for total RNA extraction and RPA analysis of enzyme expression.

Reverse Transcriptase-Polymerase Chain Reaction—Total RNA was extracted from primary cultures and freshly excised human airway epithelial cells, as previously described (55). Oligonucleotide primers were generated from the sequence of human ecto 5′-NT (CD73), cytosolic AMP-specific 5′-nucleotidase (CN-I), and the four AP isoforms (Table I). First strand cDNA was synthesized by RT reaction with 8 μg of total RNA and the outer antisense (AS2) primer, in a 20-μl reaction containing 10 mM dNTPs, 0.1 mM dithiothreitol, 40 units of RNase inhibitor, and 400 units of Superscript II RT in supplied buffer (Invitrogen). The reaction was allowed to proceed for 1 h at 42 °C and stopped by heating at 70 °C for 10 min. The cDNA was amplified by PCR with the inner antisense (AS1) and the sense (S) primers by the "hot start" technique: 36 cycles (1 min at 94 °C, 2 min at 57 °C, 3 min at 72 °C), followed by 10 min at 72 °C. In the case of CN-I, the AS primer was poly(dT) (16) and the PCR product mixture contained glyceraldehyde-3-phosphate dehydrogenase primers (BD Biosciences) as internal control. The PCR was run in the presence of 2.5% Me_2SO in the following conditions: 35–55 cycles (1 min at 94 °C, 50 s at 45 °C, 25 s at 72 °C), followed by 10 min at 72 °C. The amplified PCR products were gel purified on 1% agarose gel for ecto 5′-NT and APs, 1.5% agarose gel for CN-I and glyceraldehyde-3-phosphate dehydrogenase (Qiagen gel purification kit). The PCR products were ligated by TA cloning into the *pCRII* vector (Invitrogen) and the ligations transformed in *One-Shot* cells (Invitrogen). Plasmid DNA from individual colonies was purified with a commercial kit (Qiagen) and screened by automatic sequencing to confirm the identity of the product, verify the absence of mutations, and determine the orientation of the insert in the vector. All PCR products were confirmed to be in 100% homology with the reported human cDNA sequences. RT-PCR reactions conducted with RNA in the absence of Superscript II RT yielded no signal on the gels, ruling out the possibility of RNA contamination by genomic DNA.

RNase Protection Assays—Plasmids containing cDNA fragments for human ecto 5′-NT and NS AP were linearized with *Eco*RV. [^{32}P]CTP-labeled antisense probes were transcribed from the plasmid SP6 promoter site, according to the manufacturer's recommendations (MAXIscript *in vitro* transcription kit; Ambion, Austin, TX). The housekeeping gene probe β-actin was prepared at low specific activity by adding 2 μl of 1 mM cold CTP to the labeling mixture and reducing the ^{32}P label to 10 μCi per reaction. The probes were purified by electrophoresis on RNase-free 5% acrylamide, 8 M urea gels (250 volts, 1 h), eluted and hybridized (8×10^4 disintegration/min) with 20 μg of total RNA (RPA III kit; Ambion). The protected fragments were separated by electrophoresis on 5% acrylamide, 8 M urea gels (250 volts, 1 h). The gels were dried on a vacuum dryer (60 °C, 4 h) and exposed to a storage phosphorscreen (Amersham Biosciences) for 4–8 days. The screen was scanned with an optical scanner (Storm; Amersham Biosciences) and the signals quantified using digital image analyzing software (ImageQuant; Amersham Biosciences). Appropriate sense strand and yeast RNA controls yielded no signal on the gels.

Statistical Analysis—All experiments were performed on cultures and tissues from at least three donors. Rates of hydrolysis were calculated from the decrease in the amount of substrate monitored by HPLC, and presented as nanomoles·min^{-1}·cm^{-2} of surface area. The values were expressed as mean ± S.E. Unpaired Student's *t* tests were used to assess the significance between means. Paired Student's *t* tests were used when comparing hydrolysis rates measured on the mucosal and serosal surfaces of the same culture. Linear regressions, curve fits, and

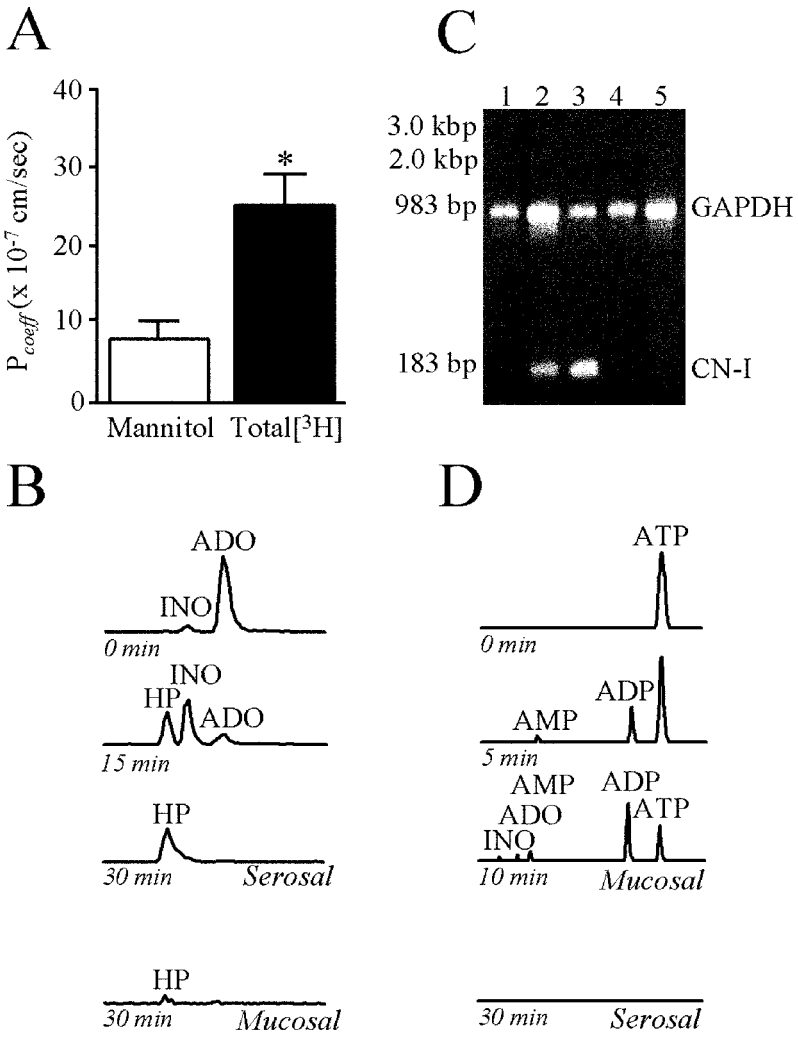

FIG. 1. **Origin of extracellular adenosine on the mucosal surface of human airway epithelia.** *A* and *B*, Ussing chamber experiments on transepithelial and paracellular adenosine transport. [^{3}H]Adenosine (1 μM) and the paracellular marker [^{14}C]mannitol were added to the serosal bath of polarized nasal epithelial cultures. Buffer samples were collected over 90 min to calculate [^{3}H]adenosine and [^{14}C]mannitol P_{coeffs}. *A*, adenosine P_{coeff} was higher than mannitol P_{coeff}. *B*, HPLC analysis of serosal and mucosal buffer samples collected over 30 min. Serosal [^{3}H]adenosine (*ADO*) was metabolized into inosine (*INO*) and hypoxanthine (*HP*). Traces of HP (but not ADO) were detected in the mucosal bath. *C*, RT-PCR agarose gel indicating the absence of mRNA for cytosolic 5′-nucleotidase (CN-I). *Lane 1*, human placenta RNA (negative control; 16); *lane 2*, human embryonic kidney cells (HEK 293) overexpressing CN-I (positive control, 16); *lane 3*, human heart RNA (positive control) (16); *lanes 4* and *5*, freshly excised bronchial epithelium from 2 donors. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)-specific product of 983 bp was produced by all reactions (internal control). *D*, production of adenosine from ATP. On the mucosal surface of human bronchial epithelial cells 0.1 mM ATP was dephosphorylated into ADP, AMP, and ADO. No substrate or product was detected on the serosal surface, and vice versa (data not shown). Values represent mean ± S.E. of five to seven independent experiments. *, $p < 0.01$.

data transformations were performed with the computer programs Origin and Sigma plot.

