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# Metabolism of P2 Receptor Agonists in Human Airways

## IMPLICATIONS FOR MUCOCILIARY CLEARANCE AND CYSTIC FIBROSIS*

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**Extracellular nucleotides are among the most potent mediators of mucociliary clearance (MCC) in human lungs. However, clinical trials revealed that aerosolized nucleotides provide only a transient improvement of MCC to patients diagnosed with cystic fibrosis (CF). In this study, we identified the mechanism that eliminates extracellular nucleotides from human airways. Polarized primary cultures of human bronchial epithelial cells were impermeable to extracellular nucleotides but rapidly dephosphorylated ATP into ADP, AMP, and adenosine. The half-life of a therapeutic ATP concentration (0.1 mM) was ~20 s within the periciliary liquid layer. The mucosal epithelial surface eliminated P2 receptor agonists (ATP = UTP > ADP > UDP) at 3-fold higher rates than the serosal surface. We also showed that mucosal (not serosal) ectoATPase activity increases toward areas most susceptible to airway obstruction (nose < bronchi ≪ bronchioles). Bronchial cultures from patients with CF, primary ciliary dyskinesia, or α1-antitrypsin deficiency exhibited 3-fold higher mucosal (not serosal) ectoATPase activity than normal cultures. Time course experiments indicated that CF enhances ATP elimination and adenosine accumulation on the mucosal surface. Furthermore, nonspecific alkaline phosphatase was identified as the major regulator of airway nucleotide concentrations in CF, primary ciliary dyskinesia, and α1-antitrypsin deficiency. The ectoATPase activity and mRNA expression of mucosally restricted nonspecific alkaline phosphatase were 3-fold higher on bronchial cultures from these patients than from healthy subjects. This study demonstrates that the duration of nucleotide-mediated MCC is limited by epithelial ectonucleotidases throughout human airways, with the efficiency of this mechanism enhanced in chronic inflammatory lung diseases, including CF.**

Healthy lungs are protected against bacterial and viral infections by mediators of mucociliary clearance (MCC)[1] mechanisms taking place on the epithelium lining the airways. The mucosal surface is covered by a mucus layer maintained above the cilia by a PCL layer (1). Infectious particles are trapped in the mucus and transported upward by coordinated cilia beating activity. Evidence derived from animal studies and human cell culture models indicates that ion transport across the epithelium regulates PCL volume (2–4) and that PCL depletion leads to mucostasis (4). Mutations of the gene encoding the cystic fibrosis transmembrane regulator (CFTR) epithelial anion transporter are associated with chronic airway obstruction, infection, and inflammation in CF patients (5).

Extracellular nucleotides regulate all major components of MCC on human airway epithelial surfaces. Through G protein-coupled P2Y receptors, they stimulate $Ca^{2+}$- and PKC-dependent mucin secretion, cilia beating activity, and ion channels that regulate PCL volume, namely $Ca^{2+}$-activated $Cl^-$ channels (6). Two members of the P2Y receptor subfamily were identified on the mucosal surface of human airway epithelia: the $P2Y_2$ receptor equally activated by ATP and UTP (not ADP or UDP) (7) and the $P2Y_6$ receptor potently activated by UDP and weakly by ADP (8, 9). The serosal surface expresses $P2Y_2$ (10) and $P2Y_1$ (ADP > ATP > UTP) (11) receptors. In addition, two members of the P2X receptor subfamily were identified by RT-PCR in cultured human airway epithelia: $P2X_4$ and $P2X_5$ (12). Calcium influx through these ligand-gated cation channels (ATP ≫ ADP) stimulated $Cl^-$ secretion (12) and cilia beating activity (13).

The physiological importance of nucleotide-mediated MCC is supported by numerous studies showing that airway epithelia release ATP under basal conditions (14, 15) and in response to various mechanical stimulations, including membrane stretch (11, 16), shear stress (17, 18), and hypotonicity-induced swelling (19–21). However, nucleotide-mediated signaling pathways do not sustain normal PCL volume under resting conditions because basal ATP levels are insufficient to activate $Ca^{2+}$-activated $Cl^-$ channels (22). On the other hand, resting airway epithelia exhibit extracellular adenosine concentrations sufficient to activate CFTR (23). Adenosine binds to G protein-coupled $A_{2B}$ receptors and mediates cAMP-dependent (24) stimulation of cilia beating (25, 26) and ion transport (23, 27–29).

Cystic fibrosis patients are unable to maintain normal PCL volume using the adenosine receptor-CFTR pathway and therefore depend on mechanically-stimulated ATP release (*i.e.* coughing, wheezing, and clapping) for $Ca^{2+}$-activated $Cl^-$ channels activation. However, clinical studies indicated that nucleotides provide only a short-term improvement of MCC in CF patients (30). Aerosolized UTP, in conjunction with amilo-

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[1] The abbreviations used are: MCC, mucociliary clearance; αAT, α1-antitrypsin deficiency; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator; HPLC, high performance liquid chromatography; KRB, Krebs buffer; NS AP, non-specific alkaline phosphatase; PCL, periciliary liquid; PBS, phosphate-buffered saline; PCD, primary ciliary dyskinesia; P1, passage 1.

ride (sodium channel blocker), increased MCC to normal levels in ~20 min in CF patients (31, 32). In polarized cultures of CF nasal epithelial cells, UTP restored normal PCL volume in <1 h (4). The transient nature of these responses suggests that nucleotide concentrations above resting levels are rapidly eliminated from human airway epithelial surfaces.

Given the importance of P2 receptor-mediated MCC for CF patients, we investigated the mechanisms of nucleotide clearance from human airway epithelial surfaces under normal and pathological conditions. We tested whether they are eliminated by paracellular permeation through the epithelial layer and/or cell surface metabolism. We also describe, for the first time, the polarity and distribution of these ectonucleotidase activities throughout human airways with epithelial cultures of nasal, bronchial, and bronchiolar cells. The impact of chronic inflammatory lung diseases on nucleotide clearance was examined with epithelial cultures from patients diagnosed with CF, PCD, or αAT. Finally, we addressed the identity of the proteins responsible for nucleotide metabolism. Three families of ectonucleotidases have been identified on mammalian cells: ectonucleotide pyrophosphatase/phosphodiesterases, alkaline phosphatases (AP), and ectonucleoside triphosphate diphosphohydrolases (33). We recently demonstrated the presence of NS AP on the mucosal surface of human airway epithelia (34). Because NS AP expression was up-regulated by the major airway pro-inflammatory cytokine, interleukin-1β (34), we tested the impact of CF, PCD, and αAT on the activity and expression of NS AP. This study demonstrates that P2 receptor agonists are rapidly eliminated from human airway epithelial surfaces by ectonucleotidases, including NS AP. Furthermore, we show that nucleotide clearance is accelerated in CF by mechanisms that involve chronic inflammation.

