Secreted and Cell-Associated Adenylate Kinase and Nucleoside Diphosphokinase Contribute to Extracellular Nucleotide Metabolism on Human Airway Surfaces

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5'-Nucleoside triphosphates (NTP) are present in the liquid covering airway surfaces and mediate important physiologic events through their interaction with P2-nucleotide receptors. Activation of airway P2Y₂ receptors, for example, stimulates ciliary beat frequency, chloride/liquid secretion, and goblet cell degranulation. We, therefore, have studied the metabolic pathways that regulate the concentration of nucleotides on airway surfaces. Stimulation of submucosal gland secretion in the nose was previously found to decrease the concentration of 5'-adenosine triphosphate (ATP) in nasal lavage samples due to the presence of a secreted 5'-nucleoside triphosphatase (NTPase). In this study, gland secretions were further studied and found to also contain adenylate kinase (AK) and nucleoside diphosphokinase (NDPK) activities. Ecto-AK and ecto-NDPK activities were also detected in well-differentiated cultures of superficial nasal epithelia, which reflected a combination of cell-associated and released (into culture media) AK and NDPK activities. This study demonstrates that "ecto-kinases" on airway surfaces (1) emanate from different enzyme families, including both AK and NDPK; (2) are expressed at superficial epithelial surfaces and in submucosal glands; and (3) may be important regulators of nucleotide concentrations on airway surfaces.

Extracellular P2X and P2Y nucleotide receptors are widely expressed throughout the body and mediate a wide array of physiologic processes. The P2Y₂ subtype appears to be the dominant apical membrane nucleotide receptor in the airway epithelia of the lung (1). This G-protein-coupled receptor activates phospholipase C and increases intracellular calcium in response to luminal adenosine 5'-triphosphate (ATP) or uridine 5'-triphosphate (UTP) (2, 3). Important cellular events that are mediated by P2Y₂ receptor activation in airway epithelia include chloride secretion (4), acceleration of ciliary beat frequency (5), and induction of mucus secretion by goblet cells (6). The net effect of these cellular events on airways physiology is accelerated mucociliary clearance (7). Consequently, the P2Y₂ receptor system has received attention as an endogenous regulator of mucociliary clearance and as a potential therapeutic target for diseases characterized by poor mu-

ciliary clearance, such as cystic fibrosis (CF). In addition, the P2X family of receptors may modulate chloride secretion and ciliary beat frequency and has been proposed as potential therapeutic targets for CF lung disease (8, 9).

If the P2Y₂ receptor is indeed involved in the endogenous regulation of mucociliary clearance, regulation of extracellular 5'-nucleotide levels is likely an important physiologic process that will reflect the balance between nucleotide release and metabolism. In a recent study, we demonstrated that the P2Y₂ receptor agonist ATP was detectable in human nasal airway surface liquid (ASL) under basal conditions at a physiologically relevant concentration. The superficial epithelium exhibited the capacity to increase ATP levels in ASL via nucleotide release and to decrease nucleoside 5'-nucleoside triphosphate (NTP) levels via cell surface nucleotidases. Interestingly, when studying the impact of submucosal gland (SMG) secretions on ASL nucleotide concentrations, a 5'-nucleoside triphosphatase (NTPase) activity was observed in nasal lavages enriched with gland secretions (10).

In the present study, we have investigated the profile of nucleotide-metabolizing enzymes present in both human SMG secretions and on superficial epithelial surfaces. In contrast to traditional paradigms of extracellular nucleotide metabolism that focus on dephosphorylation of nucleotides, our data suggest that adenylate kinase (AK) and nucleoside diphosphokinase (NDPK) play a role in the interconversion and metabolism of extracellular nucleotides on airway surfaces.

Materials and Methods

Subjects

Submucosal gland secretions were collected as outlined below from normal ($n = 5$; age 32.6 ± 1.7 yr) subjects without visible nasal inflammation or recent nasal symptoms after obtaining informed consent in accordance with the University of North Carolina Human Rights Committee.