Materials—All 5′-nucleotides and adenosine were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Concanavalin A, erythro-9-[2-hydroxyl-3-nonyl]adenine, β-glycerophosphate, levamisole, L-phenylalanine, L-leucine, α,β-met-ADP, EDTA, KH_2PO_4, TBASH, Tricine, CHES, and HEPES were obtained from Sigma. HPLC grade water was purchased from Fisher Scientific (Pittsburgh, PA). Cell culture media, bovine serum albumin, bovine pituitary extract, epidermal growth factor, gentamicin, penicillin, retinoic acid, and streptomycin were bought from Invitrogen and human cytokine IL-1β (rhIL-1β) from R&D Systems. [^{3}H]Adenosine (20 Ci/mmol) and [^{3}H]AMP (10 mCi/mmol) were obtained from Amersham Biosciences, D-[1-^{14}C]mannitol (0.1 mCi/mmol) and [α-^{32}P]CTP (10 mCi/mmol) were from PerkinElmer Life Sciences. Salts and solvents were of analytical grade.

RESULTS

Adenosine Accumulation on the Apical Surface of Human Airway Epithelia—First, we explored the possibility that serosal adenosine could reach the mucosal surface. Ussing chamber experiments were performed on polarized cultures of human nasal epithelial cells. When [^{3}H]adenosine (0.01 μCi/μl; 1 μM) was added to the serosal bath, tritiated compounds accumulated in the mucosal bath with a significantly higher P_{coeff} than for [^{14}C]mannitol (Fig. 1*A*). However, HPLC analysis of the tritiated species revealed that serosal [^{3}H]adenosine was converted into [^{3}H]inosine and [^{3}H]hypoxanthine within 30 min in the serosal compartment (Fig. 1*B*). The only radiolabeled molecule detected on the mucosal surface over 30 min was [^{3}H]hypoxanthine. These results suggest that interstitial fluid does not constitute a source of adenosine for the mucosal surface of airway epithelia.

We then tested whether adenosine could be generated within the cytosol of columnar epithelial cells and released onto the mucosal surface. To evaluate this possibility, we performed RT-PCR experiments for the expression of CN-I (16, 17). Experiments performed with total RNA from human heart or human embryonic kidney cells (HEK 293) produced a positive band of expected size for CN-I (Fig. 1*C*), as previously reported (16). In contrast, no reaction product was obtained with total RNA from freshly excised human bronchial epithelial cells or human placenta (negative control; Ref. 16), even after 55 PCR cycles (not shown). These experiments indicated that extracellular adenosine, detected on the mucosal surface of human airway epithelia does not originate from intracellular compartments.

Numerous cell types have the ability to release ATP (56) and to transform the nucleotide into adenosine by cell surface enzymes (19). Because human airway epithelial cells were reported to release ATP (20–28) and to accumulate all adenine nucleotides on the mucosal surface (ATP, ADP, AMP) (29), we tested whether extracellular adenosine could be generated from mucosal cell surface nucleotide metabolism. Fig. 1*D* shows that the mucosal surface of bronchial epithelial cultures dephosphorylated 100 μM ATP into ADP, AMP, and adenosine. No nucleotide or nucleoside was detected in the opposite compartment, whether we administered ATP on the mucosal or

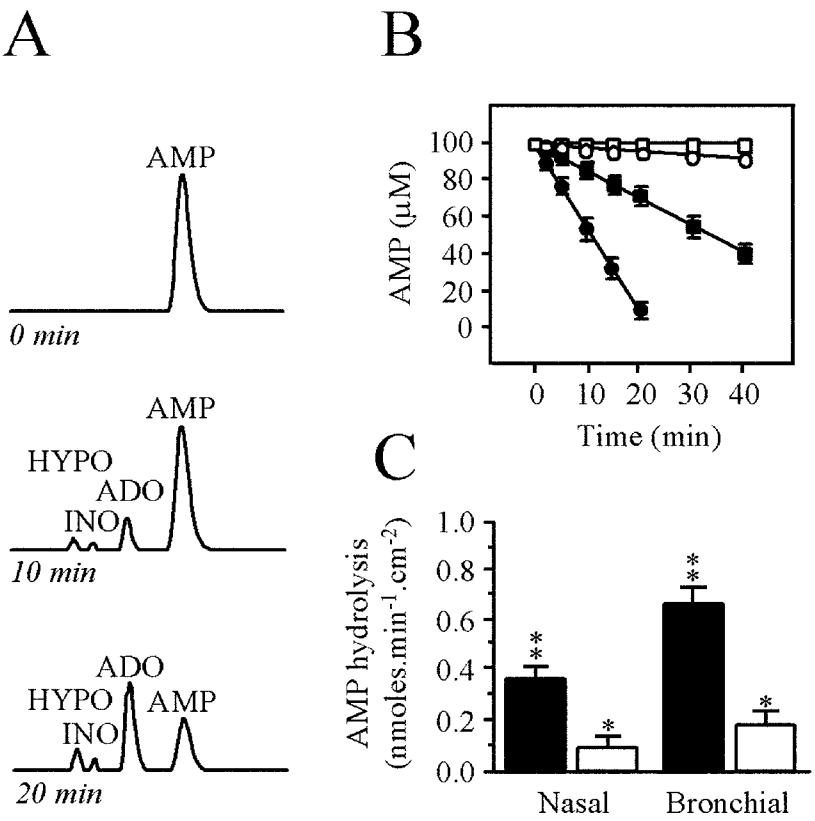

FIG. 2. **Characterization of cell surface adenosine production.** *A*, HPLC analysis for the conversion of extracellular AMP into adenosine. Mucosal 0.1 mM AMP was converted into adenosine (*ADO*), inosine (*INO*), and hypoxanthine (*HP*) on bronchial cultures. *B*, AMP-hydrolyzing ectoenzymes are cell-associated. The mucosal surface (●) hydrolyzed AMP at higher rates than the serosal (■) surface. KRB conditioned during 60 min on the mucosal (○) or serosal (□) surface hydrolyzed 0.1 mM AMP at <5% of total AMPase activity. *C*, AMP hydrolysis on nasal and bronchial epithelial cells. Mucosal activities (*filled bars*) were 3-fold higher than serosal activities (*open bars*). Reaction rates were higher on bronchial cultures. Values represent mean ± S.E. of 4–12 independent experiments. *, $p < 0.05$; **, $p < 0.01$.

serosal surface. These results were consistent with the data showing that adenosine does not permeate through tight junctions (see above).