## EXPERIMENTAL PROCEDURES

*Cell Culture*—Polarized cultures of human airway epithelial cells from healthy donors and patients with CF, PCD, or αAT were grown as previously described (35). In brief, the cells were isolated by protease digestion (36) and plated on porous Transwell Col filters (well diameter, 12 mm; pore size, 0.45 μM) in air-liquid interface medium (50:50 mixture of LHC Basal and Dulbecco's modified Eagle's medium-H, 0.5 ng/ml epithelial 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/μl streptomycin) (37). The confluent cultures were maintained in air-liquid interface with medium added only to the serosal compartment. Passage 1 (P1) cultures were obtained by plating the digested cells on a collagen-coated tissue culture dish (5–10 days) in LHC9 medium (36) containing 25 ng/ml epithelial growth factor, 50 nM retinoic acid, 40 μg/ml gentamicin, 0.5 mg/ml bovine serum albumin, 0.8% bovine pituitary extract, 50 units/ml penicillin, 50 μg/μl streptomycin, and 0.125 mg/ml amphotericin. Once they reached confluence, the cells were trypsinized and subpassaged on porous Transwell Col filters. After 4 weeks, primary and P1 cultures were composed of columnar ciliated cells (>90%) and secretory cells covering a layer of basal-like cells (38). Experiments were conducted on cultures of transepithelial electrical resistance ≥300 Ω/cm$^2$. Lactate dehydrogenase activity was employed as a test of cellular integrity.

*Ectonucleotidase Assays*—Epithelial cultures were washed three times with phosphate-buffered saline (PBS) and once 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 then preincubated in KRB (0.35 ml of mucosal/serosal) for 30 min at 37 °C (5% $CO_2$/95% $O_2$). All reactions were initiated by the addition of the substrate (dissolved (10×) in 0.035 ml of KRB) to the mucosal and/or serosal buffer and stopped by transferring 10-μl aliquots to tubes containing 0.3 ml of ice-cold water. The samples were boiled 5 min, filtered, and analyzed by reversed-phase paired-ion HPLC.

*PCL Layer Assays*—Primary cultures of human bronchial epithelial cells were assayed for the metabolism of 0.1 mM ATP within the PCL, as previously described (4). In brief, several wells of the same culture were washed three times with PBS and preincubated for 60 min to restore normal PCL height. The substrate was prepared by suspending 0.1 mg of ATP and 3 μCi (3 μl) of [$^3$H]ATP into 0.2 ml of perfluorocarbon (FC-72). The mixture was sonicated for 10 min, and 10 μl was added to the mucosal surface of each well. Perfluorocarbon evaporates in seconds, leaving the substrate within the PCL layer. The reactions were ended at different time intervals by washing the epithelial surface with PBS (0.3 ml) and transferring the entire volume to a tube containing 40 μl of 50 mM EDTA. The samples were boiled for 3 min, filtered, and analyzed by HPLC.

*Synthesis of [$^3$H]ADP and [$^3$H]UDP*—Tritiated ADP was obtained from [$^3$H]ATP by an hexokinase reaction, as described previously (8). In brief, 50 μCi of [$^3$H]ATP were incubated with 10 units/ml hexokinase (30 min, 37 °C) in 0.2 ml of KRB. The samples were boiled for 3 min to eliminate the hexokinase activity, and full conversion of [$^3$H]ATP into [$^3$H]ADP was confirmed by HPLC.

*Enzyme Release Assays*—Mucosal epithelial surfaces were washed three times with PBS and once with KRB and then incubated in bilateral KRB (350 μl of mucosal/serosal) for 0, 30, or 60 min at 37 °C (5% $CO_2$/95% $O_2$). The conditioned buffer was collected, centrifuged at 14,000 × *g* (4 °C; 20 min) to remove detached cells and debris. Released ATPase activity was measured by transferring 315 μl to a tube containing 1 mM ATP in 35 μl of KRB (37 °C) and collecting aliquots over time for HPLC analysis. These rates of ATP hydrolysis were compared with measurements performed the next day on the mucosal surface of the same wells to determine the contribution of released enzyme to total surface activity.

*HPLC Separation of Nucleotides*—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 tetrabutyl ammonium hydrogen sulfate (TBASH), pH 5.3) from 0–10 min, buffer B (100 mM $KH_2PO_4$, 8 mM TBASH, and 10% MeOH, pH 5.3) from 10–20 min, and buffer A from 20–30 min. Absorbance was monitored at 254 nm with an online Model 490 multiwavelength detector (Shimadzu Scientific Instruments, Inc., Columbia, MD), and radioactivity was determined online with a Flo-One radiomatic detector (Packard, Canberra, Australia) as described previously (39).

*RNase Protection Assays*—Total RNA and RNase protection assay probes for NS AP were prepared as previously described (34). In brief, total RNA was extracted from primary cultures of human bronchial epithelial cells and amplified by RT-PCR with primers generated from the sequence of human NS AP. The [$\alpha^{32}$P]CTP-labeled RPA probes were prepared from plasmids containing the NS AP cDNA. The probes were purified by electrophoresis, eluted, and hybridized with 20 μg of total RNA (RPA III kit; Ambion). The protected fragments were separated by electrophoresis and the gels dried on a vacuum dryer (60 °C, 4 h) and exposed to a storage phosphor screen (Amersham Biosciences). The screen was scanned with an optical scanner (Storm; Amersham Biosciences) and the signals quantified by digital image-analyzing software (ImageQuant; Amersham Biosciences). Sense strand and yeast RNA controls yielded no signal on the gels.