Collection of Nasal Submucosal Gland Secretions

To study enzyme activities in SMG secretions, a baseline nasal lavage was performed by delivering a total of 2 ml of 0.9% saline to one nasal cavity with a medication dispenser (100 μ l/actuation). The lavage was recovered by expelling the liquid from the nose into a sterile specimen cup after every five actuations. To obtain a lavage enriched with SMG secretions, the same nostril was dosed with methacholine via nebulizer (30 mg/dose; 100 mg/ml in 0.9% NaCl), followed two minutes later by a lavage as described above. To test the specificity of methacholine-induced effects, the opposite nostril was pretreated with ipratropium bromide

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Abbreviations: adenylate kinase, AK; airway surface liquid, ASL; adenosine triphosphate, ATP; cystic fibrosis, CF; epithelial growth factor, EGF; high-performance liquid chromatography, HPLC; nucleoside diphosphokinase, NDPK; 5'-nucleoside triphosphate(s), NTP; 5'-nucleotide triphosphatase, NTPase; submucosal gland, SMG; uridine 5' triphosphate, UTP.

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(168 μ g; 42 μ g/actuation), an acetylcholine receptor antagonist, followed 15 min later by dosing with methacholine (30 mg). Two minutes after methacholine administration, the nostril was lavaged as described above. The volume of each lavage return was measured, mixed 1:1 with a Tris buffer solution (in mM: Tris HCl 100, MgCl₂ 2, DTT 3; pH 7.4), and incubated (37°C; 30 min) with intermittent vortexing to aid the dissolution of mucinous secretions. The solutions were then centrifuged (5 min; 600 $\times$ g) to remove cells and undissolved particulate matter.

Enzyme Assays in Nasal Lavage Samples

All assays were performed using 0.1 ml of lavage solution at 37°C. Reactions were started with the addition of substrate and stopped with 0.5 ml of cold 5% trichloroacetic acid (TCA). Inhibitors were added 15 min before the start of the reaction. TCA was extracted from the samples using 8 vol of ethyl ether. Initial rates of reaction were determined using conditions that ensured < 10% metabolism of the added substrate had occurred.

Adenylate Kinase Assays

The forward ($[\gamma^{33}\text{P}]\text{ATP} + \text{AMP} \rightarrow [\gamma^{33}\text{P}]\text{ADP} + \text{ADP}$) and reverse ($[\text{H}]\text{ADP} \rightarrow [\text{H}]\text{AMP} + [\text{H}]\text{ATP}$) adenylate kinase reactions were studied in lavage fluids using high-performance liquid chromatography (HPLC). To measure maximal reaction rates, $[\text{H}]\text{ADP}$ was added to lavage solutions (0.5 mM final concentration; 0.5 μ Ci/sample) at 37°C. Aliquots were assayed at multiple time points ($t = 0, 2, 5$, and 10 min) to ensure that initial rates of reaction were obtained. The rate of $[\text{H}]\text{AMP}$ accumulation directly reflected adenylate kinase activity because (1) no ADPase activity was observed in nasal lavage samples after inhibiting AK with diadenosine pentaphosphate (AP₅A) (Figure 2C), and (2) no significant AMP hydrolysis was observed over comparable time intervals (Figure 2B). $[\text{H}]\text{UDP}$ as an enzyme substrate was tested in an identical fashion. The effect of AP₅A (1mM), a specific adenylate kinase inhibitor (11), was also tested.

Nucleoside Diphosphokinase Assays

The formation of $[\text{H}]\text{ATP}$ from unlabeled ATP (0.5 mM) and $[\text{H}]\text{ADP}$ (0.1 mM) was assessed with HPLC and quantitated as an indicator of NDPK activity. AP₅A (1 mM) was included in these reactions to eliminate $[\text{H}]\text{ATP}$ formation via AK. Multiple samples ($t = 0, 0.5, 1, 2, 5$, and 15 min) were collected to measure the initial rate of reaction in nasal lavages performed after designated treatments. The presence of NDPK was further assessed by the formation of $[\gamma^{33}\text{P}]\text{UTP}$ from $[\gamma^{33}\text{P}]\text{ATP}$ (0.5 mM) and UDP (0.1 mM).