Because these experiments indicated that adenosine accumulated in the mucosal compartment primarily because of extracellular ATP hydrolysis, we investigated the identity and polarity of the ectoenzyme(s) responsible for the conversion of extracellular AMP into adenosine on human airway epithelial surfaces. The mucosal surface of human bronchial epithelial cultures metabolized 0.1 mM AMP with a half-life of ~15 min (Fig. 2*A*). The nucleotide was converted into adenosine, inosine, and hypoxanthine (Fig. 2*A*). The adenosine deaminase inhibitor, 10 μM erythro-9-[2-hydroxyl-3-nonyl]adenine (57), prevented the accumulation of inosine and hypoxanthine (data not shown), supporting cell surface generation from adenosine. The properties of AMP hydrolysis on airway epithelial surfaces corresponded to those of membrane-bound ectonucleotidases for the following reasons. First, the substrate was hydrolyzed by intact cells and the product was released into the buffer (Fig. 2*B*). Second, AMP hydrolysis followed a linear relationship over time on both epithelial surfaces (Fig. 2*B*), making it unlikely that a significant amount of the substrate had been hydrolyzed after entering the cells and products released in the ectodomain. Third, KRB buffer conditioned by a 60-min exposure to mucosal or serosal surfaces hydrolyzed 0.1 mM AMP at rates that corresponded to <5% of total cell surface activity (Fig. 2*B*). Collectively, these experiments demonstrate that membrane-bound ectoenzymes are responsible for adenosine production at the surface of human airway epithelia.

The AMP hydrolytic activity measured on human bronchial epithelial cells was also detected with epithelial cultures derived from human nasal turbinate (Fig. 2*C*). Hydrolysis rates were 2–3-fold higher on bronchial than on nasal cultures. In addition, both cell types exhibited enzyme activities that were 3–5-fold higher on the mucosal surface than on the serosal surface. These results suggest that the conversion of extracellular AMP into adenosine occurs throughout airways, and that this activity predominates on the epithelia surface facing the airway lumen.

Biochemical Properties of AMP Hydrolysis—Human bronchial epithelial cells were examined for their substrate specificity, divalent cation, and pH sensitivity. The cultures displayed broad substrate specificity for nucleoside monophosphates. On the mucosal surface, hydrolysis rates were in the following order: AMP ≥ UMP = CMP > GMP ≥ IMP (Fig. 3*A*). Mucosal AMPase activity was not affected by Ca^{2+}, but was significantly increased by Mg^{2+} (Fig. 3*B*). Hydrolytic rates were 30% higher in buffers containing 3 mM Mg^{2+} or 3 mM Mg^{2+} and 3 mM Ca^{2+} than with 3 mM Ca^{2+} alone (Table II). Assays conducted on the serosal surface were not influenced by the buffer composition in divalent cations (Table II). The pH dependence profile of mucosal AMP hydrolysis followed a bimodal pattern, with optimal pH around 7.5 and 9.0 (Fig. 3*C*). Serosal AMPase activity produced a single activity peak at pH 7.5. The fact that mucosal and serosal surfaces displayed different cation and pH sensitivities suggested the presence of more than one AMP-hydrolyzing enzyme on human airway epithelial surfaces.

Identification of the Ectoenzymes—We investigated the identity of the enzymes responsible for AMP hydrolysis on human bronchial epithelial surfaces under physiological conditions (pH 7.4). The non-hydrolyzable analog of ADP, α,β-met-ADP, has been described as a competitive inhibitor of ecto 5′-NT and AP that has no effect on cytosolic 5′-NT (32). Fig. 4*A* shows that α,β-met-ADP inhibited the hydrolysis of 0.1 mM AMP in a concentration-dependent manner on both epithelial surfaces. AMP hydrolysis was completely abolished by 0.1 mM α,β-met-ADP, which ruled out the contribution of released cytosolic 5′-NT. This finding is in agreement with the absence of mRNA for CN-I (Fig. 1*C*).

Two types of ectonucleotidases could be involved in the metabolism of extracellular AMP: ecto 5′-NT (32) and APs (33). Their activity at the surface of human bronchial epithelial cells

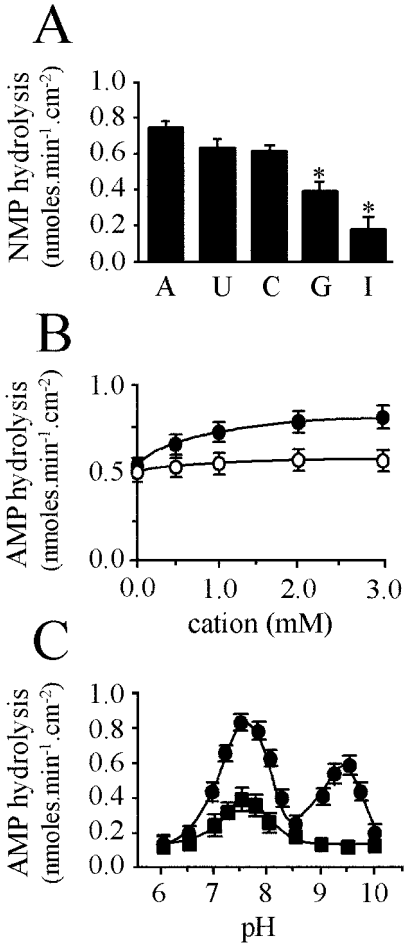

FIG. 3. **Biochemical properties of AMP hydrolysis by human bronchial epithelial cells.** *A*, substrate specificity. The mucosal surface was assayed with 0.1 mM nucleoside monophosphate in KRB, and buffer aliquots were analyzed by HPLC. All substrates were hydrolyzed: AMP (*A*) ≥ UMP (*U*) = CMP (*C*) > GMP (*G*) > IMP (*I*). *B*, cation sensitivity. Assays were performed on the mucosal surface with 0.1 mM AMP in KRB containing various concentrations of Ca^{2+} (○) or Mg^{2+} (●). The reaction was insensitive to Ca^{2+} and stimulated by Mg^{2+}. *C*, pH dependence. The profile for 0.1 mM AMP was bimodal on the mucosal surface (●), with a single peak on the serosal surface (■). Values represent mean ± S.E. of three to five independent experiments. *, $p < 0.05$.

TABLE II
Impact of divalent cations on AMP hydrolysis by mucosal and serosal surfaces of human bronchial epithelial cells

Assays were conducted with 0.1 mM AMP in the absence/presence of 3 mM Ca^{2+} and/or 3 mM Mg^{2+}. Values represent means (±S.E.) of four experiments.

Buffer composition (divalent cations)	Mucosal	%	Serosal	%
	nmol min^{-1} cm^{-2}		*mol min^{-1} cm^{-2}*	
None	1.18 ± 0.04	100	0.74 ± 0.04	100
Ca^{2+}	1.22 ± 0.04	102	0.71 ± 0.05	96
Mg^{2+}	1.54 ± 0.05	131[a]	0.76 ± 0.04	103
Ca^{2+} and Mg^{2+}	1.59 ± 0.05	134[a]	0.79 ± 0.03	106

[a] Values significantly different from rates obtained with buffer devoid of divalent cation ($p < 0.05$, Student's paired *t* test).

was detected with concanavalin A (48) and β-glycerophosphate (49), respectively. Dose-response curves were constructed with 5 mM AMP and various inhibitor concentrations. On the mucosal surface, both compounds reduced the rate of AMP hydrolysis in a saturable manner (Fig. 4*B*). Concanavalin A inhibited 21 ± 4% of total AMPase activity, with an IC_{50} of 9.0 μM. In contrast, β-glycerophosphate reduced AMP hydrolysis by 84 ± 5%, with an IC_{50} of 26 μM. Similar results were obtained when β-glycerophosphate was replaced by levamisole. This non-competitive AP inhibitor reduced the rate of AMP hydrolysis by 81 ± 3%, with an IC_{50} of 17 μM (Fig. 4*B*). Based on IC_{50} values reported for levamisole on NS AP (30 μM), PLA AP (1.7 mM), and I AP (6.8 mM) (50), the AP isoform expressed on human bronchial epithelial cells is likely to be NS AP. The identity of the AP isoforms expressed in human airways was further addressed with specific amino acids. The rate of AMP hydrolysis was not significantly reduced by 10 mM L-phenylalanine (PLA AP and I AP inhibitor) or by 10 mM L-leucine (G AP inhibitor) (34, 35), respectively (data not shown). Collectively, these experiments indicate that ecto 5′-NT and NS AP are responsible for the production of adenosine on human airway epithelial cells.