*Materials*—All 5′-nucleotides and adenosine were purchased from Roche Applied Science. $KH_2PO_4$, tetrabutyl ammonium hydrogen sulfate, and HEPES were obtained from Sigma and perfluorocarbon (FC-72) from 3 M (St. Paul, MN). HPLC-grade water was bought from Fisher Scientific (Pittsburgh, PA). Cell culture media, bovine serum albumin, bovine pituitary extract, epithelial growth factor, gentamicin, penicillin, retinoic acid, and streptomycin were bought from Invitrogen. [2,8$^3$H]ATP (40–50 Ci/mmol) and [2,8$^3$H]UTP (40–50 Ci/mmol) were from Amersham Biosciences. Salts and solvents were of analytical grade.

*Data Analysis*—Enzyme assays and RNase protection assays were conducted on polarized cultures of airway epithelial cells from at least three healthy donors or three patients diagnosed with CF, PCD, or αAT, unless stated otherwise. Nucleotide metabolism was calculated from the initial linear rate of substrate decay monitored by HPLC and presented as nmoles·min$^{-1}$·cm$^{-2}$. Values were expressed as means ± S.E. of the mean. Unpaired Student's *t* tests were used to assess the significance between two independent data sets. Paired *t* tests were used for comparisons between mucosal and serosal surfaces of the same culture or between conditioned buffer and the corresponding mucosal surface. All linear regressions, curve fits, and data transformations were performed with PC computer programs Excel, Origin, and Sigma plot.

## RESULTS

*Mechanism of Nucleotide Clearance*—We first determined whether extracellular nucleotides are eliminated from human airways by permeation through the epithelial layer and/or by cell surface metabolism. The P2 receptor agonist 0.1 mM

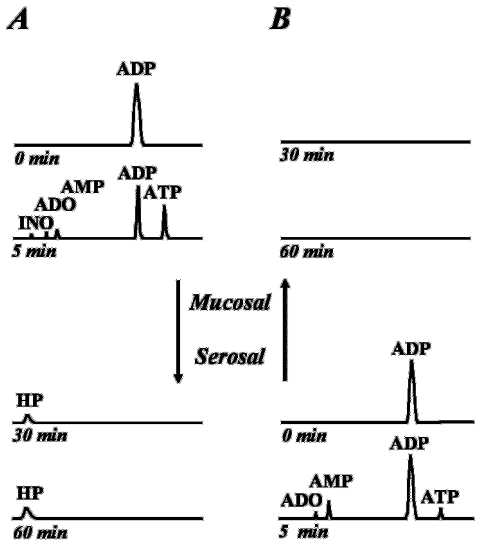


FIG. 1. **Mucosal and serosal epithelial surfaces are separate compartments.** Primary bronchial cultures were incubated in 0.35 ml of bilateral KRB (pH 7.4) containing mucosal (*A*) or serosal (*B*) 0.1 mM [$^3$H]ADP (0.5 $\mu$Ci). Buffer samples were collected from both sides over 60 min and analyzed by HPLC. *A* and *B*, mucosal and serosal [$^3$H]ADP were converted into [$^3$H]ATP, [$^3$H]AMP, [$^3$H]adenosine (*ADO*), and [$^3$H]inosine (*INO*). No radiolabeled compound except hypoxanthine (*HP*) crossed the epithelial layer. HPLC traces are typical results from four experiments. The [$^3$H] designation was omitted for clarity.

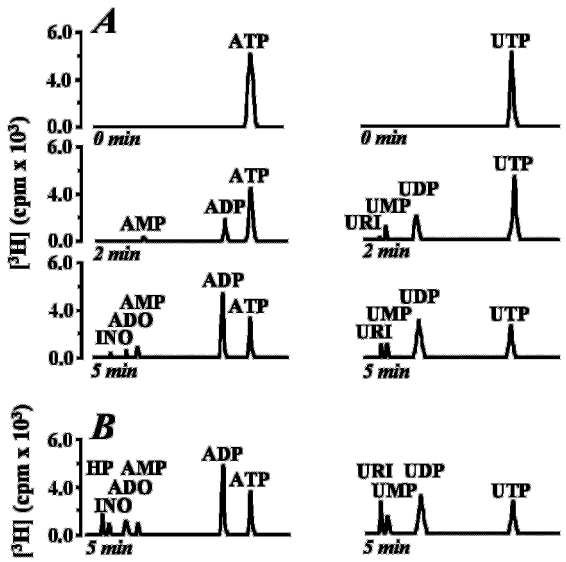


FIG. 2. **Nucleotide metabolism on human airway epithelia.** Primary bronchial cultures incubated in 0.35 ml of bilateral KRB (pH 7.4) and assayed on the mucosal surface with 0.1 mM [$^3$H]ATP (0.5 $\mu$Ci) or 0.1 mM [$^3$H]UTP (0.5 $\mu$Ci), without (*A*) or with (*B*) nucleoside transporter inhibitors (2 mM phloridzin + 0.1 mM dipyridamole). *A,* mucosal KRB samples collected over 5 min and analyzed by HPLC showed sequential dephosphorylation of [$^3$H]ATP into [$^3$H]ADP, [$^3$H]AMP, and [$^3$H]adenosine (*ADO*) and of [$^3$H]UTP into [$^3$H]UDP, [$^3$H]UMP, and [$^3$H]uridine (*URI*). *B*, transporter blockers enhanced inosine (*INO*) and hypoxanthine (*HP*) production. HPLC traces are typical results from six experiments. The [$^3$H] designation was omitted for clarity.

[$^3$H]ADP was added to the mucosal surface of bronchial cultures, and buffer samples collected through time were analyzed by HPLC. Fig. 1*A* indicates that no radiolabeled compound accumulated in the serosal compartment over 60 min, except for traces of [$^3$H]hypoxanthine. Similar results were obtained with 0.1 mM [$^3$H]ATP added to the mucosal surface of human bronchial cultures (34). Furthermore, reciprocal experiments conducted with 0.1 mM [$^3$H]ADP added to the serosal surface showed that all radiolabeled compounds were confined to the serosal compartment (Fig. 1*B*). Taken together, these experiments demonstrate that nucleotide clearance in human airways does not involve permeation through the epithelial layer.