Cell Culture

Well-differentiated primary cultures of the superficial nasal epithelia lining were grown as previously described (12). In brief, primary nasal epithelial cells were harvested from resected surgical specimens by protease digestion, plated on porous Transwell Col filters (well diameter, 12 mm; pore size, 0.45 μ m; Corning Costar, Aston, MA) and grown under air-liquid interface conditions. The culture medium contained a 50:50 mixture of LHC Basal (Biofluids, Inc., Rockville, MD) and Dulbecco's modified Eagle's medium-High glucose media (Biofluids, Inc.), 5 μ g/ml insulin, 0.072 μ g/ml hydrocortisone, 0.5 ng/ml epithelial growth factor (EGF), 10 nM T₃, 10 μ g/ml transferrin, 0.6 μ g/ml epinephrine, 0.5 μ M phosphoethanolamine, 0.5 μ M ethanolamine, 50 nM retinoic acid, 0.5 mg/ml bovine serum albumin, 0.8% bovine pituitary extract, trace elements (1X; Biofluids Inc.), Stock 4 (1X; Biofluids, Inc.), Stock 11 (Biofluids, Inc.), 50 U/ml penicillin, and 50 μ g/ml streptomycin. Enzyme assays were performed after 4 wk of confluence. Under these conditions cultures were composed mainly of ciliated cells (> 90%) and exhibited a transepithelial electrical resistance of at least 300 Ω /cm².

Enzyme Assays on Cell Cultures

The apical surface of primary cultures of human nasal epithelial cells was rinsed three times with Krebs buffer (KBR [in mM]: 140 Na⁺, 120 Cl⁻, 5.2 K⁺, 25 HCO₃⁻, 2.4 HPO₄⁻, 1.6 Ca²⁺, 1.6 Mg²⁺, 5.2 glucose, and 25 HEPES [pH 7.4]), and preincubated with KBR (0.35 ml mucosal/serosal) for 30 min at 37°C (5% CO₂/95% O₂). AK and NDPK activity on the mucosal surface was then measured. AK activity was quantitated after adding 0.1 mM $[\gamma^{32}\text{P}]\text{ATP} + 0.1$ mM AMP. NDPK activity was measured using 0.3 mM $[\gamma^{32}\text{P}]\text{ATP} + 0.1$ mM UDP in the presence of 1 mM Ap₅A to inhibit AK activity. All reactions were initiated by the addition of the substrate(s) and stopped by transferring 10 μ l aliquots to tubes containing 0.3 ml ice-cold water. The samples were boiled for 3 min, filtered, and analyzed by HPLC.

Cell Culture Enzyme Release Assays

Experiments were conducted to investigate whether nasal epithelial cells released ecto-enzymes into the mucosal bath. Cell surfaces were rinsed three times with KBR and incubated (0.35 ml mucosal/serosal) at 37°C (5% CO₂/95% O₂). The conditioned medium was collected at various time points and assayed for AK and NDPK activities as described above for cell surface assays.

HPLC Analysis

Nucleotides were separated by HPLC via a Hypersil SAX column (Bodman, Aston, PA) using a two solvent system consisting of buffer A (5 mM NH₄H₂PO₄, pH 2.8) and buffer B (375 mM NH₄H₂PO₄, pH 3.7). A linear gradient was developed from 100% buffer A to 80% buffer B over the first 13 min, followed by an additional 7 min of 100% buffer A. Absorbance at 264 nm was monitored with an SPD10A UV detector (Shimadzu; Columbia, MD), and radioactivity was measured online with a Flo-One detector (Packard; Meridan, CT).

Materials

All NTPases, including radiolabeled ATP and UTP, were obtained from Amersham Pharmacia (Piscataway, NJ). Tritium labeled ADP and UDP were prepared from the corresponding $[\text{H}]\text{NTP}$ precursor by incubating the appropriate substrate with hexokinase (Roche Molecular Biochemicals; Indianapolis, IN) and glucose (25 mM) for 1 h at 37°C, followed by heat inactivation of hexokinase by boiling for 2 min. Complete conversion to the appropriate $[\text{H}]\text{-nucleoside diphosphate (NDP)}$ was confirmed using HPLC. For *in vivo* nasal studies, methacholine was purchased from Amend Drug and Chemical (Irvington, NJ), and ipratropium bromide from Boehringer Ingelheim (Ridgefield, CT). All other reagents were purchased from Sigma (St. Louis, MO).

