

Human Airway Ecto-adenylate Kinase

A MECHANISM TO PROPAGATE ATP SIGNALING ON AIRWAY SURFACES*

Received for publication, August 7, 2002, and in revised form, January 15, 2003
Published, JBC Papers in Press, January 27, 2003, DOI 10.1074/jbc.M208071200

Maryse Picher‡ and Richard C. Boucher

From the Cystic Fibrosis/Pulmonary Research and Treatment Center, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599

Mechanically induced ATP release from human airway epithelial cells regulates mucociliary clearance through cell surface nucleotide receptors. Ecto-enzymes detected on these cells were recently shown to terminate ATP-mediated responses by sequential dephosphorylation of extracellular ATP into ADP, AMP, and adenosine. We now demonstrate that an ecto-adenylate kinase (ecto-AK) contributes to the metabolism of adenine nucleotides on human airway epithelial surfaces by the reversible reaction: $ATP + AMP \rightleftharpoons 2ADP$. This phosphotransferase exhibited a bilateral distribution on polarized primary cultures of human bronchial epithelial cells with a 4-fold higher activity on the mucosal surface. Ecto-AK presented an absolute requirement for magnesium and adenine-based nucleotides. UMP, GMP, and CMP could not substitute for AMP as γ -phosphate acceptor, and UDP could not replace ADP. Apparent K_m and V_{max} values were $23 \pm 5 \mu M$ and $1.1 \pm 0.1 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$ for ATP and $43 \pm 6 \mu M$ and $0.5 \pm 0.1 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$ for ADP. Ecto-AK accounted for 20% of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ dephosphorylation, and the impermeant AK inhibitor, diadenosine pentaphosphate, reduced ADPase activity by more than 70% on both epithelial surfaces. Time course experiments on ATP metabolism demonstrated that ecto-AK significantly prolongs effective ATP and ADP concentrations on airway epithelial surfaces for P2 receptor signaling and reduces by 6-fold adenosine production. Our data suggest a role for this nucleotide entrapment cycle in the propagation of purine-mediated mucociliary clearance on human airway epithelial surfaces.

Mucociliary clearance (MCC)¹ represents the first line of defense against lung infection. Inhaled bacteria are trapped within a mucus blanket covering the epithelium and mechanically cleared by coordinated cilia beating activity (for a review, see Ref. 1). Extracellular nucleotides are well recognized as regulators of the epithelial functions supporting MCC, includ-

ing mucus secretion, cilia beat frequency (CBF), and ion channel activities involved in the maintenance of epithelial surface liquid volume (for a review, see Ref. 2). Nucleotides are released by the epithelium under basal conditions (3, 4) and in response to membrane stretch (5, 6), shear stress (7, 8), and hypo-osmotic cell swelling (9–12). On the mucosal surface, they interact with two members of the G-protein-coupled P2Y receptor (P2YR) family: P2Y₂ (13) and P2Y₆ (14). P2Y₂ receptors are equally activated by ATP and UTP, but not by ADP or UDP, whereas P2Y₆ receptors are potentially activated by UDP and weakly activated by ADP. The serosal epithelial surface expresses P2Y₂ (15) and P2Y₁ (ADP > ATP > UTP) (6) receptors. Taylor *et al.* (17) demonstrated that human airway epithelial cultures also express members of the ionotropic P2X receptor (P2XR) family: P2X₄ and P2X₅. Patch clamp and Ussing chamber experiments indicated that ATP-induced Ca²⁺ entry through these channels stimulates luminal Cl⁻ secretion. In rabbit trachea, epithelial P2XRs contribute to ATP-mediated increase in CBF via Ca²⁺ influx (18).

Over the last decade, several studies have demonstrated the role of cell surface nucleotide metabolism in the regulation of MCC-related epithelial functions (9, 10, 19, 22). ATP and UTP elicited large transient increases in CBF on human nasal explants (19). However, ATP also produced a post-peak sustained increase in CBF that was prevented by the nonspecific adenosine receptor antagonist, 8-*p*-sulfophenyltheophylline. Hypotonicity-induced cell swelling triggered ATP release and activation of volume-sensitive Cl⁻ channels by a mechanism that required cell surface conversion of ATP into adenosine (10). In a human bronchial cell line lacking P2Y₂ receptors (Calu-3 (23)), the basal ion channel activity of the cystic fibrosis transmembrane regulator (CFTR) was inhibited by 8-*p*-sulfophenyltheophylline and by AMPCP (22), an inhibitor of the cell surface conversion of AMP into adenosine (for a review, see Ref. 24). Incidentally, adenosine has been shown to trigger MCC-related epithelial responses, including CBF (19, 25) and ion transport (26–28).

We reported that human nasal and bronchial epithelial surfaces express metabolic enzymes that sequentially dephosphorylate ATP into ADP, AMP, and adenosine and UTP into UDP, UMP, and uridine (29–31). Lazarowski *et al.* (32) proposed that extracellular nucleotide metabolism in human airways also involves transphosphorylating enzymes. They identified a nucleoside diphosphate kinase (NDPK) activity on the mucosal surface of human nasal epithelial cells. Classically described as ubiquitous intracellular enzymes, NDPKs catalyze the transfer of γ -phosphate groups between nucleoside di- and triphosphates by the reversible reaction: $NTP + NDP \rightleftharpoons NDP + NTP$ (for a review, see Ref. 33). Transphosphorylation events taking place in most intracellular compartments combine the activities of NDPKs and adenylate kinases (AKs; ATP:AMP-phos-

* This work was supported by Grants CFF R026 and CFF Picher 00G0 from the Cystic Fibrosis Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: School of Medicine, University of North Carolina, 7010 Thurston-Bowles Bldg., Chapel Hill, NC, 27510. Tel.: 919-966-7047; Fax: 919-966-7248; E-mail: pichm@med.unc.edu.

¹ The abbreviations used are: MCC, mucociliary clearance; Ecto-AK, ecto-adenylate kinase; CF, cystic fibrosis; CBF, ecto-adenylate kinase; AMPCP, α,β -methyleneADP; NDPK, nucleoside diphosphate kinase; KRB, Krebs-Ringer buffer; TBASH, tetrabutyl ammonium hydrogen sulfate; P2YR, P2Y receptor; P2XR, P2X receptor; HPLC, high pressure liquid chromatography.

phototransferases). In contrast to the broad substrate specificity of NDPKs, AKs transfer phosphate groups between adenine-based nucleotides by the reaction: $\text{MgATP} + \text{AMP} \rightleftharpoons \text{MgADP} + \text{ADP}$ (for a review, see Ref. 34). Cell surface AK (ecto-AK) activity was recently detected on *Sartorius* frog muscles (35, 36), rat brain synaptosomes (37), human umbilical vein endothelial cells (38, 39), and human nasal epithelial cells (40). However, the physiological role of cell surface adenine nucleotide transphosphorylation has not been explored.

In the present study, we describe the biochemical and kinetic properties, as well as the polarity, of ecto-AK expressed at the surface of human bronchial epithelia. We measured the impact of ecto-AK on the availability of adenine nucleotides for P2 (ATP and ADP) and P1 (adenosine) receptors regulating MCC. We tested the hypothesis that ecto-AK could extend the duration of locally released ATP to promote airway clearance of noxious agents. Biochemical assays were performed on polarized primary cultures of bronchial epithelial cells from normal donors and patients diagnosed with cystic fibrosis (CF), an inherited obstructive lung disease characterized by impaired MCC, chronic infection, and inflammation (for a review, see Ref. 41). This study provides evidence that ecto-AK plays a major role in the prolongation of P1 and P2 receptor-mediated MCC functions on human airway epithelial surfaces.

EXPERIMENTAL PROCEDURES

Cell Culture—Well differentiated primary cultures of human bronchial epithelial cells were grown as described previously (42). In brief, the cells were isolated from freshly excised main stem bronchi by protein digestion (43) 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-high glucose, 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 $\mu\text{g}/\mu\text{l}$ streptomycin) (44). Once they reached confluence, the cultures were maintained in air-liquid interface with 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 with transepithelial electrical resistance ≥ 300 ohms $\cdot\text{cm}^{-2}$. Lactate dehydrogenase activity was employed as a test of cellular integrity.