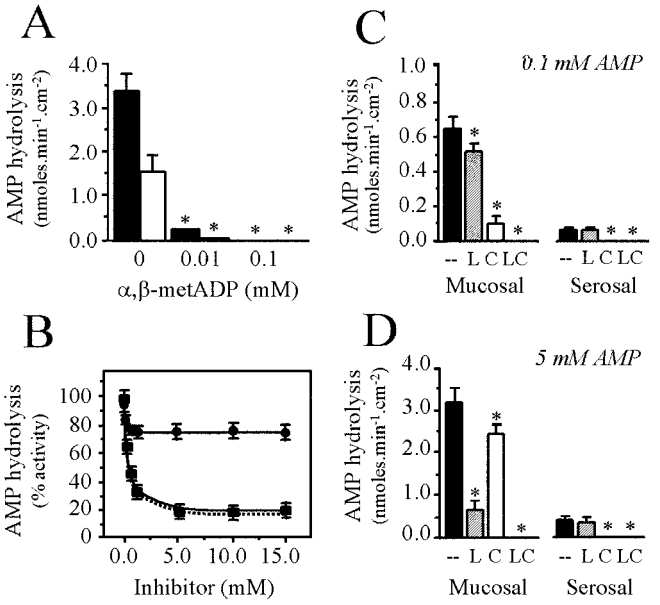

FIG. 4. **Identification of the AMP-hydrolyzing ectoenzymes on human bronchial epithelial cells.** *A*, concentration-dependent inhibition of AMP hydrolysis by α,β-met-ADP. Reactions were initiated with 0.1 mM AMP and 0, 0.01, or 0.1 mM α,β-met-ADP in KRB on mucosal (*filled bars*) and serosal (*open bars*) surfaces, and buffer aliquots were analyzed by HPLC. *B*, detection of mucosal ecto 5′-NT and AP activities. Reaction rates for 5 mM AMP decreased with increasing concentrations of concanavalin A (●), β-glycerophosphate (■), or levamisole (···). *C* and *D*, polarity of ecto 5′-NT and AP. On the mucosal surface, levamisole (*L*) and concanavalin A (*C*) reduced reaction rates by 23 ± 3 and 78 ± 5% with 0.1 mM AMP (*C*) and by 83 ± 4 and 28 ± 3% with 5 mM AMP (*D*). Serosal reactions (*C* and *D*) were insensitive to levamisole and completely inhibited by concanavalin A. The two inhibitors together (*LC*) abolished the reactions on both surfaces. Values represent mean ± S.E. of four to seven independent experiments. *, $p < 0.05$.

The polarity of ecto 5′-NT and NS AP on human bronchial epithelial cultures was addressed with concentrations of concanavalin A (5 mM) and levamisole (10 mM) that produced maximal inhibition (Fig. 4*B*). With 0.1 mM AMP, levamisole and concanavalin A reduced mucosal rates of hydrolysis by 23 ± 3 and 78 ± 5%, respectively (Fig. 4*C*). The two enzymes added together completely abolished AMP hydrolysis. On the serosal surface, levamisole had no significant effect on AMP hydrolysis, whereas concanavalin A completely inhibited the reaction. These results indicate that ecto 5′-NT was expressed on both mucosal and serosal surfaces, whereas NS AP activity was restricted to the mucosal surface. The relative contribution of the two enzymes on mucosal surfaces was strongly influenced by substrate concentration. Assays conducted with 5 mM AMP generated total activities 5 times higher than with 0.1 mM substrate. Under these conditions, levamisole and concanava-

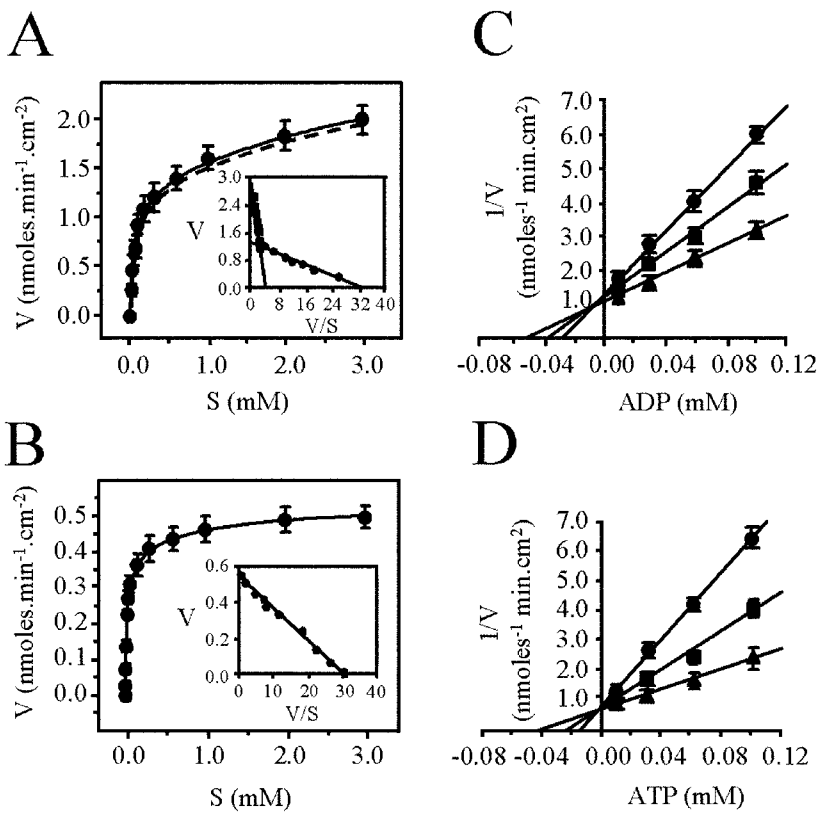

FIG. 5. **Kinetic analysis of ecto 5′-NT and NS AP activities on human bronchial epithelial cells.** *A*, properties of NS AP. Reactions initiated with 0.001–3 mM [^{3}H]AMP in the absence (- - -) or presence (—) of 5 mM concanavalin A. *Inset*, Woolf-Augustinson Hoftsee transformation fitted to two regressions (r = 0.96–0.99), with K_m, V_{max}, and Cat_{eff} of 717 ± 49 μM, 2.8 ± 1.2 nmol·min^{-1}·cm^{-2}, and 0.006 ± 0.001 min^{-1}, and 6 ± 8 μM, 1.2 ± 0.2 nmol·min^{-1}·cm^{-2}, and 0.021 ± 0.005 min^{-1}, respectively. *B*, properties of ecto 5′-NT. Reactions initiated with 0.001–3 mM [^{3}H]AMP and 10 mM levamisole. *Inset*, Woolf-Augustinson Hoftsee transformation fitted to a single regression (r = 0.99), with K_m, V_{max}, and Cat_{eff} of 14 ± 3 μM, 0.52 ± 0.05 nmol·min^{-1}·cm^{-2}, and 0.041 ± 0.006 min^{-1}, respectively (n = 6; S.E. < 5% of the mean). *C* and *D*, impact of ADP and ATP on UMP hydrolysis. Reactions initiated with UMP 10 μM (●), 30 μM (■), or 100 μM (▲) and ADP (10, 30, 60, or 100 μM) or ATP (10, 30, 60, or 100 μM). Buffer aliquots were analyzed by HPLC. Dixon plots showed (*C*) ADP and (*D*) ATP as competitive inhibitors of UMP hydrolysis, with correlation coefficients of 0.96–0.99. Values represent mean ± S.E. of five independent experiments.

lin A reduced mucosal rates of AMP hydrolysis by 83 ± 4 and 28 ± 3%, respectively (Fig. 4*D*). Whereas ecto 5′-NT activity was already maximal with 0.1 mM AMP, NS AP activity was 5 times higher with 5 mM AMP. These results suggested distinct kinetic properties for the two AMP-hydrolyzing enzymes.