Statistics

Unless otherwise stated, data are reported as mean $\pm$ SE. Comparisons were made with the Student's *t* test, with *P* < 0.05 considered significant.

Results

5'-Nucleoside Triphosphate and Diphosphate Metabolism in Gland Secretions

We have previously shown that the concentration of ATP in nasal lavages obtained after stimulating SMG secretion is markedly reduced due to the presence of a secreted NTPase (10). To further characterize the metabolic fate of exogenously added nucleoside triphosphates, $[\text{H}]\text{ATP}$ and $[\text{H}]\text{UTP}$ were added to lavages enriched with SMG secretions (Figure 1). After the addition of $[\text{H}]\text{ATP}$, ADP and then AMP were formed, with little adenosine formation,

Figure 1. The pattern of NTP metabolism in SMG secretions. (A) [^3H]ATP or (B) [^3H]UTP (100 μM) were added to nasal lavages samples collected after stimulating nasal submucosal gland secretion with methacholine. The formation of metabolic products was monitored with HPLC over time (closed squares, NTP; open circles, NDP; closed triangles, NMP; open triangles, adenosine/uridine).

over time. In contrast, the addition of [^3H]UTP led mainly to the accumulation of [^3H]UDP with little [^3H]UMP formation. The different rates of AMP and UMP formation from their respective NTPs, despite similar disappearance rates of ATP and UTP, suggested that SMG secretions might contain a complex mixture of enzymes, rather than a single enzyme.

Adenylate Kinase Activity in Nasal Submucosal Gland Secretions

To further investigate the nature of NDP metabolism in SMG secretions, [^3H]ADP and [^3H]UDP (100 μM) were added as starting substrates. As predicted from the patterns of ATP and UTP metabolism (Figure 1), the metabolism of [^3H]ADP occurred much more rapidly than that of [^3H]UDP ($54.7 \pm 2.3\%$ versus $6.6 \pm 3.6\%$ metabolized over 15 min, respectively) (Figure 2). Interestingly, the disappearance of [^3H]ADP was associated not only with [^3H]AMP formation, but also with the appearance of a second peak that ran precisely at the location of [^3H]ATP. Based on this observation, we hypothesized that AK was responsible for ADP metabolism in nasal lavage samples.

To test this notion further, we preincubated lavage solutions with diadenosine pentaphosphate (AP_5A), a competitive inhibitor of adenylate kinase (11), before examining ADP metabolism. AP_5A itself was not metabolized (data not shown), but did completely block the metabolism of

[^3H]ADP (Figure 2C). [^3H]UDP metabolism was notably slower than that of [^3H]ADP and was also inhibited by AP_5A (Figures 2E and 2F).

As another test for the presence of AK activity in SMG secretions, we added [$\gamma\text{-}^{33}\text{P}$]ATP and either AMP or UMP to lavage solutions. The formation of [^{33}P]ADP from [$\gamma\text{-}^{33}\text{P}$]ATP and AMP did indeed occur in an AP_5A sensitive manner (Figures 3A–3C), confirming the presence of a NMPK activity. The formation of [^{33}P]UDP from [$\gamma\text{-}^{33}\text{P}$]ATP and UMP was very slow (Figure 3D) and blocked by AP_5A (Figure 3E). The transphosphorylation of AMP by ATP (Figure 3B), with poor UMP utilization (Figure 3D), is consistent with the substrate specificity of most AK isoforms (AK1, AK2, AK4, and AK5) and rules out other nucleoside monophosphate kinase family members (13).

Using [^3H]ADP as the starting substrate, we quantified AK activity in SMG secretions (Figure 3F). With saturating substrate concentrations and short time intervals, the production of [^3H]AMP directly reflected AK activity because ADP metabolism was entirely AP_5A sensitive and no AMP degradation occurred under these conditions (Figure 2). Note that the formation of ATP from ADP did not quantitatively reflect AK activity due to the concomitant presence of an NTPase (Figure 1). The AK activity in lavages enriched with SMG secretions was ~ 9 -fold higher than in control lavages ($P < 0.001$). Pretreatment with ipratropium markedly reduced the methacholine-induced increase in AK activity, ($P = 0.01$).