These experiments also showed that less phosphorylated nucleotides accumulated on the epithelial surface exposed to [$^3$H]ADP (Fig. 1, *A* and *B*), suggesting that P2 receptor agonists may be eliminated by cell surface metabolism. To test this hypothesis, the mucosal surface of bronchial cultures was incubated with 0.1 mM [$^3$H]ATP or [$^3$H]UTP in KRB, and buffer samples collected over 5 min were analyzed by HPLC. Fig. 2*A* shows that [$^3$H]ATP gradually disappeared and [$^3$H]ADP, [$^3$H]AMP, [$^3$H]adenosine, and [$^3$H]inosine accumulated. Likewise, the epithelial surface eliminated [$^3$H]UTP by a mechanism that generated [$^3$H]UDP, [$^3$H]UMP, and [$^3$H]uridine. The inhibition of concentrative (2 mM phloridzin) (40) and equilibrative (100 $\mu$M dipyridamole) (41) nucleoside/nucleobase transporters accelerated the accumulation of [$^3$H]adenosine, [$^3$H]inosine, and [$^3$H]hypoxanthine from [$^3$H]ATP and of [$^3$H]uridine from [$^3$H]UTP (Fig. 2*B*), supporting their cell surface production. Accordingly, total radioactivity measured after 5 min corresponded to >90% of initial [$^3$H]ATP or [$^3$H]UTP counts (Fig. 2*A*). Collectively, these results suggest that P2 receptor agonists are eliminated from human airway epithelia by cell surface dephosphorylation.

*Mucosal and Serosal Surfaces: Two Distinct Compartments*—The permeability experiments suggested that extracellular nucleotide metabolism occurs on both mucosal (Fig. 1*A*) and serosal (Fig. 1*B*) surfaces of human airway epithelia. We therefore investigated whether the distinct nucleotide and nucleoside compositions reported for mucosal (adenosine ≫ AMP > ADP ≫ ATP) and serosal (ADP = AMP > adenosine [tmt] ATP) surfaces (42) may reflect local differences in cell surface metabolism. Time course experiments showed that 0.1 mM [$^3$H]ATP, [$^3$H]ADP, and [$^3$H]AMP are all sequentially dephosphorylated on both epithelial surfaces (Fig. 3). However, all three nucleotides were eliminated at 3-fold higher rates on the mucosal surface. Furthermore, mucosal and serosal profiles for [$^3$H]ATP metabolism differed with respect to [$^3$H]ADP and [$^3$H]adenosine production. On the mucosal surface, [$^3$H]ATP dephosphorylation resulted in a transient production of [$^3$H]ADP and a sustained accumulation of [$^3$H]adenosine. The nucleoside represented more than 80% of total reaction products by the end of the incubation period. On the serosal surface, although [$^3$H]ADP and [$^3$H]adenosine concentrations increased steadily, [$^3$H]ADP remained the major reaction product throughout the experiment. These results suggest that local ATP release and metabolism are responsible for the distinct nucleotide/nucleoside composition of human airway epithelial surfaces.

Further examination of the time course experiments revealed an additional discrepancy between mucosal and serosal nucleotide metabolism with respect to adenosine production. On the mucosal surface, reactions initiated with 0.1 mM [$^3$H]AMP generated higher adenosine levels than with [$^3$H]ATP or [$^3$H]ADP as substrate (Fig. 3). These data are consistent with our recent finding that ATP and ADP are competitive inhibitors of mucosal ectoAMPase activities on human airway epithelia (34). In contrast, ATP and ADP did not interfere with adenosine production on the serosal surface. Collectively, the above experiments clearly demonstrate that in human airways, mucosal and serosal epithelial surfaces constitute separate and distinct compartments for nucleotide metabolism.

*Cell-associated and Secreted Ectonucleotidases*—These experiments were designed to determine whether extracellular nucleotide metabolism on human airway epithelial surfaces results from the activities of cell-associated or soluble ecto-

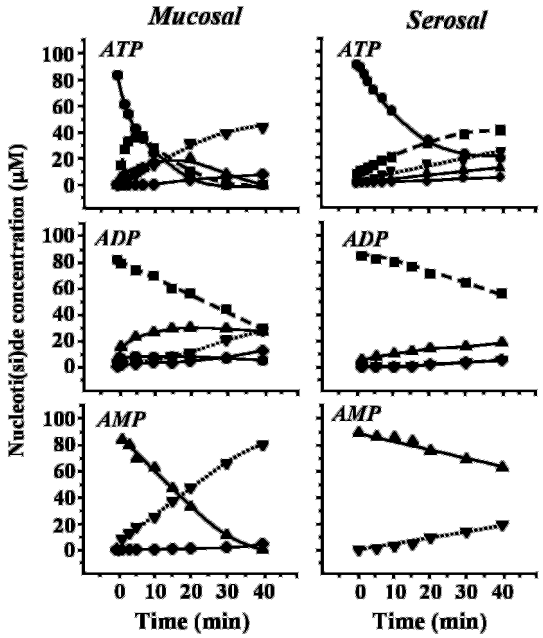


FIG. 3. **Polarity of nucleotide metabolism on human airway epithelial surfaces.** Primary cultures were incubated in 0.35 ml of lateral KRB (pH 7.4) containing mucosal or serosal 0.1 mM [$^3$H]ATP (0.5 $\mu$Ci), [$^3$H]ADP (0.5 $\mu$Ci), or [$^3$H]AMP (0.5 $\mu$Ci). The major product of ATP metabolism was adenosine and ADP on mucosal and serosal surfaces, respectively. ATP and ADP inhibited adenosine production on the mucosal (not serosal) surface. [$^3$H]ATP (●), [$^3$H]ADP (■), [$^3$H]AMP (▲), [$^3$H]adenosine (▼), and [$^3$H]inosine (♦). The data are mean results from five experiments (S.E. <10% of the mean). The [$^3$H] designation was omitted for consistency.