Kinetic Properties of the AMP-hydrolyzing Enzymes—The kinetic properties of AMP hydrolysis were examined on the mucosal surface of human bronchial epithelial cultures. Fig. 5*A* shows that the rate of AMP hydrolysis decreased with increasing substrate concentration. Hydrolytic rates did not saturate with millimolar AMP, suggesting the presence of at least two catalytic sites with different substrate affinities.

The profile was not significantly modified when ecto 5′-NT activity was inhibited by 5 mM concanavalin A (Fig. 5*A*), suggesting that AP activity dominated at high AMP concentrations, as shown in Fig. 4*D*. Woolf-Augustinson Hoftsee transformation revealed two kinetic components for AP: a high affinity and a low affinity activity. The K_m and V_{max} values of the high affinity activity were 36 ± 8 μM and 1.2 ± 0.2 nmol·min^{-1}·cm^{-2}, respectively (Fig. 5*A*, *inset*). The K_m and V_{max} values of the low affinity activity were 717 ± 49 μM and 2.8 ± 1.2 nmol·min^{-1}·cm^{-2}, respectively. The Cat_{eff} of the high affinity activity was 4-fold higher than the Cat_{eff} of the low affinity activity, with 0.021 ± 0.005 min^{-1} and 0.006 ± 0.001 min^{-1}, respectively.

Experiments conducted in the presence of the AP inhibitor (10 mM levamisole) revealed a simple Michaelis-Menten equation for the ecto 5′-NT reaction rate reaching saturation around 1 mM AMP (Fig. 5*B*). Woolf-Augustinson Hoftsee transformation indicated a single high affinity activity, with K_m and V_{max} values of 14 ± 3 μM and 0.52 ± 0.05 nmol·min^{-1}·cm^{-2}, respectively (Fig. 5*B*, *inset*). Calculated Cat_{eff} was 0.041 ± 0.006 min^{-1}, which is 2-fold higher than the value obtained for the high affinity AP activity.

We investigated the impact of ATP and ADP on the hydrolysis of nucleoside monophosphates by human bronchial epithelial cells. The assays were conducted with UMP instead of AMP to distinguish the substrate from the inhibitors and their metabolites on the HPLC chromatograms (see "Experimental Procedures"). The rate of UMP hydrolysis was inversely related to ATP and ADP concentrations. Dixon plot analysis indicated a competitive pattern of inhibition for both ADP (Fig. 5*C*) and ATP (Fig. 5*D*), with K_i values of 7 and 10 μM, respectively. These results suggest that extracellular ATP and ADP participate in the control of adenosine concentrations on human airway epithelial cells.

Tissue Measurements of Ecto 5′-NT and NS AP—The relative contributions of ecto 5′-NT and NS AP activities to AMP hydrolysis were examined on the epithelial surface of freshly excised bronchial sections. Fig. 6*A* shows that the polarized cultures closely reproduced the *in vivo* morphologic characteristics displayed by freshly excised bronchial epithelia. Both preparations exhibited a layer of columnar ciliated and secretory epithelial cells covering cuboidal basal-like cells. The epithelial surface of the bronchial sections was assayed with 0.01–5.0 mM AMP, in the absence/presence of the ecto 5′-NT (5 mM concanavalin) or NS AP (10 mM levamisole) inhibitor (see "Experimental Procedures"). Ecto 5′-NT activity was 5-fold higher than NS AP activity when assayed with 0.01 mM AMP (Fig. 6*B*). In contrast, NS AP activity dominated with 0.1 and 5 mM AMP. These results are in agreement with the cell culture model (Fig. 4, *C* and *D*), suggesting that the two AMP-hydrolyzing enzymes address distinct pools of nucleotides on human airway epithelial surfaces.

Expression of Ecto 5′-NT and NS AP in Cultured and Excised Epithelia—The identity of the AP isoforms expressed on human airway epithelial cells was addressed by RT-PCR with primers specific for NS AP, PLA AP, I AP, and G AP (Table I). These experiments were performed with total RNA extracted from primary cultures of human nasal, bronchial, and bronchiolar epithelial cells. Control reactions were conducted with commercial human total RNA (BD Biosciences): liver (NS AP),

small intestine (I AP), placenta (PLA AP), and testis (G AP) (35). Only two AP isoforms were detected in human airway epithelial cells: NS AP and PLA AP. Agarose gels exhibited strong mRNA signals for NS AP in all epithelial samples (Fig. 7*A*). In contrast, PLA AP was not detected in nasal and bronchial epithelial cells and produced a weak signal in bronchiolar epithelial cells. These results are consistent with the inhibition data indicating that the AP activity, detected on bronchial epithelial surfaces, corresponded to the NS AP isoform (Fig. 4*B*). Similar results were obtained for RT-PCR performed with total RNA from freshly excised human airway epithelia (*data not shown*).

The co-expression of ecto 5′-NT and NS AP in primary cultures of human nasal and bronchial epithelial cells was confirmed by RPA. Both enzymes displayed mRNA levels that were 2–3 times higher in bronchial than in nasal epithelial cells (Fig. 7*B*), findings consistent with the biochemical measures of their relative cell surface AMPase activities (Fig. 2*C*).

Impact of Environmental Conditions on Adenosine Production—Because the airways are continuously exposed to inhaled bacteria, we investigated whether the aseptic and stable environment provided by the cell culture conditions affected the expression of ecto 5′-NT and NS AP. Fig. 8*A* shows that ecto 5′-NT mRNA levels in epithelial cultures and freshly excised bronchial epithelia were not significantly different. In contrast, NS AP mRNA was 10-fold lower in culture ($p < 0.01$). These results suggest that the contribution of NS AP to adenosine production in human airways would be underestimated by experiments performed on epithelial cultures. To further test this notion, we compared the mRNA levels of ecto 5′-NT and NS AP in human epithelia freshly excised from the nose, trachea, bronchi, and bronchioles. Fig. 8*B* shows that the two ectoenzymes were detected throughout the respiratory tract. Ecto 5′-NT and NS AP were expressed at comparable levels in nasal turbinate and tracheal epithelia. In lower airways, ecto 5′-NT expression gradually decreased toward alveoli, whereas NS AP mRNA increased with airway generation. These results, combined with the *ex vivo* assays conducted on bronchial sections (Fig. 6*B*), support a major role for NS AP in the elimination of high (> 0.01 mM) nucleotide concentrations below the tracheobronchial tree.