Nucleoside Diphosphokinase Activity in Nasal Submucosal Gland Secretions

NDPK, which catalyzes the transfer of the γ -phosphate group from a NTP donor molecule to a NDP acceptor, is generally known as an intracellular enzyme responsible for balancing the intracellular pool of available NTPs (14). Recent work by Lazarowski and colleagues, however, demonstrated that an NDPK activity is present on the apical surface of a variety of cell lines, including a human bronchial epithelial cell line, and is able to interconvert NTPs in the extracellular milieu (15, 16). We hypothesized that SMG secretions might also contain this enzyme. We therefore performed NDPK assays on control lavages (no methacholine treatment), after methacholine treatment, and after ipratropium bromide plus methacholine.

NDPK activity was present in nasal lavages, as demonstrated by the transfer of the terminal phosphate group from [^{33}P]ATP to UDP, forming [^{33}P]UTP, and by the formation of [^3H]ATP from [^3H]ADP + ATP (in the presence of AP_5A , to eliminate competition with AK) (Figures 4A–4D). Because an NTPase is also present in SMG secretions, the measured formation of labeled NTP underestimates the true NDPK activity present in these lavage solutions. Despite this limitation, [^3H]ADP was converted to [^3H]ATP in the presence of unlabeled ATP and excess AP_5A at 10.7 nmol/min/ml in lavages enriched with SMG secretions (Figure 4E). This represents a 4-fold increase in comparison with control lavages ($P < 0.001$). Once again, pretreatment with ipratropium bromide inhibited the subsequent increase in NDPK activity in lavages after methacholine treatment ($P = 0.02$).

Figure 2. NDP Metabolism and in SMG Secretions. (A) $[^3\text{H}]\text{ADP}$ or (D) $[^3\text{H}]\text{UDP}$ (0.1 mM) were added to nasal lavages enriched with SMG secretions at $t = 0$ min. (B) The conversion of $[^3\text{H}]\text{ADP}$ to $[^3\text{H}]\text{ATP}$ and $[^3\text{H}]\text{AMP}$ at $t = 15$ min is shown, whereas (E) minimal $[^3\text{H}]\text{UDP}$ metabolism occurred over this time interval. The AK inhibitor AP_5A (500 μM) blocked the metabolism of (C) $[^3\text{H}]\text{ADP}$ and (F) $[^3\text{H}]\text{UDP}$.

AK and NDPK Activity on Superficial Airway Epithelial Surfaces

The presence of AK and NDPK activity in SMG secretions suggested that these enzymes might play a role in superficial airway epithelial extracellular nucleotide metabolism. We therefore used well-differentiated airway epithelial cultures to determine whether AK and NDPK were also cell surface-associated ecto-nucleotidases. Using AP_5A as a probe for AK activity, $[^3\text{H}]\text{ADP}$ conversion to $[^3\text{H}]\text{AMP}$ was inhibited by 59% (0.51 ± 0.05 versus 0.21 ± 0.03 nmol/min/cm²), the difference (0.30 nmol/min/cm²) reflecting AK activity. To

more directly demonstrate AK activity, the formation of $[^{33}\text{P}]\text{ADP}$ from AMP and $[\gamma^{33}\text{P}]\text{ATP}$ was further quantitated on cell surfaces. Although specific for the AK reaction, this assay likely underestimates AK activity due to the concomitant presence of AP_5A -insensitive ADP metabolism on cell surfaces. Despite this limitation, significant AK activity was indeed detected at the apical surface of nasal surface epithelial cells (Figure 5A).

To test whether the observed AK activity was strictly cell-associated or the result of released/shed enzymes, the formation of $[^{33}\text{P}]\text{ADP}$ from $[\gamma^{33}\text{P}]\text{ATP}$ and AMP was