Ectonucleotidase Assays—Primary cultures of human bronchial epithelial cells were rinsed three times with Krebs-Ringer buffer (KRB): 140 mM Na^+ , 120 mM Cl^- , 5.2 mM K^+ , 25 mM HCO_3^- , 2.4 mM HPO_4^- , 1.6 mM Ca^{2+} , 1.6 mM Mg^{2+} , 5.2 mM glucose, and 25 mM HEPES (pH 7.4), and preincubated in KRB (350 μl mucosal/serosal) for 30 min at 37 °C (5% $\text{CO}_2/95\%$ O_2). All reactions were initiated by the addition of the substrate(s), dissolved in 35 μl of KRB, to the mucosal or serosal bath, and stopped by transferring 10- μl aliquots to tubes containing 300 μl of ice-cold water. The samples were boiled for 5 min, filtered, and analyzed by reversed-phase paired-ion HPLC. ATPase activity was measured with [^3H]ATP (0.5 μCi) or [$\gamma\text{-}^{32}\text{P}$]ATP (0.1 μCi) as substrates. ADPase and UDPase activities were assayed with [^3H]ADP (0.5 μCi) and [^3H]UDP (0.5 μCi), respectively. The forward ecto-AK reaction ($\text{ATP} + \text{AMP} \rightarrow 2\text{ADP}$) was initiated with equal concentrations of [$\gamma\text{-}^{32}\text{P}$]ATP (0.1 μCi) and AMP mixed previously and then quantified by the rate of [$\beta\text{-}^{32}\text{P}$]ADP production. The reverse ecto-AK reaction ($2\text{ADP} \rightarrow \text{ATP} + \text{AMP}$) was measured by the rate of [^3H]ATP production from [^3H]ADP (0.5 μCi).

Synthesis of [^3H]ADP and [^3H]UDP—Tritiated ADP and UDP were obtained from their respective nucleoside triphosphates by hexokinase reaction, as described previously (46). In brief, 50 μCi of either [^3H]ATP or [^3H]UTP (40–50 Ci/mmol) 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 hexokinase activity, and full conversion into [^3H]ADP or [^3H]UDP was confirmed by HPLC.

Enzyme Release Assays—Mucosal epithelial surfaces were rinsed three times with KRB and incubated in KRB (350 μl of mucosal/serosal) during 90 min at 37 °C (5% $\text{CO}_2/95\%$ O_2). The conditioned buffer was collected and centrifuged at 14,000 $\times g$ (4 °C; 20 min) to remove detached cells, debris, and large organelles. The supernatant was transferred to tubes containing 1 mM [$\gamma\text{-}^{32}\text{P}$]ATP (0.1 μCi) or 1 mM [$\gamma\text{-}^{32}\text{P}$]ATP (0.1 μCi) + 1 mM AMP in 35 μl KRB for assessment of

ATPase and ecto-AK activities in a shaker bath (37 °C). These values were compared with enzyme activities measured the following day on the same cultures to ascertain the contribution of released enzymes to total epithelial surface activity.

Kinetic Properties of Human Airway Ecto-AK—We established the kinetic properties of the forward and reverse ecto-AK reactions for ATP and ADP, respectively. For the forward reaction, the assays were initiated with 1 mM AMP + 0.001–1 mM [$\gamma\text{-}^{32}\text{P}$]ATP (0.1 μCi), and reaction rates were calculated from [$\beta\text{-}^{32}\text{P}$]ADP production. For the reverse reaction, the assays were initiated with 0.001–1 mM [^3H]ADP (0.5 μCi), and reaction rates were calculated from [^3H]ATP production. All experiments were conducted on the mucosal surface of human bronchial epithelial cultures, as described above for enzyme assays. Samples were collected after incubation periods that limited substrate hydrolysis to $\leq 10\%$ and were analyzed by HPLC. Michaelis-Menten constants (K_m) and maximal velocities (V_{max}) were obtained from the slope and the ordinate of Woolf-Augustinson Hofstee transformations, respectively. Catalytic efficiency (Cat_{eff}) was calculated from the velocity (V_o) at K_m divided by K_m (47).

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 to 10 min, buffer B (100 mM KH_2PO_4 , 8 mM TBASH, and 10% 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 Scientific Instruments, Columbia, MD), and radioactivity was determined on-line with a Flo-One Radiomatic β detector (Packard, Canberra, Australia), as described previously (48).

Materials—All 5'-nucleotides and adenosine were purchased from Roche Molecular Biochemicals. KH_2PO_4 , TBASH, and HEPES were obtained from Sigma. HPLC-grade water was bought from Fisher Scientific. Cell culture media, bovine serum albumin, bovine pituitary extract, epidermal growth factor, gentamicin, penicillin, retinoic acid, and streptomycin were bought from Invitrogen. [$2,8\text{-}^3\text{H}$]ATP (40–50 Ci/mmol), [$2,8\text{-}^3\text{H}$]UTP (40–50 Ci/mmol), and [$\gamma\text{-}^{32}\text{P}$]ATP (3000 Ci/mmol) were obtained from Amersham Biosciences. Salts and solvents were of analytical grade.

Data Analysis—All enzyme assays were performed on primary cultures of human bronchial epithelial cells obtained from at least five different donors or CF patients. Rates of dephosphorylation were calculated from the initial linear rate of substrate decay monitored by HPLC and presented as $\text{nmol}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$. Forward and reverse ecto-AK activities were calculated from initial linear rates of accumulation of the phosphorylated product. Values were expressed as means \pm S.E. Unpaired Student's *t* tests were used to assess the significance between measurements performed on different cultures. Paired *T* tests were used for comparisons between mucosal and serosal surfaces of the same culture or between conditioned buffer and the corresponding epithelial surface. All linear regressions, curve, fits and data transformations were performed with the PC computer programs Origin and Sigma plot.

RESULTS

Detection of Ectonucleotidase and Ecto-AK Activities on Airway Epithelia—Polarized bronchial epithelial cultures were assayed for nucleotide metabolism. Fig. 1A presents typical HPLC traces for samples collected 0, 2, and 5 min after the addition of 0.1 mM [^3H]ATP to the mucosal surface. The nucleotide was converted into less phosphorylated compounds ([^3H]ADP, [^3H]AMP, [^3H]adenosine) and [^3H]inosine. Time course analysis showed that the disappearance of [^3H]ATP occurred during the accumulation of [^3H]ADP and [^3H]AMP (Fig. 1B). [^3H]Adenosine concentration increased over the first 40 min and accounted for 50% of all radioactive species by the end of the experiment. In contrast, reactions initiated with 0.1 mM [^3H]ADP indicated that adenine nucleotide metabolism involves other activities besides dephosphorylation. As observed with [^3H]ATP, [^3H]ADP was converted into less phosphorylated compounds ([^3H]AMP, [^3H]adenosine) and [^3H]inosine (Fig. 1C). The HPLC traces also revealed the presence of [^3H]ATP, suggesting that [^3H]ADP was phosphorylated. Time course analysis showed transient accumulation of [^3H]AMP and [^3H]ATP during the first 30

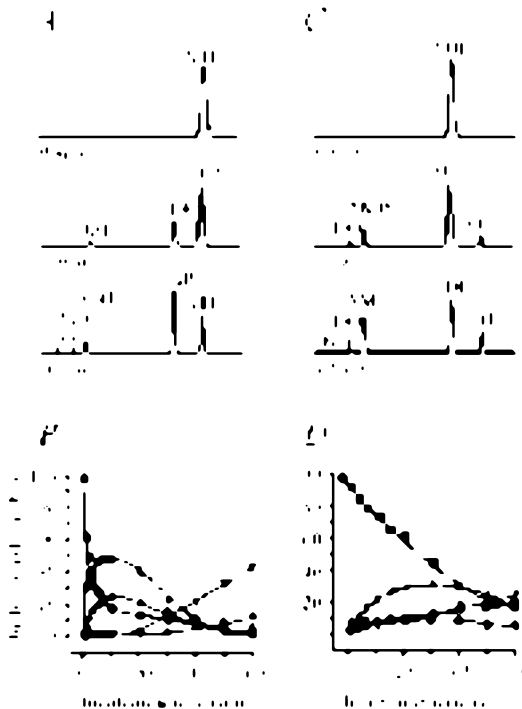


FIG. 1. ATP and ADP metabolism at the surface of human bronchial epithelial cells. The epithelial cultures were incubated with KRB (pH 7.4; 0.35 ml mucosal/serosal) containing mucosal 0.1 mM [^3H]ATP (0.5 μCi) (A and B) or 0.1 mM [^3H]ADP (0.5 μCi) (C and D). Buffer samples collected over 60 min and analyzed by HPLC revealed [^3H]ATP (A) and [^3H]ADP (C) dephosphorylation, as well as phosphorylation of [^3H]ADP into [^3H]ATP (C and D). Time course analyses indicated significant differences in the patterns of [^3H]ATP (B) and [^3H]ADP (D) metabolism on bronchial epithelial cells: [^3H]ATP (\bullet), [^3H]ADP (\blacksquare), [^3H]AMP (\blacktriangle), [^3H]adenosine (\blacktriangledown), [^3H]inosine (\blacklozenge). HPLC traces represent typical results obtained from six independent experiments. Metabolic patterns represent mean results from all experiments (S.E. < 10% of the mean).

min, whereas adenosine and inosine concentrations increased during the entire incubation period (Fig. 1D). These results support a complex interplay between dephosphorylating and phosphorylating activities on human airway epithelial surfaces.