We also tested the impact of the inflammatory mediator IL-β on the expression of ecto 5′-NT and NS AP. Primary cultures of human bronchial epithelial cells were exposed to serosal 0.1–1.0 ng/ml IL-1β in air-liquid interface medium during 24 h and then processed for RPA. Fig. 9 shows that NS AP mRNA increased with IL-1β concentration, whereas ecto 5′-NT mRNA was unaffected. Collectively, these two sets of experiments

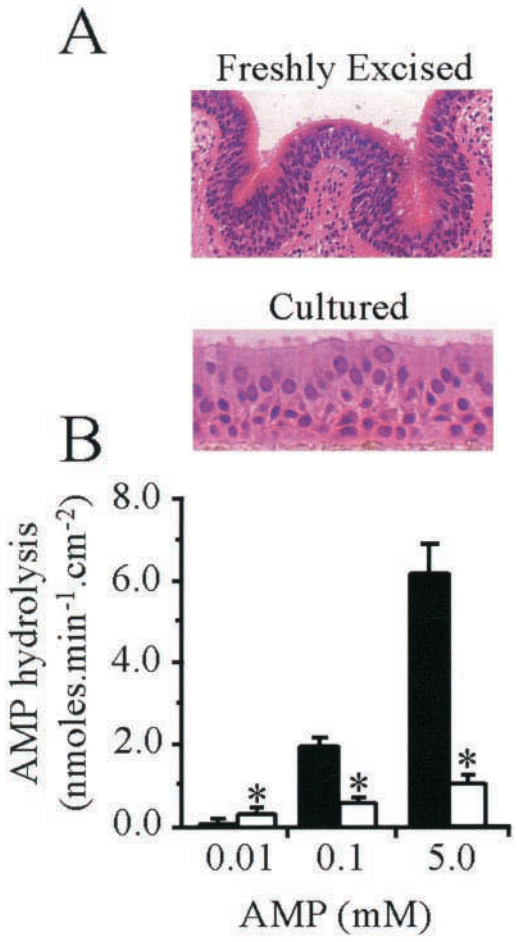

FIG. 6. **Tissue measurements of ecto 5′-NT and NS AP activities.** *A*, typical brightfield (H&E) sections of human airway epithelium from freshly excised main bronchus and polarized bronchial cultures. *B*, AMP hydrolysis on the epithelial surface of bronchus sections. Mucosal NS AP (*filled bars*) and ecto 5′-NT (*open bars*) activities toward 0.01–5 mM AMP assayed with 5 mM concanavalin A and 10 mM levamisole, respectively. Values represent mean ± S.E. of four independent experiments. *, $p < 0.05$.

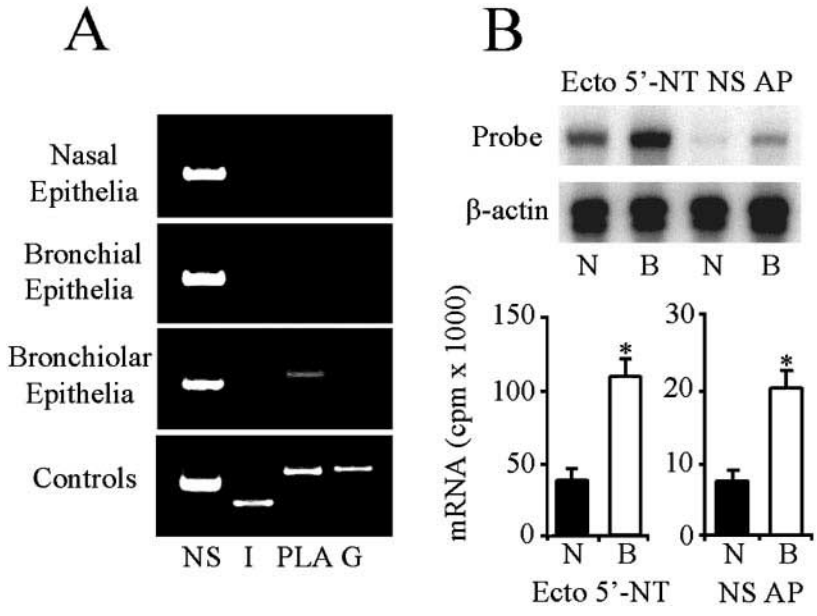

FIG. 7. **Expression of ecto 5′-NT and NS AP in primary cultures of human airway epithelial cells.** *A*, identification of the AP isoform by RT-PCR. Reactions conducted with total RNA from cultured airway epithelial cells and primers for NS AP, I AP, PLA AP, and G AP (Table I). Controls: commercial human RNA (BD Biosciences) from liver (NS AP), small intestine (I AP), placenta (PLA AP), and testis (G AP). *B*, co-expression of ecto 5′NT and NS AP detected by RPA in nasal and bronchial cultures. The mRNA levels, normalized with β-actin, were 2–3 times lower in nasal (*N*, *filled bars*) than bronchial (*B*, *open bars*) epithelial cells. Values corrected for the number of G-C binding sites represent mean ± S.E. of three independent experiments. *, $p < 0.05$.

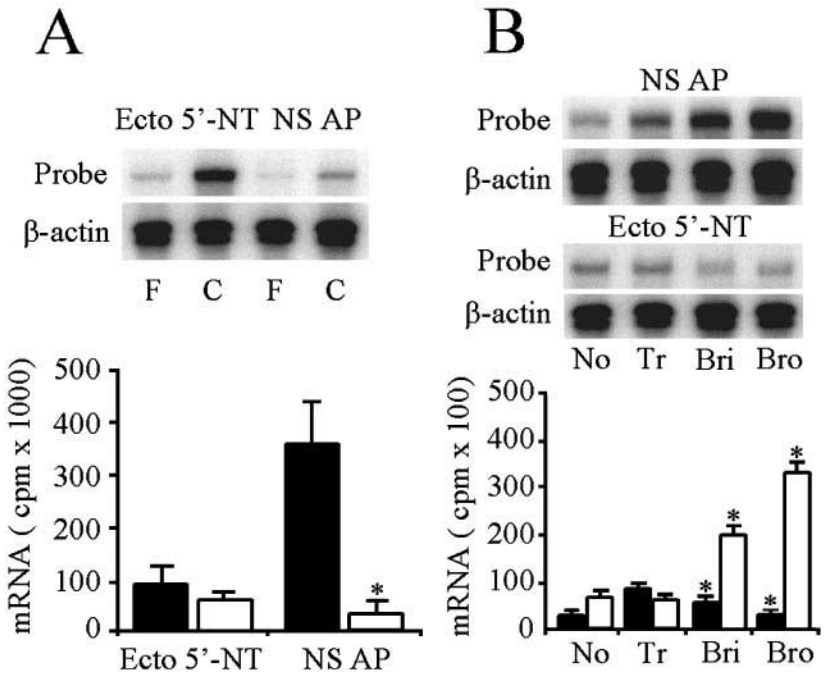

FIG. 8. **Cell culture *versus* tissue expression of ecto 5′-NT and NS AP.** *A*, selective down-regulation by the culture conditions. RPAs conducted with total RNA from freshly excised (*F*, *filled bars*) and cultured (*C*, *open bars*) human bronchial epithelial cells. NS AP (but not ecto 5′-NT) mRNA was higher *in vivo* than in culture. *B*, opposite distributions for ecto 5′-NT (*filled bars*) and NS AP (*open bars*) in human airways. RPAs conducted with total RNA from freshly excised human nasal, tracheal, bronchial, and bronchiolar epithelial cells. Values normalized with β-actin and corrected for the number of G-C binding sites represent mean ± S.E. of four independent experiments. *, $p < 0.05$.