Figure 3. AK activity in SMG secretions: $\text{ATP} + \text{AMP} \leftrightarrow 2 \text{ADP}$. The formation of $[^{33}\text{P}]\text{ADP}$ from $[\gamma^{33}\text{P}]\text{ATP}$ (0.1 mM) + AMP (0.1 mM) confirmed the presence of AK in SMG secretions. (A) At $t = 0$ min only $[\gamma^{33}\text{P}]\text{ATP}$ was seen. (B) At $t = 30$ min, the transfer of the γ^{33} -phosphate group from ATP to AMP resulted in the formation of $[^{33}\text{P}]\text{ADP}$. (C) The addition of AP_5A (0.5 mM) to lavages completely blocked the formation of $[^{33}\text{P}]\text{ADP}$; $t = 30$ min. (D) Minimal $[^{33}\text{P}]\text{UDP}$ is produced from $[\gamma^{33}\text{P}]\text{ATP}$ (0.1 mM) + UMP (0.1 mM) at $t = 30$ min, which was blocked (E) by the addition of AP_5A (0.5 mM). Note the liberation of free PO_4^{3-} (B, C, D, and E) as the result of NTPase activity in SMG secretions. (F) AK activity was quantitated in lavages collected under control conditions (Cont); after stimulating SMG secretion with methacholine (Meth) (* $P < 0.001$ versus Cont); and with methacholine treatment after ipratropium bromide pretreatment (Ipra + Meth) ($^{\dagger}P = 0.01$ versus Meth) using $[^3\text{H}]\text{ADP}$ (0.5 mM) as the starting substrate.

secretion with methacholine (Meth) (* $P < 0.001$ versus Cont); and with methacholine treatment after ipratropium bromide pretreatment (Ipra + Meth) ($^{\dagger}P = 0.01$ versus Meth) using $[^3\text{H}]\text{ADP}$ (0.5 mM) as the starting substrate.

was quantitated in lavages collected under control conditions (Cont); after stimulating SMG secretion with methacholine (Meth) (* $P < 0.001$ versus Cont); and with methacholine treatment after ipratropium bromide pretreatment (Ipra + Meth) († $P = 0.02$ versus Meth).

monitored in parallel experiments using conditioned media collected from the apical surface of cultures. After a 30-min preincubation in KBR, a majority of the AK activity was cell-associated, although a significant fraction of the total activity was contained in the conditioned media (Figure 5A). With increasing incubation times, conditioned media was found to contain progressively greater amounts of AK activity (Figure 5B).

NDPK activity was also assayed on the mucosal surface of well-differentiated airway epithelial cultures using the $[\gamma^{33}\text{P}]\text{ATP} + \text{UDP} \rightarrow \text{ADP} + [\gamma^{33}\text{P}]\text{UTP}$ reaction. Like AK, a majority of the NDPK activity was cell associated after a 30-min preincubation in KBR, although significant activity was also present in conditioned media collected from the apical compartment (Figure 5A). Once again, the amount of NDPK activity in conditioned media increased

over time (Figure 5B). Of note, NTPase activity on cells confounded NDPK activity measurements, causing our measurements to underestimate total activity.

Discussion

Extracellular nucleotides, via their interaction with airway P_2Y_2 receptors, play an important role in regulating elements of the mucociliary clearance apparatus. The regulation of extracellular nucleotide concentrations is physiologically important, therefore, and involves both nucleotide release and metabolic pathways.

General schemes of extracellular nucleotide metabolism have included a role for (1) the E-NTPDase family (e.g., CD39 and CD39L1–CD39L5) in NTP and NDP hydrolysis; (2) the ecto-phosphodiesterase/nucleotide pyrophosphatase (PDNP) family for ATP, ADP, and dinucleotide metabolism; (3) alkaline phosphatase, with the capacity to dephosphorylate nucleoside 5'-tri-, di- and monophosphates; and (4) ecto-5'-nucleotidase, which hydrolyzes NMP to nucleoside (17). Once fully dephosphorylated, the resulting nucleosides may be scavenged by nucleoside transporters (18). In airways, a full characterization of the relevant ecto-nucleotidases has not been performed. Recent studies found evidence for an ecto-alkaline phosphodiesterase activity that was responsible for dinucleotide metabolism on well-differentiated airway cultures (19), and Lazarowski and colleagues demonstrated that ecto-NDPK activity is present on the apical and basolateral surfaces of primary airway epithelial cultures (20).