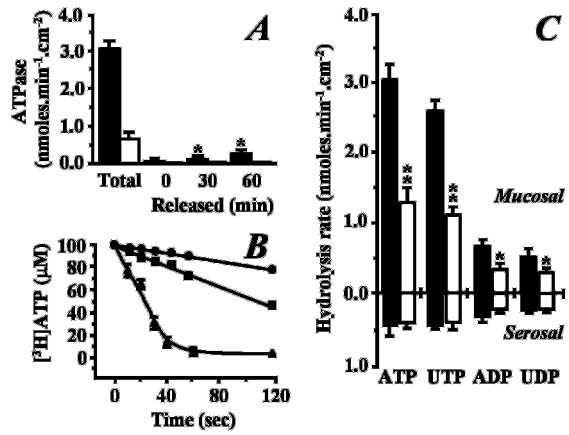


FIG. 4. **Human airway ectonucleotidases are cell-associated and predominantly mucosal.** *A*, cell-associated ectoATPases. KRB buffer (pH 7.4; 0.35 ml) conditioned for 0, 30, or 60 min on mucosal (*filled bars*) or serosal (*open bars*) epithelial surfaces was collected and assayed for released activity with 1 mM ATP. Mucosal and serosal surfaces were assayed after 24 h for total activity. Values were calculated from linear rates of substrate decay determined by HPLC. *B*, impact of liquid height on ectoATPase activity. Mucosal surface assayed with 0.1 mM [$^3$H]ATP in 0 (▲), 0.2 (■), or 0.35 ml (●) KRB. EctoATPase activity is inversely related to PCL volume. *C,* polarity of nucleotide metabolism. Bronchial (*filled bars*) and nasal (*open bars*) cultures incubated in bilateral 0.35 ml of KRB with 1 mM ATP, UTP, ADP, or UDP. Hydrolysis rates were higher on mucosal than serosal surfaces and higher on bronchial than nasal cultures. Values are mean ± S.E. of 3–8 experiments (*, $p < 0.05$. **, $p < 0.01$).

nucleotidases. Because all enzymatic assays conducted on the epithelial cultures involved a 30-min preincubation followed by a 30- to 40-min incubation period, enzyme release was assessed in KRB collected after a 0–60-min conditioning period on a mucosal or serosal surface (see "Experimental Procedures"). Primary cultures of bronchial epithelial cells were assayed for total and released activities with 1 mM ATP (Fig. 4*A*). On the mucosal surface, ectoATPase activity released in conditioned KRB increased with exposure time and represented <8% of total surface activity after 60 min. The enzyme fraction remaining on the epithelial surface after conditioned KRB was collected could not be removed by excessive wash (five times) with PBS. On the serosal surface, no significant enzyme activity was detected in conditioned KRB over 60 min. These results suggest that the enzymes supporting nucleotide metabolism on human airway epithelia are cell-associated ectonucleotidases.

*Impact of PCL Volume on ATP Metabolism*—The biochemical characterization of extracellular nucleotide metabolism on the epithelial cultures required an artificially large liquid volume (350 $\mu$l). Indeed, the mucosal surface of human airway epithelia in culture is covered by a thin PCL layer of ~5–10 $\mu$m in height (4), corresponding to an average volume of 10 $\mu$l over a 1-cm$^2$ Transwell. To test the impact of PCL volume on nucleotide metabolism, the mucosal surface of bronchial epithelial cultures was assayed with 0.1 mM [$^3$H]ATP in 0, 0.2, or 0.35 ml of KRB (see "Experimental Procedures"). The assays conducted in the absence of KRB also documented for the first time the *in vivo* properties of ATP metabolism within the PCL layer. Fig. 4*B* shows that the half-life ($t_{1/2}$) of [$^3$H]ATP increased with the PCL volume. In the absence of KRB, [$^3$H]ATP concentration was reduced below 1 $\mu$M within 1 min ($t_{1/2} \cong 20$ s). These experiments clearly demonstrate that ectonucleotidases are remarkably efficient in eliminating micromolar concentrations of P2 receptor agonists within the PCL layer.

*Distribution of Ectonucleotidases in Human Airways*—The distribution and polarity of ectonucleotidase activities in proximal airways was examined with primary cultures of human nasal and bronchial epithelial cells. Fig. 4*C* shows that both cell types displayed bilateral ectonucleotidase activities toward P2 receptor agonists (1 mM; ATP ≥ UTP > ADP > UDP). However, all reaction rates were 3- to 5-fold higher on mucosal than on serosal surfaces. On the mucosal surface, bronchial cultures hydrolyzed all four nucleotides at significantly higher rates than nasal cultures. Interestingly, no significant difference in metabolic rates was noted between the two cell types on the serosal surface.

Because distal airways constitute the main target for aerosolized treatments of chronic obstructive lung diseases (43), we tested their ability to eliminate therapeutic concentrations of P2 receptor agonists. Because of the limited availability of bronchiolar epithelial cells, we tested the possibility of using P1 cultures. Fig. 5*A* shows that primary and P1 bronchial cultures preserved the *in vivo* morphologic characteristics of bronchial epithelia, with columnar ciliated and secretory cells facing the mucosal surface (38). These cultures were assayed with bilateral 1 mM ATP or ADP. Although mucosal metabolism occurred at a significantly lower rate on P1 than on primary cultures, the ATP/ADP hydrolysis ratio was preserved through passage (Fig. 5*B*). Therefore, P1 cultures were used to investigate the relationship between airway generation and cell surface nucleotide metabolism. Fig. 5*C* indicates that mucosal epithelial surfaces hydrolyzed ATP and ADP with the efficiency order: nasal < bronchial << bronchiolar cells. In contrast, there was no significant difference between serosal activities on the three culture types. Collectively, these experiments demonstrate that the capacity of airway mucosa to eliminate P2 receptor agonists increases significantly toward areas most susceptible to airway obstruction.