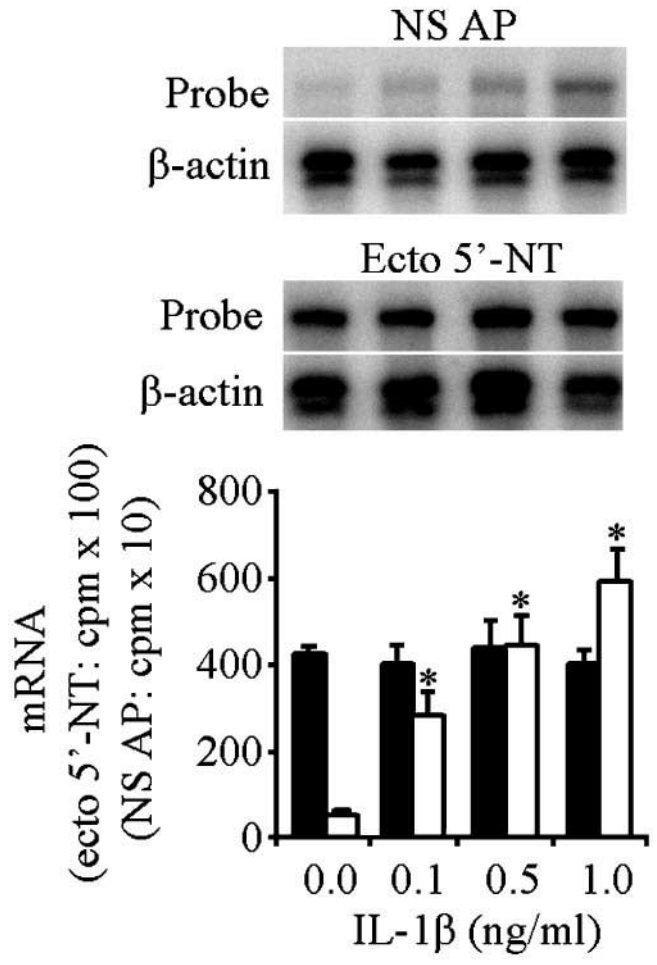

FIG. 9. **Impact of IL-1β on the expression of human airway ecto 5′-NT and NS AP.** Primary cultures of human bronchial epithelial cells were exposed to serosal 0.0–1.0 ng/ml IL-1β during 24 h. RPAs on total RNA showed that NS AP (*open bars*), but not ecto 5′-NT (*filled bars*), was up-regulated by IL-1β. Values normalized with β-actin and corrected for the number of G-C-binding sites represent mean ± S.E. of three independent experiments. *, $p < 0.05$.

suggest that the high affinity ecto 5′-NT modulates physiological nucleotide concentrations, independently of the environmental conditions. In contrast, the fact that NS AP was down-regulated in cell culture, but up-regulated by IL-1β, suggests that the ectoenzyme could be recruited by inflammatory mediators released on epithelial surfaces in response to infection.

DISCUSSION

The current understanding of airway defense mechanisms against bacterial infection acknowledges a complex interplay between ATP and adenosine receptor-mediated epithelial functions (2–15). However, despite numerous studies demonstrating that ATP is released from the mucosal surface of human airway epithelia (20–28), the origin of extracellular adenosine has not been established. In the present work, we considered three possible sources of adenosine for the mucosal surface: 1) paracellular or transepithelial transport from the interstitium; 2) cytosolic formation and release from epithelial cells facing the lumen; and 3) cell surface metabolism of locally released ATP. First, Ussing chamber experiments demonstrated that serosal [^{3}H]adenosine did not permeate into the mucosal compartment, but was locally converted into [^{3}H]inosine and [^{3}H]hypoxanthine (Fig. 1, *A* and *B*). This observation was confirmed by applying ATP to the serosal compartment and searching by HPLC for the appearance of purine nucleotides/nucleosides on the mucosal surface (Fig. 1*D*). Second, we provided evidence that adenosine was not generated intracellularly for transport to the mucosal surface. Specifically, we sought evidence for the mRNA encoding the cytosolic enzyme for adenosine production, CN-I (16, 17), and no evidence for expression of this transcript was found by RT-PCR (Fig. 1*C*). Moreover, we recently reported that extracellular adenosine was actively removed from the mucosal surface of human nasal epithelial cultures by a concentrative Na^+-dependent nucleoside transporter (58). These properties are consistent with the vectorial transport system described for adenosine across intestinal epithelia (59, 60). Taken together, these findings indicate that interstitial and cytosolic pools of nucleosides do not contribute to the endogenous adenosine concentrations detected on the mucosal surface of human airway epithelia (29).

The abundant literature describing basal and stimulated ATP release from the mucosal surface of airway epithelia (20–

28) raised the possibility that released ATP could represent the major source of adenosine through cell surface metabolism. Indeed, cell surface conversion of extracellular ATP into adenosine has been reported in most mammalian tissues (19). In the present study, we have shown that ATP is sequentially dephosphorylated into ADP, AMP, and adenosine on airway epithelial surfaces. The physiological importance of constitutive ATP release and conversion to adenosine was emphasized by the substantial inhibitory effect (>70%) of 0.3 mM α,β-met-ADP on the basal cystic fibrosis transmembrane regulator activity of Calu-3 cells (11). This compound has been described as a competitive inhibitor of ecto 5′-NT and AP, preventing cell surface conversion of AMP to adenosine (32). The fact that ATP, ADP, AMP, and adenosine were confined to the epithelial surface on which ATP was administered suggested that mucosal and serosal surfaces represent distinct compartments with respect to nucleotide pools and purine-mediated signaling pathways. Patch clamp studies on Calu-3 cells demonstrated the close proximity between the site of ATP release, the enzymes responsible for adenosine production, A_{2B} receptors, G proteins, and cystic fibrosis transmembrane regulator (11). Taken together, these findings support local ATP release and metabolism as the major source of adenosine for P1 receptor activation on the mucosal surface of human airway epithelia.

The present work identifies the ectoenzymes directly responsible for the production of adenosine from AMP on the mucosal surface of human airway epithelia. The conversion of extracellular AMP into adenosine was detected on nasal and bronchial epithelial cells, suggesting widespread expression throughout human airways. Several ectoenzymes have been reported to dephosphorylate AMP in the ectodomain: ecto 5′-NT and APs. The biochemical properties of AMP hydrolysis on primary cultures of human bronchial epithelial cells supported the functional expression of more than one ectoenzyme. Mucosal surfaces hydrolyzed nucleoside monophosphates with a substrate specificity (AMP ≥ UMP = CMP > GMP ≥ IMP) intermediate between that of purified ecto 5′-NT (AMP = UMP > CMP > GMP > IMP (48)) and purified AP (AMP = UMP = CMP = GMP = IMP (33)). Mammalian ecto 5′-NT and AP have been reported to display different cation and pH sensitivities. Purified ecto 5′-NT from rat glioblastoma was insensitive to Ca^{2+} and Mg^{2+} (47). In contrast, human liver AP (61) and kidney AP (62) activities were enhanced 2–3-fold by Mg^{2+}, suggesting that an AP could be responsible for the Mg^{2+}-sensitive AMPase activity we detected on the mucosal surface of human bronchial epithelial cells (Fig. 3*B*). In the presence of Mg^{2+}, the optimum pH of purified ecto 5′-NT and AP were reported in the range 7.5–8.0 (47, 63) and 9–10 (33, 61, 62), respectively. We showed that assays conducted in the presence of Ca^{2+} and Mg^{2+} generated two peaks of activity (pH 7.5 and 9.0) on the mucosal surface and a single peak (pH 7.5) on the serosal surface (Fig. 3*C*). Bimodal pH dependence profiles (pH 8.0 and 10.0) were also reported for AMPase activities measured on intact cells or plasma membrane preparations, the alkaline peak of activity exhibiting an absolute requirement for Mg^{2+} (64, 65). Collectively, these results suggest that ecto 5′-NT was responsible for the activity peak detected on both surfaces around pH 7.5, whereas the alkaline activity restricted to the mucosal surface corresponded to APs.