We explored the possibility that an ecto-AK was responsible for the phosphorylation of ADP into ATP at the surface of human bronchial epithelial cells. By definition, an AK supports the reversible reaction: $\text{ATP} + \text{AMP} \rightleftharpoons 2\text{ADP}$ (for a review, see Ref. 34). We already demonstrated that these epithelial cultures support the reverse reaction ($2\text{ADP} \rightarrow \text{ATP} + \text{AMP}$; Fig. 1, C and D). In the following experiments, we investigated the forward AK reaction ($\text{ATP} + \text{AMP} \rightarrow 2\text{ADP}$). Fig. 2A shows typical HPLC traces obtained from buffer samples collected 0, 2, and 5 min after the addition of 0.1 mM [$\gamma\text{-}^{32}\text{P}$]ATP to the mucosal surface. The dephosphorylation of [$\gamma\text{-}^{32}\text{P}$]ATP was detected by the accumulation of inorganic phosphate (^{32}P] P_i). Reactions initiated with 0.1 mM [$\gamma\text{-}^{32}\text{P}$]ATP + 0.1 mM AMP produced an additional radioactive compound that co-migrated with ADP standards (Fig. 2B), suggesting that the γ -phosphate group of [$\gamma\text{-}^{32}\text{P}$]ATP was transferred to AMP to produce [$\beta\text{-}^{32}\text{P}$]ADP. These experiments demonstrated that human airway epithelial surfaces exhibit both forward and reverse AK activities.

Cell-associated Ecto-AK and ATPase Activities—The mucosal surface of human bronchial epithelial cultures and conditioned KRB were assayed for ATPase and AK activities with 1 mM [$\gamma\text{-}^{32}\text{P}$]ATP and 1 mM [$\gamma\text{-}^{32}\text{P}$]ATP + 1 mM AMP, respectively (see "Experimental Procedures"). Similar levels of ATPase and

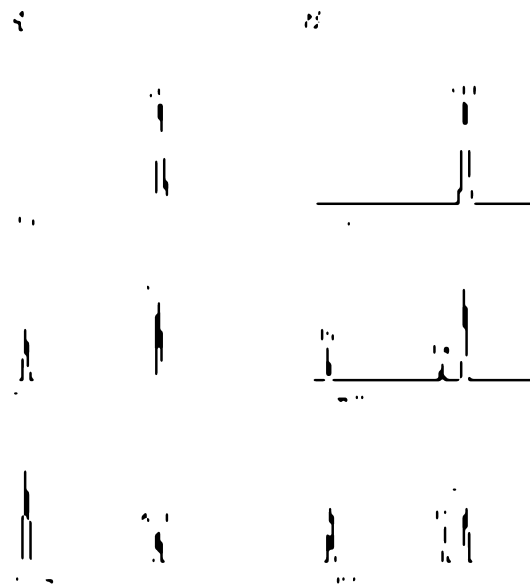


FIG. 2. Detection of ecto-AK activity on human bronchial epithelial cells. The epithelial cultures were incubated with KRB (pH 7.4; 0.35 ml mucosal/serosal) containing mucosal 0.1 mM [$\gamma\text{-}^{32}\text{P}$]ATP (0.1 μCi) (A) or 0.1 mM [$\gamma\text{-}^{32}\text{P}$]ATP (0.1 μCi) + 0.1 mM AMP (B). Buffer samples (10 μl) collected over 10 min were analyzed by HPLC. HPLC traces are presented for buffer samples collected after 0, 2, and 5 min. As shown in A, [$\gamma\text{-}^{32}\text{P}$]ATP dephosphorylation was detected by [^{32}P]inorganic phosphate (^{32}P] P_i) release. As shown in B, addition of 0.1 mM [$\gamma\text{-}^{32}\text{P}$]ATP + 0.1 mM AMP generated [^{32}P] P_i by dephosphorylation and [$\beta\text{-}^{32}\text{P}$]ADP by ecto-AK phosphorylation. HPLC traces represent typical results from six independent experiments.

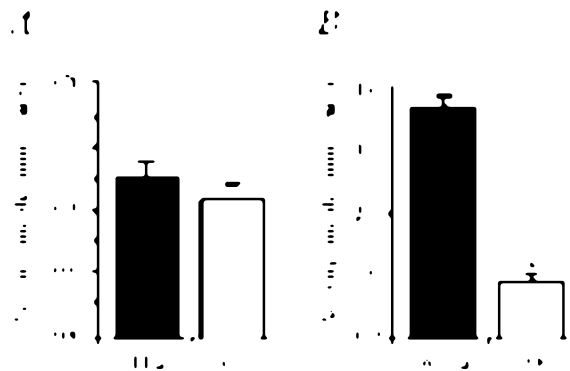


FIG. 3. Contribution of released enzymes to cell surface ecto-AK and ATPases. A, enzyme activities released from the mucosal surface. KRB was conditioned by a 90-min exposure to the mucosal surface. B, cell-associated enzyme activities. The mucosal surface was assayed for total enzyme activities. ATPase (filled bars) and ecto-AK (open bars) activities were assayed in KRB (pH 7.4) with 0.1 mM [$\gamma\text{-}^{32}\text{P}$]ATP (0.1 μCi) and 0.1 mM [$\gamma\text{-}^{32}\text{P}$]ATP (0.1 μCi) + 0.1 mM AMP, respectively. Buffer samples (10 μl) collected over 10 min were analyzed by HPLC. Released enzyme activities accounted for <15% of total epithelial surface activity. Values represent mean \pm S.E. of five independent experiments (*, $p < 0.01$).

AK activities were detected in the conditioned KRB (Fig. 3A). On the epithelial surface, the rate of [$\gamma\text{-}^{32}\text{P}$]ATP dephosphorylation by ATPase activity was 5-fold higher than the rate of AMP phosphorylation by AK activity (Fig. 3B). Importantly, released ATPase and AK activities represented $\sim 3 \pm 1\%$ and $11 \pm 2\%$ of total surface activity, respectively. The cell surface enzymes, remaining on the bronchial culture after the conditioned KRB was collected, were not eluted by subsequent washes (five rapid changes in KRB). These experiments indicate that the ectoenzymes responsible for the interconversion of phosphate groups between extracellular nucleotides were pre-

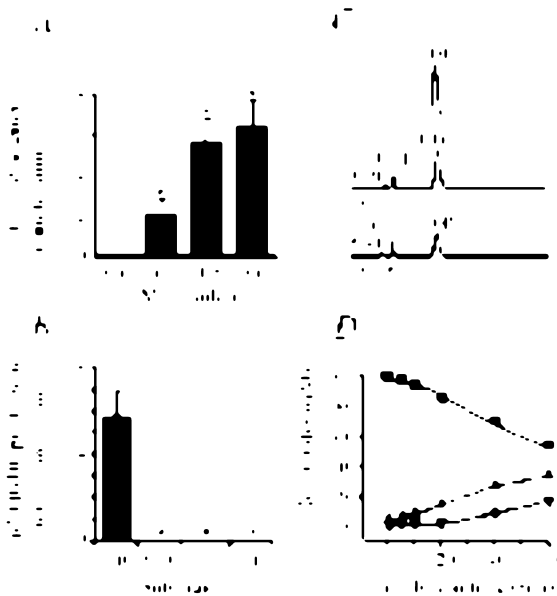


FIG. 4. Biochemical properties of ecto-AK on human bronchial epithelial cells. A, absolute requirement for Mg^{2+} . Epithelial cultures were incubated in KRB (pH 7.4) containing 0.1 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (0.1 μCi) + 0.1 mM AMP in the mucosal bath. Buffer samples (10 μl) collected over 10 min were analyzed by HPLC. KRB contained 1.5 mM Ca^{2+} + 0, 0.5, or 1.5 mM Mg^{2+} . B–D, ecto-AK specificity for adenine-based nucleotides. As shown in B, mucosal 0.1 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (0.1 μCi) phosphorylated 0.1 mM AMP but not UMP, CMP, or GMP. C, HPLC traces for the metabolism of mucosal 0.1 mM $[\text{H}]\text{UDP}$ (0.1 μCi). As shown in D, time course assays showed that $[\text{H}]\text{UDP}$ (■) was dephosphorylated into $[\text{H}]\text{UMP}$ (▲) and $[\text{H}]\text{uridine}$ (▼) but not phosphorylated into $[\text{H}]\text{UTP}$. Bar diagrams show means \pm S.E. of 6–8 independent experiments (*, $p < 0.05$). HPLC traces represent typical results and metabolic patterns mean results (S.E. < 10% of the mean) from all experiments.

dominantly associated to the epithelial surface of human bronchial epithelial cells.