*Metabolism of P2 Receptor Agonists in MCC-deficient Lungs*—We tested the impact of chronic airway obstruction on the regulation of nucleotide-mediated MCC. Primary cultures of bronchial epithelial cells from healthy donors and CF pa-

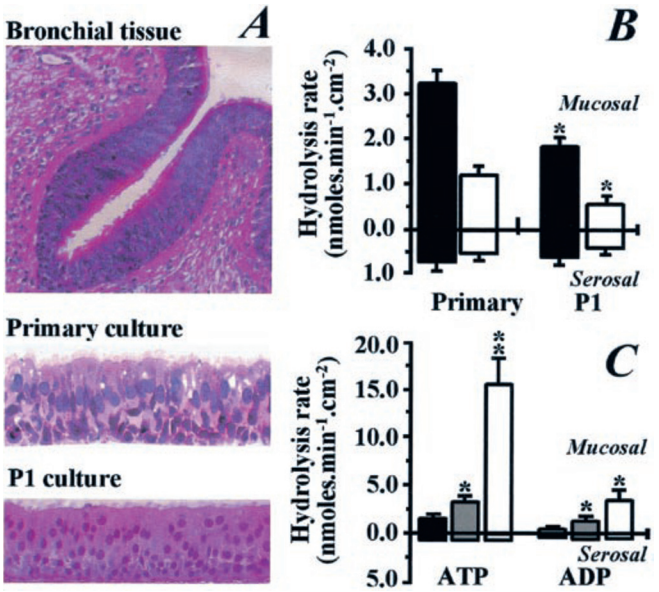


FIG. 5. **Increasing efficiency of nucleotide metabolism with airway generation.** *A*, typical brightfield hematoxylin and eosin staining sections of main bronchial tissue and primary and P1 cultures. *B*, impact of passage. Primary or P1 bronchial cultures assayed in bilateral 0.35 ml of KRB (pH 7.4) with 1 mM ATP (*filled bars*) or ADP (*open bars*). The ATPase/ADPase activity ratio was preserved by passage. *C*, widespread distribution of nucleotide metabolism. P1 cultures of human nasal (*filled*), bronchial (*gray*), or bronchiolar (*open*) epithelial cells assayed in bilateral 0.35 ml of KRB with 1 mM ATP or ADP. Hydrolysis rates calculated from linear rates of substrate decay were determined by HPLC. Values are mean ± S.E. of four experiments *, $p < 0.05$. **, $p < 0.01$.

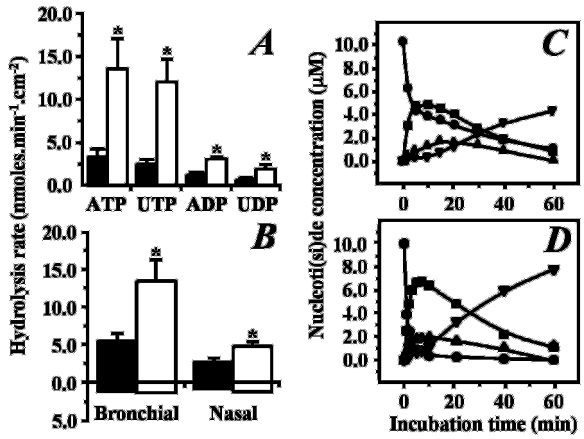


FIG. 6. **Cystic fibrosis up-regulates nucleotide metabolism in human airways.** *A*, enhanced clearance of P2 receptor agonists. Normal (*filled bars*) and CF (*open bars*) bronchial cultures assayed in bilateral 0.35 ml of KRB (pH 7.4) with mucosal 1 mM ATP, UTP, ADP, or UDP. *B*, CF effects confined to the mucosal surface. Nasal and bronchial cultures from healthy donors (*filled bars*) and CF patients (*open bars*) assayed with bilateral 1 mM ATP as in *panel A*. Hydrolysis rates calculated from linear rates of substrate decay determined by HPLC. Values are mean ± S.E. of nine experiments (*, $p < 0.05$). *C–D*, impact of CF on the metabolic patterns. Bronchial cultures from healthy donors (*C*) and CF (*D*) patients assayed with mucosal 0.01 mM [$^3$H]ATP as in *panel A*. CF enhances ADP and adenosine accumulation. [$^3$H]ATP (●), [$^3$H]ADP (■), [$^3$H]AMP (▲), [$^3$H]adenosine (▼), and [$^3$H]inosine (♦). Mean results from five experiments (S.E. <10% of the mean).

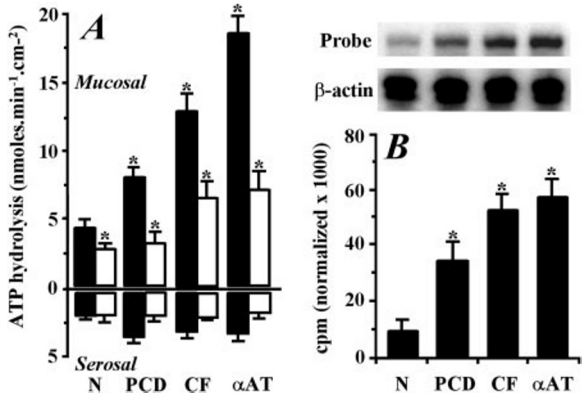


FIG. 7. **Airway nucleotide clearance is enhanced by NS AP in chronic lung diseases.** *A*, acceleration of nucleotide clearance. Bronchial cultures of epithelial cells from healthy donors (*N*) or patients with PCD, CF, or αAT assayed in bilateral 0.35 ml of KRB (pH 7.4) with bilateral 1 mM ATP (*filled bars*) or 1 mM ATP + 10 mM levamisole (NS AP inhibitor; *open bars*). Hydrolytic rates calculated from linear decay in substrate concentrations determined by HPLC. Values are mean ± S.E. of five experiments (*, $p < 0.05$). *B*, up-regulation of NS AP expression. RNase protection assays conducted on the epithelial cultures assayed in *panel A*. Values, normalized with β-actin, represent mean ± S.E. of three-five experiments (*, $p < 0.05$).

tients were assayed with 1 mM ATP, UTP, ADP, or UDP on the mucosal surface. Fig. 6*A* shows that CF epithelia hydrolyzed all nucleotides at significantly higher rates than normal cultures while maintaining a normal rank order of efficiency: ATP ≥ UTP > ADP > UDP. Similar results were obtained with ATP on the mucosal surface of nasal cultures (Fig. 6*B*). Interestingly, the impact of CF on nucleotide metabolism was restricted to the mucosal surface of nasal and bronchial epithelia (Fig. 6*B*). These results support a widespread and polarized up-regulation of nucleotide metabolism throughout the airways of CF patients.