The identity of the AMP-hydrolyzing enzymes expressed on human bronchial epithelial cells at pH 7.4 was further investigated with specific inhibitors. The activity of ecto 5′-NT was revealed with concanavalin A, a lectin reported to have no effect on APs (48). When reactions were initiated with 0.1 mM AMP, this compound inhibited 80 and 100% of total activity measured on mucosal and serosal surfaces, respectively. The remaining mucosal AMPase activity was completely inhibited by β-glycerophosphate and levamisole, non-competitive (49) and competitive (50) inhibitors of APs, respectively. Conversely, serosal AMP hydrolysis was insensitive to levamisole. These results were in agreement with the polarity of ecto 5′-NT and AP activities suggested by the pH and cation sensitivity experiments.

Levamisole has been used to discriminate between NS AP (IC_{50} = 30 μM), PLA AP (IC_{50} = 1.7 mM), and I AP (IC_{50} = 6.8 mM) (50). The high sensitivity of the mucosal AMPase activity to levamisole (IC_{50} = 17 μM; Fig. 4*B*) suggests that NS AP would be the major AP isoform expressed on human airway epithelial cells. In addition, the reaction was resistant to 10 mM L-phenylalanine (PLA and I AP inhibitor) and 10 mM L-leucine (G AP inhibitor) (34, 35). This identification of NS AP as the dominant AP isoform expressed in proximal airways was consistent with mRNA expression studies. Two AP isoforms were detected by RT-PCR in total RNA extracted from cultured or freshly excised human nasal, bronchial, and bronchiolar epithelial cells: NS AP and PLA AP (Fig. 7*A*). Whereas strong signals were obtained for NS AP in all RNA fractions, PLA AP mRNA was limited to bronchiolar epithelial cells. The localization of NS AP and PLA AP in human lungs has been investigated by histochemical and immunocytochemical procedures (36, 37). L-*p*-Bromotetramisole-sensitive L-phenylalanine-resistant NS AP activity was detected on the epithelial surface lining the entire respiratory system (36). In contrast, PLA AP distribution was restricted to peripheral lung parenchyma: respiratory bronchioli, alveolar ducts, alveolar sacs, and alveoli (37). Therefore, the identity of the human airway AP isoforms provided in this work by functional assays and RT-PCR was supported by studies of their tissue distribution.

The polarized primary cultures of human nasal and bronchial epithelial cells exhibited the *in vivo* morphologic characteristics of proximal airway epithelia (45), with columnar ciliated and secretory cells covering basal-like cells. Based on the biochemical properties of AMP hydrolysis on these cells, ecto 5′-NT would be expressed on mucosal and serosal surfaces of airway epithelia, whereas NS AP would be restricted to the mucosal surface. In rat nasal respiratory epithelium, ecto 5′-NT displayed a polarity consistent with these results (66). Ecto 5′-NT was localized by histochemistry to the mucosal surface of columnar epithelial cells and the underlining basal cells. Human airway NS AP was detected predominantly on the apical plasma membrane of columnar epithelial cells (36). These *in vivo* studies support the polarity we obtained for ecto 5′-NT and NS AP activities on the culture model.

Novel information on the tissue distribution of the AMP-hydrolyzing ectoenzymes was provided by quantitative analysis of mRNA levels throughout human airways with cultured and freshly excised epithelial preparations. First, ecto 5′-NT and NS AP expression were 2–3 times higher in bronchial than in nasal epithelial cultures. Second, we demonstrated with freshly excised epithelial cells that the two ectoenzymes exhibit opposite expression patterns throughout airways. Ecto 5′-NT mRNA levels gradually declined from nasal to bronchiolar epithelia, whereas the expression of NS AP increased with airway generation. This constitutes the first report of ecto 5′-NT in mammalian airway epithelia below the nasal cavity. Interestingly, opposite gradient distributions for the two ectoenzymes were also reported for mammalian intestinal mucosa, with decreasing ecto 5′-NT immunostaining (67) and increasing AP activity (68) from the small intestine to the colon.

The relative contribution of the two AMP-hydrolyzing ectoenzymes on the mucosal surface of human bronchial epithelial cells was strongly influenced by substrate concentration.

Experiments conducted on epithelial cultures (Fig. 4, *C* and *D*) and freshly excised bronchial tissues (Fig. 6*B*) indicated that 5 mM AMP was eliminated 5–6 times more rapidly by NS AP than by ecto 5′-NT. In contrast, ecto 5′-NT activity dominated 5-fold over NS AP when assayed with 10 μM AMP (Fig. 6*B*). Further analysis of the substrate concentration-enzyme activity relationship revealed different kinetic properties for the two ectoenzymes (Fig. 5, *A* and *B*). Ecto 5′-NT presented a single high affinity activity for AMP, with a K_m of 14 μM, a value that falls within the range reported for purified ecto 5′-NT (5–20 μM) (47, 63). In contrast, NS AP exhibited two kinetic components: a high affinity (K_m = 36 μM) and a low affinity (K_m = 717 μM) activity. These findings are consistent with the kinetic properties reported for NS AP purified from rat osseous plates at pH 7.5, with K_m values of 82 μM and 1.3 mM for ATP (69). Human osteosarcoma NS AP assayed at pH 7.4 also displayed high and low affinity activities, with K_m values of 25 and 780 μM, respectively (51). The fact that ecto 5′-NT and NS AP possess high affinity activities suggests that they both participate in the metabolism of nucleotide concentrations ($\leq$10 μM) detected locally following ATP release from human airway epithelial cells (70–72). However, the 2-fold higher Cat_{eff} of ecto 5′-NT predicts that this enzyme would be more efficient than NS AP at producing adenosine from physiological AMP concentrations, as demonstrated by their respective contribution to the hydrolysis of 10 μM AMP on bronchial cultures and tissues. Finally, AMP hydrolysis was competitively inhibited by ATP (K_i = 7 μM) and ADP (K_i = 10 μM) on human bronchial epithelial cells (Fig. 5, *C* and *D*), as previously reported for purified rat renal ecto 5′-NT (K_i = 0.03–30 μM) (63, 73, 74). Consequently, the conversion of AMP into adenosine would represent a rate-limiting step in the production of P1 receptor agonists from ATP release on airway surfaces.

The purinergic modulation of MCC on human airway epithelial surfaces involves a complex interplay between nucleotide and nucleoside concentrations, ectonucleotidases, P2 and P1 receptors. Mechanical stimulation, such as coughing-induced shear stress, raises local cell surface ATP to concentrations (1–10 μM) (70–72) that initiate P2 receptor-mediated MCC functions (2–9). Desensitization of the $P2Y_2$ receptors by prolonged exposure to micromolar nucleotides (4, 75) might be avoided by the rapid dephosphorylation of ATP and ADP into AMP we reported on human airway epithelial surfaces (21, 31). Through the conversion of extracellular AMP into adenosine, ecto 5′-NT and NS AP would provide the agonist (adenosine) required for the smaller but more sustained MCC responses, mediated by A_{2B} receptor regulation of CBF (4, 5) and ion transport (12–15).

In summary, we have demonstrated that ecto 5′-NT and NS AP are responsible for the production of adenosine on the mucosal surface of human airway epithelial cells. The relatively high efficiency of ecto 5′-NT suggests that this enzyme would play a major role in the regulation of adenosine-mediated epithelial functions. On the other hand, all APs dephosphorylate not only AMP but also ADP and ATP (33). Under pathological conditions, trauma generates quantities of extracellular nucleotides that may cause damage to the epithelium. For example, airway epithelial cultures have been reported to express $P2X_7$ receptors (8), an ATP-gated channel known to induce apoptosis (76). The high-capacity NS AP could protect these airways against the deleterious effects of high ATP concentrations. The fact that IL-1β enhanced 5-fold NS AP activity and expression supports a role for this enzyme in airway defenses during periods of inflammation. We would note, however, that the broad substrate specificity of APs suggests that NS AP could be involved in other airway functions, including bacterial endotoxin neutralization (77) and sphingosine 1-phosphate receptor signaling (78, 79).

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