Cation Dependence and Substrate Specificity of Ecto-AK—Human airway ecto-AK exhibited an absolute requirement for divalent cations and adenine nucleotides, as reported for intracellular AKs (34, 49). Enzyme assays were conducted on the mucosal surface of human bronchial epithelial cultures with 0.1 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ + 0.1 mM AMP. The kinase activity was not detected in Mg^{2+} -free KRB containing millimolar Ca^{2+} (Fig. 4A). The rate of AMP phosphorylation was proportional to the Mg^{2+} concentration. Fig. 4B shows that UMP, GMP, and CMP could not substitute for AMP as phosphate acceptor for the forward ecto-AK reaction. In addition, UDP could not substitute for ADP as phosphate donor or acceptor for the reverse ecto-AK reaction (Fig. 4, C and D). Fig. 4C presents typical HPLC traces for buffer samples collected 0, 5, and 10 min after the addition of 0.1 mM $[\text{H}]\text{UDP}$ to the mucosal surface. The nucleotide was gradually dephosphorylated into $[\text{H}]\text{UMP}$ and $[\text{H}]\text{uridine}$ but was not phosphorylated into $[\text{H}]\text{UTP}$ (Fig. 4D). Taken together, these results indicate the presence of a Mg^{2+} -dependent ecto-AK activity specific for adenine nucleotides at the surface of human bronchial epithelial cells.

Kinetic Properties of Human Airway Ecto-AK—Experiments were designed to compare the kinetic properties of the forward and reverse ecto-AK reactions on the mucosal surface of human bronchial epithelial cells. The forward reaction was measured with 1 mM AMP + 0.001–1 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Fig. 5A shows that the rate of $[\beta\text{-}^{32}\text{P}]\text{ADP}$ production increased with substrate concentration and saturated with 0.6 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The reverse reaction was measured with 0.001–3 mM $[\text{H}]\text{ADP}$. The production of $[\text{H}]\text{ATP}$ saturated at a higher substrate concen-

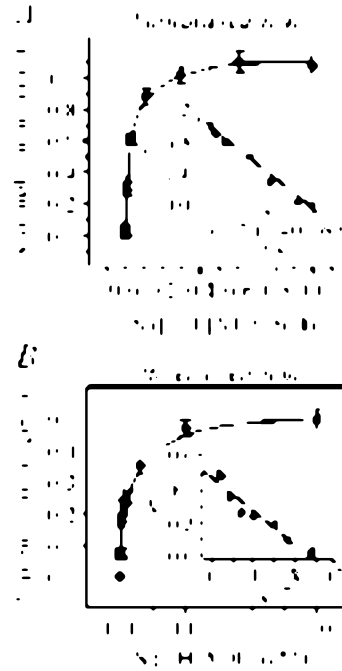


FIG. 5. Kinetic properties of human bronchial ecto-AK. A, catalytic properties of the forward reaction, initiated with 1 mM AMP + 0.001–1 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (0.1 μCi). $[\beta\text{-}^{32}\text{P}]\text{ADP}$ production saturated with 0.6 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Inset, Woolf-Augustinson Hoftsee transformation fitted to one regression ($r = 0.96\text{--}0.99$) with K_m , V_{max} , and catalytic efficiencies of $23 \pm 5 \mu\text{M}$, $1.1 \pm 0.1 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$, and $0.025 \pm 0.005 \text{ min}^{-1}$, respectively. B, catalytic properties of the reverse reaction, initiated with 0.001–3 mM $[\text{H}]\text{ADP}$ (0.5 μCi). Inset, Woolf-Augustinson Hoftsee transformation fitted to one regression ($r = 0.97\text{--}0.99$) with K_m , V_{max} , and catalytic efficiencies of $43 \pm 6 \mu\text{M}$, $0.5 \pm 0.1 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$, and $0.014 \pm 0.002 \text{ min}^{-1}$, respectively. Samples were collected after incubation periods that limited substrate hydrolysis to $\leq 10\%$ and then analyzed by HPLC. Values represent mean \pm S.E. of five independent experiments.

tration than the forward reaction (Fig. 5B; $p < 0.05$). Woolf-Augustinson Hoftsee analysis indicated that the K_m for $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was 2-fold lower than for $[\text{H}]\text{ADP}$ with corresponding values of $23 \pm 5 \mu\text{M}$ and $43 \pm 6 \mu\text{M}$, respectively (Fig. 5, A and B, insets; $p < 0.05$). The V_{max} of the forward reaction was 2-fold higher than for the reverse reaction with values of $1.1 \pm 0.1 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$ and $0.5 \pm 0.1 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$, respectively (Fig. 5, A and B, insets; $p < 0.05$). Calculated Cat_{eff} were $0.025 \pm 0.005 \text{ min}^{-1}$ and $0.014 \pm 0.002 \text{ min}^{-1}$ for $[\beta\text{-}^{32}\text{P}]\text{ADP}$ and $[\text{H}]\text{ATP}$ production, respectively ($p < 0.05$).

Polarity and Directionality of Ecto-AK on Airway Epithelial Surfaces—Nucleotide transphosphorylation was detected on both mucosal and serosal surfaces of human bronchial epithelial cultures (Table I). Enzyme assays, performed with saturable substrate concentrations, indicated that forward and reverse activities were 4-fold higher on the mucosal surface (Table I, reactions 1–2; $p < 0.01$). These experiments also showed that ecto-AK favored AMP phosphorylation by 3-fold over $[\text{H}]\text{ADP}$ phosphorylation on both epithelial surfaces ($p < 0.01$). These results were in agreement with the higher affinity of the enzyme for ATP over ADP (Fig. 5, A and B). The directionality of the reversible ecto-AK reaction suggested that the enzyme could partially circumvent the loss of high energy phosphate groups resulting from dephosphorylation of AMP into adenosine.

Contribution of Ecto-AK to ADP Metabolism—We evaluated the contribution of ecto-AK to the metabolism of ADP on human bronchial epithelial surfaces with the non-permeant AK inhibitor, diadenosine pentaphosphate (Ap_5A (37)). This dinu-

TABLE I
Measurements of ecto-adenylate kinase activity at the surface of human bronchial epithelial cells

Values represent means (\pm S.E.) of six experiments.

Enzyme reaction	Substrates (1 mM)	Parameter	Mucosal	Serosal
			<i>nmol/min · cm²</i>	
1. AK forward	[γ - ³² P]ATP + AMP	[γ - ³² P]ADP production	1.19 \pm 0.14	0.34 \pm 0.09
2. AK reverse	2[³ H]ADP	[³ H]ATP production	0.53 \pm 0.08	0.11 \pm 0.06
3. Total ADPase	2[³ H]ADP	2[³ H]ADP hydrolysis	0.92 \pm 0.10	0.46 \pm 0.07
4. ADPase-AK	2[³ H]ADP + Ap ₅ A	2[³ H]ADP hydrolysis	0.26 \pm 0.03	0.33 \pm 0.07
5. AK	Reactions 3–4		0.66 \pm 0.05	0.13 \pm 0.03
6. Total UDPase	2[³ H]UDP	2[³ H]UDP hydrolysis	0.73 \pm 0.08	0.29 \pm 0.04
7. UDPase-DPEs	2[³ H]UDP + Ap ₅ A	2[³ H]UDP hydrolysis	0.59 \pm 0.12	0.27 \pm 0.03
8. DPEs _(Ap₅A) ^a	Reactions 5–6		0.14 \pm 0.02	0.02 \pm 0.01
9. AK corrected for DPEs _(Ap₅A)	Reactions 5–7		0.52 \pm 0.17	0.11 \pm 0.03

^a DPEs_(Ap₅A), Ap₅A-sensitive non-AK dephosphorylating enzyme activities.

cleotide was shown to act as competitive inhibitor for the forward AK reaction (ATP + AMP \rightarrow 2ADP) and non-competitive inhibitor for the reverse AK reaction [2ADP \rightarrow ATP + AMP] (50). We tested the impact of Ap₅A on the phosphorylation of ADP by human bronchial ecto-AK. Dose-response curves were constructed on mucosal and serosal surfaces with 2 mM [³H]ADP and a range (0–0.5 mM) of Ap₅A concentrations. Fig. 6A shows that [³H]ATP production was inhibited in a dose-dependent manner and completely abolished by 0.5 mM Ap₅A. In the absence of ecto-AK activity, the pattern of [³H]ADP metabolism (Fig. 6B) closely resembled the pattern of [³H]UDP metabolism (Fig. 4D). This Ap₅A concentration reduced [³H]ADP metabolism by 60–70% on mucosal and serosal surfaces (Fig. 6C).