Time course experiments were performed to determine the impact of CF on the metabolism of an airway ATP concentration generated locally from mechanical stimulation (44–46). Normal and CF cultures of bronchial epithelial cells were monitored by HPLC over 60 min after the addition of 0.01 mM [$^3$H]ATP to the mucosal surface. On normal cultures, this nucleotide concentration produced a metabolic pattern (Fig. 6*C*) similar to that of 0.1 mM ATP (Fig. 3), characterized by transient ADP and AMP accumulations and a gradual increase in adenosine concentration. In contrast, the CF cultures generated higher rates of ATP decay and transiently enhanced ADP accumulation and 2-fold higher rates of adenosine production (Fig. 6*D*). These experiments demonstrate that CF enhances all enzymatic reactions involved in the stepwise conversion of mucosal ATP to adenosine.

Other chronic obstructive lung diseases affect the regulation of P2 receptor agonists in human airways. Fig. 7*A* shows that bronchial cultures from PCD, CF, and αAT patients eliminated mucosal 1 mM ATP at 2–4-fold higher rates than cultures from healthy donors. Furthermore, as observed with CF cultures, no significant difference in ectoATPase activity was detected on the serosal surface between normal, PCD, and αAT epithelial cultures. To identify the ectonucleotidase(s) up-regulated in these diseases, the mucosal assays were repeated with 1 mM ATP in the presence 10 mM levamisole, a specific inhibitor of NS AP (34). Expressed exclusively on the mucosal surface of human airway epithelia, this ectonucleotidase dephosphorylates ATP into ADP, AMP, and adenosine (34). We show that NS AP activity (Fig. 7*A*) and mRNA expression (Fig. 7*B*) were 3–6-fold higher in cultures from PCD, CF, and αAT patients than from healthy donors. Taken together, these data suggest that chronic airway obstruction accelerates the removal of aerosolized P2 receptor agonists, mainly through an up-regulation of NS AP.

## DISCUSSION

Clinical studies have shown that aerosolized UTP temporarily relieves CF patients from chronic airway obstruction (<1 h) (30–32). We tested the hypothesis that the duration of nucleotide-mediated MCC is limited by the rapid clearance of P2 receptor agonists from airway epithelial surfaces. Aerosolized nucleotides could be eliminated by paracellular permeation through the epithelial barrier and/or by surface metabolism. In the present study, we clearly showed that therapeutic concentrations (0.1–1.0 mM) (30) of [$^3$H]ADP are unable to cross the epithelial layer in either direction. In addition, primary cultures of human airway epithelia were impermeable to mucosal 0.1 mM [$^3$H]ATP (34). These results indicate that P2 receptor agonists are not eliminated from human airways by permeation through the epithelium.

The observation that several radiolabeled species accumulated following the addition of [$^3$H]ADP suggested the presence of metabolic activities on human airway epithelial surfaces. We reported earlier that mucosal [$\gamma^{32}$P]ATP levels on primary cultures of human nasal epithelial cells are maintained by a balance between basal release and surface metabolism, detected by the production of radiolabeled inorganic phosphate ($^{32}$P) (15). Unfortunately, the non-radioactive products of [$\gamma^{32}$P]ATP metabolism were not identified by UV detection. In the present study, a thorough investigation of extracellular nucleotide metabolism was conducted by time course experiments monitoring the buffer composition in tritiated compounds following the addition of 0.1 mM [$^3$H]ATP or [$^3$H]UTP to the mucosal surface. The radioisotope is positioned on the base, which is carried by all nucleotides and nucleosides generated from the metabolism of these substrates. We showed that [$^3$H]ATP is gradually replaced by [$^3$H]ADP, followed by [$^3$H]AMP and finally [$^3$H]adenosine. Similar results were obtained with [$^3$H]UTP. The directionality of nucleotide metabolism was confirmed with [$^3$H]ADP as substrate, which was dephosphorylated into [$^3$H]AMP, followed by [$^3$H]adenosine. The metabolism of [$^3$H]ADP also generated small amounts of [$^3$H]ATP, most likely by the ectoadenylate kinase activity (2ADP $\leftrightarrow$ ATP + AMP) recently identified at the surface of human nasal and bronchial epithelial cells (47, 48). Finally, assays initiated with [$^3$H]AMP generated [$^3$H]adenosine. Collectively, these experiments demonstrate that P2 receptor agonists (ATP, UTP, ADP, and UDP) are eliminated from human airway epithelial surfaces by sequential dephosphorylation.

Dephosphorylating ectonucleotidases have been reported in several human tissues, either as soluble or cell-associated proteins (33). We evaluated the physiological importance of airway ectonucleotidases by addressing their localization, polarity, and biochemical properties. Our data indicate that ATP metabolism is conducted primarily (>90%) by cell-associated ectonucleotidases on both epithelial surfaces. Similar findings were reported for AMP dephosphorylation by NS AP and ecto 5′-nucleotidase (34), and for ectoadenylate kinase activity (47, 48) on primary cultures of human airway epithelial cells. These data suggest that extracellular nucleotide metabolism could locally limit the duration of P2 receptor activation.

Mucociliary clearance in human airways is acutely stimulated by respiratory irritants (49). Mechanical stress, induced by physical interaction of an air contaminant or a microbe with the epithelium, causes a local burst of ATP release (11, 16–18), which then stimulates P2 receptor-mediated MCC (9). Because P2 receptors desensitize in response to sustained activation (50, 51), the ability of the epithelium to detect the next threat may depend on the time required to restore basal ATP level. Incidentally, we showed that high ATP concentrations (0.1 mM) are eliminated in less than a minute ($t_{1/2}$ = 20 s) within *in vivo* PCL volume. Such remarkable efficiency supports a pivotal role for ectonucleotidases in the maintenance of alertness of airways against infectious particles.