The possibility remained that this inhibition reflected, at least in part, interactions between Ap₅A and ADP-dephosphorylating enzymes reported on these cells (4, 29–32). To address this question, we took advantage of the substrate specificity and ion requirements of ecto-AK (Fig. 4). The hydrolysis of 2 mM [³H]UDP was reduced from 0.73 nmol·min⁻¹·cm⁻² to 0.59 nmol·min⁻¹·cm⁻² by 0.5 mM Ap₅A on the mucosal surface (Table I). Since [³H]UDP is not a substrate of ecto-AK (Fig. 4C), the difference between these two reaction rates would represent inhibition of dephosphorylating enzymes by Ap₅A (Table I, reaction 8). Similar results were obtained when [³H]ADP metabolism was assayed in Mg²⁺-free KRB (data not shown), which does not support ecto-AK activities (Fig. 4A). Reverse AK activity, calculated from Ap₅A-sensitive ADPase activity (Table I, reaction 5) and corrected for nonspecific Ap₅A interactions (Table I, reaction 8), was not significantly different (Table I, reaction 9) from values calculated by ATP production (Table I, reaction 2). Altogether, these experiments clearly demonstrate that ADP metabolism occurs mostly (~60%) by transphosphorylation on human airway epithelial surfaces.

Impact of Ecto-AK on the Metabolism of ATP—We designed time course experiments to investigate the impact of ecto-AK on ATP and other receptor agonists (ADP and adenosine) generated during cell surface metabolism. Since dephosphorylating ectoenzymes hydrolyze ATP and UTP (for a review, see Ref. 51), whereas ecto-AK is specific for adenine nucleotides (for a review, see Ref. 34) (Fig. 4, C and D), the phosphotransferase activity would be responsible for any discrepancy between the patterns of ATP and UTP metabolism through time. These experiments were conducted with substrate concentrations (10 μ M) relevant to stimulated nucleotide release and P2 receptor activation (for a review, see Ref. 52). Fig. 7, A and B, shows that initial hydrolytic rates for [³H]UTP were lower than for [³H]ATP with values of 7.9 \pm 1.2 nmol·min⁻¹·cm⁻² and 12.1 \pm 2.3 nmol·min⁻¹·cm⁻², respectively ($p < 0.01$). However, whereas [³H]UTP and [³H]UDP were undetectable after 30 min (Fig. 7A), [³H]ATP and [³H]ADP concentrations remained above 1 μ M throughout the experiment (Fig. 7B). Uridine con-

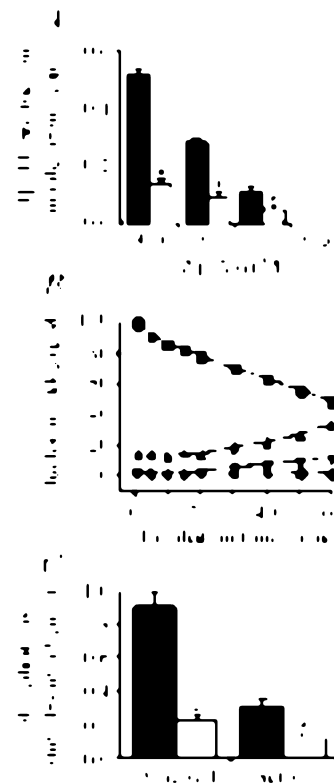


FIG. 6. Contribution of ecto-AK to ADP metabolism on human bronchial epithelial cells. Epithelial cultures were incubated with KRB (pH 7.4) containing bilateral 2 mM [³H]ADP (0.5 μ Ci), and buffer samples collected over 60 min were analyzed by HPLC. A, concentration-dependent inhibition of [³H]ATP production on mucosal (filled bars) and serosal (open bars) surfaces by Ap₅A. B, in the presence of 0.5 mM Ap₅A, the metabolic pattern of 0.1 mM ADP was similar to that of 0.1 mM UDP (Fig. 4D). ■, ADP; ▲, AMP; ▼, adenosine; ◆, inosine. C, ADPase and ecto-AK activities detected on mucosal and serosal surfaces. Enzyme activities measured without (filled bars, total ADPase) or with (open bars, ADPase without ecto-AK) 0.5 mM Ap₅A were 2–3-fold higher on the mucosal surface ($p < 0.01$). Complete inhibition of ecto-AK with 0.5 mM Ap₅A reduced the rate of ADP hydrolysis by 70% on both surfaces (*, $p < 0.01$). Values represent \pm S.E. of 5–7 independent experiments.

centration rapidly plateaued at 5–6 μ M within 20 min, whereas adenosine gradually reached 4 μ M by the end of the experiment.

The reversible transphosphorylating activity of ecto-AK on adenine nucleotides could be responsible for the differences in UTP and ATP metabolism. The initial faster rates of [³H]ATP degradation and [³H]ADP accumulation could reflect the combination of [³H]ATP dephosphorylation into [³H]ADP and phosphorylation of [³H]AMP into [³H]ADP. During the following 15 min, the reversible ecto-AK reaction competed with dephosphorylating enzymes to prolong the pools of [³H]ATP, [³H]ADP,

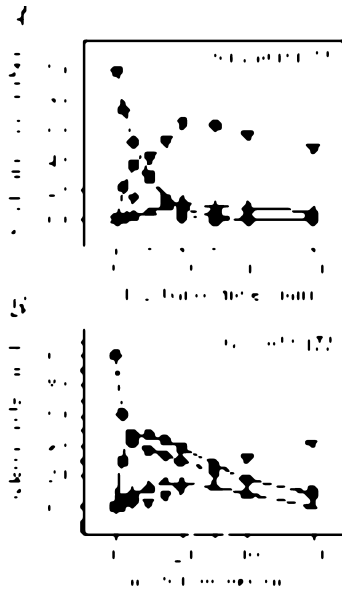


FIG. 7. Contribution of ecto-AK to UTP and ATP metabolism on human bronchial epithelial cells. The mucosal surface was assayed in KRB (pH 7.4) containing mucosal 0.01 mM [^3H]UTP (0.5 μCi) (A) or 0.01 mM [^3H]ATP (0.5 μCi) (B). Mucosal buffer samples collected over 20 min were analyzed by HPLC. ●, [^3H]UTP and [^3H]ATP; ■, [^3H]UDP and [^3H]ADP; ▲, [^3H]UMP and [^3H]AMP; ▼, [^3H]uridine and [^3H]adenosine; ◆, inosine. The patterns represent mean results from seven independent experiments (S.E. < 10% of the mean).

and [^3H]AMP. Adenine nucleotide concentrations eventually decreased below the micromolar range, suggesting that phosphate groups were escaping the nucleotide entrapment cycle, most likely through conversion of AMP into adenosine by the high affinity ecto-5'-nucleotidase activity ($K_m = 14 \mu\text{M}$) we described on these cells (30). Consequently, ecto-AK would prolong P2 receptor agonist availability and delay $\text{A}_{2\text{B}}$ receptor activation over a finite period of time following ATP release.

Lazarowski and Boucher (53) reported the presence of AMP on the mucosal surface of human airway epithelial cultures. We hypothesized that endogenous AMP concentrations would be sufficient to support transphosphorylation events initiated by the addition of $10 \mu\text{M}$ [$\gamma\text{-}^{32}\text{P}$]ATP. Fig. 8A shows HPLC traces for KRB samples collected 0, 1, and 2 min after the addition of [$\gamma\text{-}^{32}\text{P}$]ATP to the mucosal surface. The reaction generated [^{32}P]inorganic phosphate and two peaks that co-migrated with ADP and UTP standards. The rapid conversion of endogenous AMP into [$\beta\text{-}^{32}\text{P}$]ADP (Fig. 8B) suggests that ecto-AK plays a significant role in the availability of extracellular adenine nucleotides supporting MCC in human airways. The production of [$\gamma\text{-}^{32}\text{P}$]UTP corroborated earlier reports of ecto-NDPK activity on these cultures (32), whereby γ -phosphate was transferred from [$\gamma\text{-}^{32}\text{P}$]ATP to endogenous UDP.

Impact of Cystic Fibrosis on Human Airway Ecto-AK—The airways of CF patients are characterized by severe inflammatory responses to chronic bacterial infection, resulting from defective MCC (for a review, see Ref. 41). We investigated the long term effects of airway obstruction, chronic infection, and inflammation on the activity of human airway ecto-AK. Primary cultures of bronchial epithelial cells from normal donors and CF patients were assayed for forward and reverse ecto-AK activities with 2 mM [^3H]ADP and 1 mM [$\gamma\text{-}^{32}\text{P}$]ATP + 1 mM AMP, respectively. Fig. 9A indicates that CF was associated with ~2-fold higher forward and reverse ecto-AK activities on the mucosal surface ($p < 0.05$). Interestingly, the disease had no significant effect on serosal ecto-AK. Fig. 9B shows that total ADPase activity was enhanced 2-fold by CF on the muco-

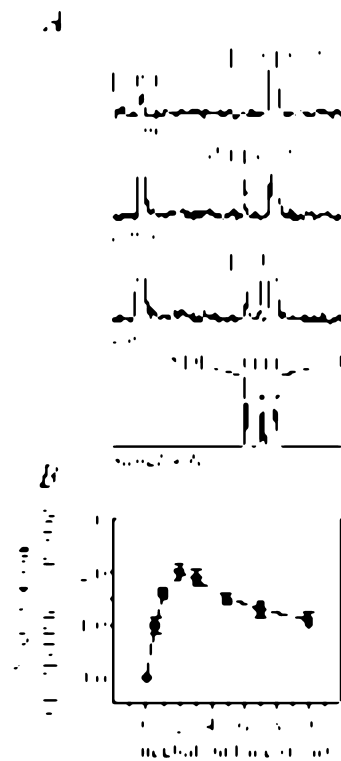


FIG. 8. Detection of ecto-AK activity with endogenous nucleotides on human bronchial epithelial surfaces. Assays were conducted in KRB (pH 7.4) containing mucosal 0.01 mM [$\gamma\text{-}^{32}\text{P}$]ATP (0.1 μCi). Buffer samples (10 μl) collected over 10 min were analyzed by HPLC. A, HPLC traces showing that ecto-AK converted endogenous AMP into [$\beta\text{-}^{32}\text{P}$]ADP. Small amounts of [$\gamma\text{-}^{32}\text{P}$]UTP were also identified by nucleotide standards, indicating ecto-NDPK activity. As shown in B, [$\beta\text{-}^{32}\text{P}$]ADP accumulation reached a maximum within 2 min. HPLC traces represent typical results (S.E. < 10% of the mean), and values (B) are mean \pm S.E. of four independent experiments.

sal epithelial surface. Assays repeated in Mg^{2+} -free KRB indicated that ecto-AK was mostly responsible for the impact of CF on ADP metabolism (Fig. 9B). These experiments demonstrated that ecto-AK activity is enhanced by CF on the mucosal surface of human airway epithelia.

DISCUSSION

In the present study, we have demonstrated that extracellular adenine nucleotide concentrations on human airway epithelial surfaces are regulated by a complex interplay between dephosphorylating and transphosphorylating ectoenzymes. Extracellular ATP was rapidly dephosphorylated by the stepwise reaction: $\text{ATP} \rightarrow \text{ADP} \rightarrow \text{AMP} \rightarrow \text{adenosine}$, as generally reported for mammalian cells (for a review, see Ref. 54). Experiments conducted with ADP as the initial substrate produced unexpected results that could not be revealed by standard phosphate assays. The reaction exhibited dephosphorylating activity by the production of AMP and adenosine, as well as phosphorylating activity by the accumulation of ATP. The observation that A_2B prevented ATP formation suggested that an ecto-AK was responsible for the phosphorylation of ADP (37). Indeed, experiments performed herein clearly showed that human bronchial epithelial surfaces display both forward ($\text{ATP} + \text{AMP} \rightarrow \text{ADP} + \text{ADP}$) and reverse ($\text{ADP} + \text{ADP} \rightarrow \text{ATP} + \text{AMP}$) reactions characteristic of an AK activity (for a review, see Ref. 34).

We first addressed the distribution of ecto-AK on human airway epithelial surfaces. The primary bronchial cultures exhibited the morphologic characteristics expressed *in vivo* (45) with a layer of columnar ciliated and secretory cells covering

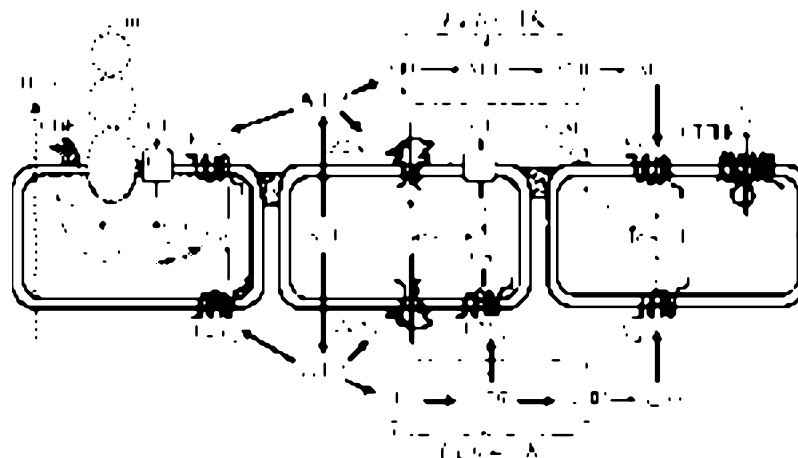
1–2 layers of basal-like cells. Both forward and reverse ecto-AK reactions were bilaterally distributed with 4-fold higher activities on the mucosal than on the serosal surface. The mucosal surface is comprised of the apical surface of the columnar cells and a thin liquid layer, *i.e.* airway surface liquid. Time course experiments indicated that more than 90% of the mucosal ecto-AK activity remained associated to the epithelial surface. Similar results were reported for human nasal epithelial cultures, whereby released AK represented ~20% of total ecto-AK activity measured on the mucosal surface (40). Given the short half-life of extracellular nucleotides within the airway surface liquid (*i.e.* 0.2 mM UTP; $t_{1/2}$ ~30 s (55)), these findings suggest that human airway ecto-AK is likely to target extracellular nucleotides released by the epithelium.

The kinetic properties of human airway ecto-AK at first glance may not suggest a significant role in the modulation of physiological nucleotide concentrations on airway surfaces.



FIG. 9. Impact of CF on human bronchial ecto-AK. As shown in A, CF enhanced ecto-AK activity on the mucosal surface but not on the serosal surface. Epithelial cultures from normal donors or CF patients were incubated with KRB (pH 7.4) containing 1 mM [γ - 32 P]ATP (0.1 μ Ci) + 1 mM AMP (Forward Reaction) or 2 mM [3 H]ADP (0.5 μ Ci; Reverse Reaction). Buffer samples (10 μ l) collected over 60 min were analyzed by HPLC for [γ - 32 P]ADP or [3 H]ATP production. As shown in B, the impact of CF on ADP metabolism was limited to ecto-AK. Assays were conducted in KRB (pH 7.4) containing 2 mM [3 H]ADP, without (0.5 μ Ci; total ADPase) or with (ADPase - AK) 0.5 mM A_2B . Values represent \pm S.E. of 5–7 independent experiments with epithelial cultures from normal donors (filled bars) or CF patients (empty bars) (*, $p < 0.05$).

FIG. 10. Ecto-AK and adenine nucleotide-mediated MCC on human airway epithelial surfaces. Mechanical stimulation induces bilateral ATP release, which is prolonged by the nucleotide entrapment cycle of ecto-AK or dephosphorylated into adenosine. Activation of bilateral $P2Y_2$ (ATP), $P2X_4$ (ATP \gg ADP), and $P2X_5$ (ATP \gg ADP) receptors, and serosal $P2Y_1$ receptors (ADP $>$ ATP), leads to cytosolic Ca^{2+} mobilization and stimulation of MCC-related cellular functions: CBF, mucin secretion, and Ca^{2+} -dependent Cl^- channels (I_{CA}). Adenosine maintains basal CBF and cystic fibrosis transmembrane regulator (CFTR) conductance by bilateral activation of A_{2B} receptors.