Two essential components of nucleotide-mediated MCC were reported to exhibit a bilateral distribution on human airway epithelial surfaces: ATP release (9) and P2 receptors (9, 12). Evidence presented here demonstrates that ectonucleotidases adopt a similar distribution, with 3- to 5-fold higher efficiency on the mucosal surface. Comparative analysis of the time course experiments revealed surface-specific patterns of ectonucleotidase activities. On the mucosal surface, ATP metabolism was characterized by the transient accumulation of ADP and AMP, followed by the sustained accumulation of adenosine. Conversely, the serosal surface produced mainly ADP from ATP throughout the experiment. The fact that the major reaction product generated from ATP metabolism on mucosal (adenosine) and serosal (ADP) surfaces corresponds to the most abundant purine measured under resting conditions (42) supports an intimate relationship between ATP release, ectonucleotidase activities, and P2 receptor-mediated MCC. For instance, we showed that the mucosal surface generates higher rates of adenosine production from AMP than from ADP or ATP as substrate. Extracellular ATP and ADP were identified as competitive inhibitors of AMP metabolism on various cell types (52, 53), including the mucosal surface of human airway epithelia (34). This regulatory mechanism suggests that ATP release could temporarily delay the transition between P2 (ATP, ADP) and P1 (adenosine) receptor-mediated events in human airways.

Given the potential importance of ectonucleotidases in the regulation of nucleotide-mediated MCC, their activities should be particularly critical in small airways, which are most susceptible to obstruction (43). The present study demonstrates, for the first time, that extracellular nucleotide metabolism extends below the tracheobronchial tree. Furthermore, comparative analysis indicated that the rates of ATP and ADP hydrolysis increase considerably toward alveoli (nose < bronchi $\ll$ bronchioles). In contrast, serosal ectonucleotidase activities were not influenced by airway generation. Recent studies suggest that the remarkable efficiency of bronchiolar epithelia may represent an adaptative response to more frequent ATP outbursts, triggered by particle deposition and bronchoconstriction-mediated membrane stretch (54). Mechanical stimulation of cultured human airway epithelial cells (Calu-3) induced ATP release from the mucosal surface, with negligible nucleotide release from the serosal surface.[2] Wiendl *et al*. (55) showed that repetitive exposures of a human epidermal cell line (A431) to an ATP concentration (70 $\mu$M) detected in proximity to a site of stimulated release (44–46) up-regulated all ectonucleotidase activities supporting the conversion of ATP to adenosine. Collectively, these studies suggest that, in human airways, mucosal nucleotide metabolism may be attuned to the intensity of mechanically induced ATP release and P2 receptor-mediated MCC.

The relationship between nucleotide metabolism and P2 receptor-mediated MCC hypothesized above for healthy lungs would predict lower ectonucleotidase activities for the mucostatic airways of CF patients. However, we provide evidence that CF accelerates nucleotide metabolism in human airways. On the mucosal surface of nasal and bronchial epithelial cultures, CF increased by 2–4-fold the hydrolysis rate of P2 receptor agonists (ATP, UTP, ADP, and UDP). Alternatively, the impact of CF on mucosal nucleotide metabolism could provide protection against ATP-mediated epithelial damage. The lungs

[2] E. R. Lazarowski, personal communication.

of CF patients may be exposed locally to unusually elevated concentrations of ATP, released from damaged epithelia, lysed bacteria, and activated leukocytes. Although not reported in normal airway epithelia, $P2X_7$ receptors were detected by RT-PCR in primary epithelial cultures from CF nasal polyps and in CF lung epithelial cell lines (12). These channels are well known to induce cell death upon activation by ATP concentrations ($EC_{50}$:0.1–1 mM) that may be reached locally on CF airway epithelia (56). These findings suggest that ectonucleotidase activities may not be regulated by nucleotide-mediated MCC but rather by mucosal ATP release.

An indirect consequence of accelerated nucleotide metabolism on the mucosal surface of CF airway epithelia is the enhanced production of the P1 receptor agonist, adenosine. Time course experiments indicated that CF up-regulates by 3-fold all reactions supporting the conversion of mucosal ATP to adenosine. Furthermore, mucosal adenosine levels reached 2-fold higher values on CF cultures. Excess adenosine is not expected to improve MCC in CF lungs because $A_{2B}$ receptor-mediated stimulation of CFTR channel activity is defective (57). However, chronically elevated adenosine induced airway inflammation in mice (58). These results suggest that enhanced mucosal ATP metabolism and adenosine production may exacerbate chronic inflammation in the lungs of CF patients.

A key finding in this work is the widespread occurrence of enhanced nucleotide metabolism on airway epithelial surfaces from patients diagnosed with chronic obstructive lung diseases. We showed that primary cultures of bronchial epithelial cells from patients with PCD, CF, or αAT displayed 2–4-fold higher rates of mucosal ATP hydrolysis than normal epithelia. Therefore, these diseases may share factors regulating airway ectonucleotidases. Chronic airway obstruction is commonly associated with recurrent cycles of inflammatory responses to bacterial infection (5). Besides the excessive ATP released under these conditions, inflammatory mediators may also regulate mucosal nucleotide metabolism. The pro-inflammatory cytokine, interleukin-1β, increased by 6-fold the mRNA expression of NS AP in primary cultures of human bronchial epithelial cells (34). In the present work, we showed that NS AP expression and mucosal activity were up-regulated 3–6-fold by PCD, CF, or αAT. Furthermore, NS AP activity among the different diseases followed the same rank order of efficiency as total ectoATPase activity (PCD < CF < αAT). Collectively, these data support a major role for NS AP in the up-regulation of nucleotide clearance in chronic obstructive lung diseases.

In summary, the present study demonstrates that P2 receptor agonists are rapidly eliminated from human airway epithelial surfaces. We provide evidence that nucleotide clearance does not involve permeation through the epithelial layer but rather extracellular metabolism by cell-associated enzymes. These ectonucleotidases display significantly higher activities on mucosal epithelial surfaces, with increasing efficiency toward areas most susceptible to airway obstruction. The rapid clearance of micromolar ATP concentrations may prevent P2 receptor desensitization (50, 51), thereby maintaining adequate nucleotide-mediated MCC of inhaled infectious particles. In chronic obstructive lung diseases, enhanced nucleotide metabolism could represent a defense mechanism against the deleterious effects of excess ATP (56). Finally, we identified NS AP as the major ectonucleotidase responsible for the termination of aerosolized nucleotide-mediated MCC in the lungs of CF patients.

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