Ecto-AK transphosphorylated ATP and ADP with K_m values of 23 and 43 μ M, respectively. Although these values are lower than for ecto-AK on rat brain synaptosomes (K_m , ATP = 460 μ M (37)) and human endothelial cells (K_m , ATP = 135 μ M (38)), ATP concentrations on airway epithelial surfaces, measured by luciferin-luciferase assays in bulk solutions, are >100-fold lower under both basal (1–10 nM) and stimulated (5–200 nM) conditions (56, 57). It is important to consider, however, that measurements of endogenously released ATP in large buffer volumes may significantly underestimate near cell surface concentrations. A biosensor technique, using $P2X_2R$ -expressing PC12 cells as probes, detected ~13 μ M ATP at the surface of osmotically challenged intestinal epithelial cells, as compared with 5 nM by the “bulk fluid” luciferin-luciferase assay (57). Single cell confocal bioluminescence luciferase assays (58) and cell-attached luciferase assays (59) also measured ATP concentrations as high as 10–80 μ M in close vicinity to the site of stimulated release. These studies suggest that ecto-AK may influence physiological nucleotide concentrations on human airway epithelial surfaces, particularly following an osmotic or mechanical stimulation.

Most dephosphorylating and transphosphorylating ectoenzymes characterized on human airway epithelial surfaces exhibit substrate affinities in the low micromolar range. Ecto-5'-nucleotidase hydrolyzed AMP into adenosine with a K_m of 14 μ M (30), and ecto-nucleotide pyrophosphatase/phosphodiesterases (ATP \rightarrow AMP + PP_i) dephosphorylated ATP with a K_m of 13 μ M (60). Nonspecific alkaline phosphatase displayed high affinity ($K_m = 36 \mu$ M) and low affinity ($K_m = 717 \mu$ M) activities toward AMP (30). Lazarowski *et al.* (32) demonstrated that an ecto-NDPK influences endogenous concentrations of purine and pyrimidine nucleotides (ATP + UDP \rightleftharpoons ADP + UTP) on human nasal epithelial surfaces. When expressed in the human 1231N1 astrocytoma cell line, this enzyme presented a higher substrate affinity for ADP ($K_m = 17 \mu$ M) than for ATP ($K_m = 93 \mu$ M) (46). Collectively, these data suggest substantial interactions between dephosphorylating and transphosphorylating reactions on human airway epithelial surfaces.

A key finding of this work is the demonstration that ecto-AK competes with dephosphorylating enzymes to maintain adenine nucleotides on human airway epithelial surfaces. The higher affinity of human bronchial ecto-AK for ATP over ADP translated into 3-fold higher rates for the forward (ATP + AMP \rightarrow ADP + ADP) than the reverse (ADP + ADP \rightarrow ATP + AMP) reaction. This directionality could reduce the loss of phosphate groups through AMP dephosphorylation. We explored this possibility by taking advantage of the substrate specificity of AKs for adenine nucleotides (Fig. 4) (for a review, see Ref. 34). Time course ex-

periments on the metabolism of ATP and UTP indicated that ecto-AK significantly extends the duration of micromolar adenine nucleotides on epithelial surfaces while reducing adenosine production (Fig. 7). The kinetic constant of ecto-AK for the forward reaction is comparable with the affinity of ecto-5'-nucleotidase for AMP on these cultures ($K_{mAMP} = 14 \mu\text{M}$ (30)). Consequently, ecto-AK could significantly interfere with the conversion of AMP into adenosine by ecto-5'-nucleotidase, delaying the transition between P2 and P1 receptor-mediated signaling pathways on airway epithelial surfaces.

Perhaps the most compelling evidence for the physiological relevance of this nucleotide entrapment cycle was obtained from assays conducted with endogenous nucleotides. Adenine nucleotide and nucleoside concentrations measured on the mucosal surface of human nasal epithelial cells by etheno derivatization assays in bulk fluid were: ATP (10 nM), ADP (40 nM), AMP (70 nM), and adenosine (200 nM) (53). Because AMP levels are significantly higher than ATP under basal conditions, stimulated ATP release would drive the forward ecto-AK reaction ($\text{ATP} + \text{AMP} \rightarrow \text{ADP} + \text{ADP}$), thus minimizing nucleotide loss through AMP dephosphorylation. The fact that $[\beta\text{-}^{32}\text{P}]\text{ADP}$ accumulated from endogenous AMP and an $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ concentration (10 μM ; Fig. 8), mimicking stimulated release (57–59), supports such a role for ecto-AK on human airway epithelial surfaces. Furthermore, since ADP metabolism occurs mostly through ecto-AK on these cells, this ADP pool could constitute a significant source of extracellular ATP through the reverse reaction ($\text{ADP} + \text{ADP} \rightarrow \text{ATP} + \text{AMP}$). Consequently, during a limited period following ATP release, ecto-AK could efficiently compete with dephosphorylating ectoenzymes to prolong the availability of ATP and ADP for P2 receptor activation.

Based on the properties of ecto-AK, the dephosphorylating ectoenzymes (4, 29–32), P2XRs and P2YRs on human airway epithelial surfaces, we propose the following model for adenine nucleotide-mediated MCC (Fig. 10). Immediately following locally stimulated ATP release, the nucleotide concentration is at its highest, in a range that activates P2XRs ($\text{EC}_{50} = 10 \mu\text{M}$; for a review, see Refs. 62 and 63). P2XRs may desensitize rapidly, *i.e.* within ~30 s (16, 20), suggesting that a prolonged ATP level may not be important for signaling. On the other hand, P2YRs are activated by lower ATP concentrations ($\text{EC}_{50} = 0.1\text{--}1 \mu\text{M}$; for a review, see Ref. 21) and desensitize less rapidly. For instance, Cl^- currents (61) and CBF (19) induced by 100 μM ATP on airway epithelial surfaces remained above 60% of maximal response for at least 15 min. Therefore, ecto-AK may have a more pronounced effect on P2Y₂R activation and P2Y₂R-linked effectors because ecto-AK may significantly prolong the duration of ATP in the relevant concentration range (1–2 μM ; Fig. 8B). As nucleotide concentrations decrease with time, the high affinity dephosphorylating activities ($K_{ms} = 5\text{--}17 \mu\text{M}$ (31)) will eventually dominate over ecto-AK ($K_{ms} = 23 \mu\text{M}$ and 43 μM), and basal MCC functions will be maintained through adenosine receptor activation (19, 25–28).

In conclusion, we have demonstrated the co-existence of ATP-consuming and ATP-generating pathways on human airway epithelial surfaces. The biochemical and kinetic properties of ecto-AK suggest that the transphosphorylase activity could participate in the propagation of MCC along airway epithelial surfaces. Following stimulated ATP release, ecto-AK would compete with dephosphorylating enzymes to maintain effective nucleotide concentrations for P2Y and possibly P2X receptor activation, resulting in enhanced Ca^{2+} -dependent Cl^- secretion, CBF, and mucus secretion and, ultimately, acceleration of MCC. Interestingly, we have shown that ecto-AK is up-regulated in chronic obstructive airway diseases like CF. As extra-

cellular nucleotides are key regulators of MCC in CF (for a review, see Ref. 1), up-regulation of AK activity may be an adaptive response in these diseases.

Acknowledgments—We thank Drs. Eduardo Lazarowski and Scott Donaldson for critical comments on the manuscript.

REFERENCES

- Boucher, R. C. (1999) *J. Physiol.* **516**, 631–638
- Donaldson, S. H., and Boucher, R. C. (1998) in *The P2 Nucleotide Receptors* (Turner, J. T., Weisman, G. A., and Fedan, J. S., eds.), pp. 413–424, Humana Press, Totowa, NJ
- Taylor, A. L., Kudlow, B. A., Marrs, K. L., Gruenert, D. C., Guggino, W. B., and Schwiebert, E. M. (1998) *Am. J. Physiol.* **275**, C1391–C1406
- Donaldson, S. H., Lazarowski, E. R., Picher, M., Knowles, M. R., Stutts, M. J., and Boucher, R. C. (2000) *Mol. Med.* **6**, 969–982
- Felix, J. A., Woodruff, M. L., and Dirksen, E. R. (1996) *Am. J. Respir. Cell Mol. Biol.* **14**, 296–301
- Homolya, L., Steinberg, T. H., and Boucher, R. C. (2000) *J. Cell Biol.* **150**, 1349–1359
- Grygorczyk, R., and Hanrahan, J. W. (1997) *Am. J. Physiol.* **272**, C1058–C1066
- Watt, W. C., Lazarowski, E. R., and Boucher, R. C. (1998) *J. Biol. Chem.* **273**, 14053–14058
- Wang, Y., Roman, R., Lidofsky, S. D., and Fitz, G. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 12020–12025
- Musante, L., Zegarra-Moran, O., Montaldo, P. G., Ponzoni, M., and Galletta, L. J. V. (1999) *J. Biol. Chem.* **274**, 11701–11707
- Lange, K. (2000) *J. Cell. Physiol.* **185**, 21–35
- Braunstein, G. M., Roman, R. M., Clancy, J. P., Kudlow, B. A., Taylor, A. L., Shylonsky, V. G., Jovov, B., Peter, K., Jilling, T., Ismailov, I. I., Benos, D. J., Schwiebert, L. M., Fitz, J. G., and Schwiebert, E. M. (2001) *J. Biol. Chem.* **279**, 6621–6630
- Mason, S. J., Paradiso, A. M., and Boucher, R. C. (1991) *Br. J. Pharmacol.* **103**, 1649–1656
- Lazarowski, E. R., Paradiso, A. M., Watt, W. C., Harden, T. K., and Boucher, R. C. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 2599–2603
- Paradiso, A. M., Mason, S. J., Lazarowski, E. R., and Boucher, R. C. (1995) *Nature* **377**, 643–646
- Lé, K.-T., Babinski, K., and Séguéla, P. (1998) *J. Neurosci.* **18**, 7152–7159
- Taylor, A. L., Schwiebert, L. M., Smith, L. M., King, C., Jones, J. R., Sorscher, E. J., and Schwiebert, E. M. (1999) *J. Clin. Invest.* **104**, 875–884
- Korngreen, A., Ma, M., Priel, Z., and Silberberg, S. D. (1998) *J. Physiol.* **508**, 703–720
- Morse, D. M., Smullen, J. L., and Davis, C. W. (2001) *Am. J. Physiol.* **280**, C1485–C1497
- Ramirez, A. N., and Kunze, D. L. (2002) *Am. J. Physiol.* **282**, H2106–H2116
- Kennedy, C., and Leff, P. (1995) *Trends Pharmacol. Sci.* **16**, 168–174
- Huang, P., Lazarowski, E. R., Tarran, R., Milgram, S. L., Boucher, R. C., and Stutts, M. J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 14120–14125
- Communi, D., Paindavoine P., Place, G., Parentier, M., and Boeynaems, J. M. (1999) *Br. J. Pharmacol.* **127**, 562–568
- Le Hir, M., and Kaissal, B. (1993) *Am. J. Physiol.* **264**, F377–F387
- Lieb, T., Wikstrom Frei, C., Frohock, J. I., Bookman, R. J., and Salathe, M. (2001) *J. Physiol.* **538**, 633–646
- Lazarowski, E. R., Mason, S. J., Clarke, L., Harden, T. K., and Boucher, R. C. (1992) *Br. J. Pharmacol.* **106**, 774–782
- Stutts, M. J., Fitz, J. G., Paradiso, A. M., and Boucher, R. C. (1994) *Am. J. Physiol.* **267**, C1442–C1451
- Clancy, J. P., Ruiz, F. E., and Sorscher, E. (1999) *Am. J. Physiol.* **276**, C361–C369
- Picher, M., and Boucher, R. C. (1999) *Pediatr. Pulmonology Suppl.* **19**, 311 (abstr.)
- Picher, M., Burch, L. H., Hirsh, A. J., Sychala, J., and Boucher, R. C. (January 30, 2003) *J. Biol. Chem.* 10.1074/jbc.M300569200
- Picher, M., and Boucher, R. C. (2001) *Drug Dev. Res.* **52**, 66–75
- Lazarowski, E. R., Boucher, R. C., and Harden, T. K. (2000) *J. Biol. Chem.* **275**, 31061–31068
- Lacombe, M.-L., Milon, L., Munier, A., Mehus, J. G., and Lambeth, D. O. (2000) *J. Bioenerg. Biomembr.* **32**, 247–258
- Van Rompay, A. R., Johansson, M., and Karlsson, A. (2000) *Pharmacol. Ther.* **87**, 189–198
- Cascalheira, J. F., and Sebastiao, A. M. (1992) *Eur. J. Pharmacol.* **222**, 49–59
- Dunkley, C. R., Manery, J. F., and Dryden, E. E. (1966) *J. Cell. Physiol.* **68**, 241–248
- Nagy, A. K., Shuster, T. A., and Delgado, V. (1989) *J. Neurochem.* **53**, 1166–1172
- Yegutkin, G. G., Henttinen, T., and Jalkanen, S. (2001) *FASEB J.* **15**, 251–260
- Yegutkin, G. G., Henttinen, T., Samburski S. S., Sychala J., and Jalkanen, S. (2002) *Biochem. J.* **367**, 121–128
- Donaldson, S. H., Picher, M., and Boucher, R. C. (2001) *Am. J. Respir. Cell Mol. Biol.* **26**, 209–215
- Wine, J. J. (1999) *J. Clin. Invest.* **103**, 309–312
- Gray, T. E., Guzman, K., Davis, C. W., Abdullah, L. H., and Nettekheim, P. (1996) *Am. J. Respir. Cell Mol. Biol.* **14**, 104–112
- Wu, R., Yankaskas, J., Cheng, E., Knowles, M. R., and Boucher, R. C. (1985) *Am. Rev. Respir. Dis.* **132**, 311–320
- Lechner, J. F., and LaVeck, M. A. (1985) *J. Tissue Cult. Methods* **9**, 43–48
- Matsui, H., Davis, C. W., Tarran, R., and Boucher, R. C. (2000) *J. Clin. Invest.* **105**, 1419–1427
- Lazarowski, E. R., Homolya, L., Boucher, R. C., and Harden, T. K. (1997)

- J. Biol. Chem.* **272**, 20402–20407
47. Fedde, K. N., Lane, C. C., and Whyte, M. P. (1988) *Arch. Biochem. Biophys.* **264**, 400–409
48. Lazarowski, E. R., Watt, W. C., Stutts, M. J., Boucher, R. C., and Harden, T. K. (1995) *Br. J. Pharmacol.* **116**, 1619–1627
49. Hamada, M., Sumida, M., Okuda, H., Watanabe, T., Nojima, M., and Kuby, S. A. (1982) *J. Biol. Chem.* **257**, 13120–13128
50. Kuby, S. A., Hamada, M., Gerber, D., Tsai, W., Jacobs, H. K., Cress, M. C., Chua, G. K., Fleming, G., Wu, L. L., Fischer, A. H., Frischat, A., and Maland, L. (1978) *Arch. Biochem. Biophys.* **187**, 34–52
51. Zimmermann, H. (2000) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **362**, 299–309
52. DUBYAK, G. R., and El-Moatassim, C. (1993) *Am. J. Physiol.* **265**, C577–C606
53. Lazarowski, E. R., and Boucher, R. C. (2001) *Pediatr. Pulmonology Suppl.* **22**, 193
54. Beaudoin, A. R., Sévigny, J., and Picher, M. (1996) in *ATPases Biomembranes* (Lee, A. G. ed.), Vol. 5, pp. 369–401, Greenwich, CT
55. Tarran, R., Grubb, B. R., Parsons, D., Picher, M., Hirsh, A. J., Davis, C. W., and Boucher, R. C. (2001) *Mol. Cell* **8**, 149–158
56. Lazarowski, E. R., and Harden, T. K. (1997) *Br. J. Pharmacol.* **127**, 1272–1278
57. Hazama, A., Shimizu, T., Ando-Akatsura, Y., Hayashi, S., Tanaka, S., Maeno, E., and Okada, Y. (1999) *J. Gen. Physiol.* **114**, 525–533
58. Newman, E. A. (2001) *J. Neurosci.* **21**, 2215–2223
59. Beigi, R., Kobatake, E., Aizawa, M., and DUBYAK, G. R. (1999) *Am. J. Physiol.* **276**, C267–C278
60. Picher, M., and Boucher, R. C. (2000) *Am. J. Respir. Cell Mol. Biol.* **23**, 255–261
61. Clarke, L. L., Harline, M. C., Otero, M. A., Glover, G. G., Garrard, R. C., Krugh, B., Walker, N. M., Gonzalez, F. A., Turner, J. T., and Weissman, G. A. (1999) *Am. J. Physiol.* **276**, C777–C787
62. Soto, F., Garcia-Guzman M., and Stühmer, W. (1997) *J. Membr. Biol.* **160**, 91–100
63. Williams, M., and Jarvis, M. F. (2000) *Biochem. Pharmacol.* **59**, 1173–1185