The complexity of nucleotide metabolic pathways in airways is further increased by the fact that ecto-nucleotidases are associated with both the superficial epithelium and submucosal gland secretions (10). In this study, we showed that SMG secretions contain robust AK and NDPK activities, in addition to the previously described NTPase activity. AK and NDPK activities were also detected on the apical surface of well-differentiated airway epithelial cultures. Interestingly, a significant proportion of this ac-

Figure 5. AK and NDPK activity on human nasal epithelial cells. (A) Total AK and NDPK activities (solid columns) on airway epithelial surfaces were determined, as well as the proportion of activity that was released into the apical media (shaded columns), after a 30 min preincubation in KBR. (B) The release of AK (solid columns) and NDPK (open columns) activities into the apical culture media over time was monitored after washing airway surfaces with KBR.

tivity was released into the culture medium over time, suggesting either shedding of membrane-associated enzymes or the presence of an enzyme secretory pathway. The commonality of enzymes found in SMG secretions and on the surface of airway epithelial cells suggests that these "ecto-kinases" (AK and NDPK) might have an important role in extracellular nucleotide metabolism, which has not previously been recognized.

Most notable, perhaps, is the finding of ecto-AK activity in SMG secretions and on airway surfaces. AK catalyzes the reversible transfer of a phosphate group between ATP and AMP, forming ADP, and has traditionally been viewed as an intracellular enzyme responsible for the control of intracellular adenine nucleotide composition. Five mammalian isoforms have been identified to date (AK1–AK5), which are encoded by separate genes and include both mitochondrial and cytosolic forms. Precedent for a plasma membrane isoform is scant and comes from reports of ecto-AK activity in human umbilic vein endothelial cells (HUVEC) (21), synaptosomal brain preparations (22), and an innervated frog muscle preparation (23). As a consequence, AK is not often considered when extracellular nucleotide metabolism is studied. Importantly, its activity can be missed when nucleotide metabolism assays rely on the liberation of free phosphate and when ATPase and/or ADPase activities are also present. Utilization of HPLC to assay reaction products, and AP₅A as a specific inhibitor of AK (11), permits detection of AK when significant activity is present.

Nucleoside diphosphokinase, another enzyme that traditionally has been viewed as an intracellular regulator of nucleotide pools, was also found to play a role in extracellular nucleotide metabolism in airways. The notion of an ecto-NDPK was initially raised by multiple reports of nm-23–anti-metastasis gene products (24), which encode NDPK isoforms expressed at the cell surface (25). Further evidence for an ecto-NDPK comes from recent reports that demonstrated NDPK activity on the surface of a variety of cells, including primary human nasal epithelial cells (20), 16HBE14o[–] human bronchial epithelial cells (16), and HUVEC cells (21). Because a single cell type may possess more than one P₂-nucleotide receptor species with different nucleotide agonist selectivity, it has been speculated that ecto-NDPK may alter the balance of extracellular nucleotide species, and thereby effect signaling through P₂ receptors (26). For example, ATP released into the extracellular milieu may donate its terminal phosphate group to a UDP molecule in the same compartment, thus forming UTP and allowing activation of the UTP-selective P₂Y₄ receptor (16).

Understanding the impact of AK and NDPK on extracellular nucleotide metabolism within the larger framework of ecto-nucleotidases on airway surfaces is an important, though complicated, issue. In the context of a complex system of enzymes, the role that individual components play will depend upon several variables, including enzyme K_m and V_{max} . The enzyme V_{max} , in turn, is proportional to the abundance of the enzyme and its K_{cat} . In addition, because AK and NDPK each utilize two substrate molecules, the concentration of individual nucleotide substrates in the local milieu is an important determinant of activity. Because the reported K_m values ($\sim 100 \mu\text{M}$) for AK (21, 27) and NDPK (15) are similar to those of other airway ecto-nucle-

otidases (28), the impact that these enzymes have on extracellular nucleotide metabolism will depend primarily on the enzyme V_{max} and substrate concentrations.

In the experimental systems we have studied (i.e., surface epithelial cells and submucosal gland secretions), the relative importance of AK and NDPK varies. On surface epithelial cells, E-NTPases have ~ 10 -fold higher activity (10) than AK and NDPK and so will likely serve as the major pathway for NTP degradation. With regard to ADP metabolism, however, $> 50\%$ of surface epithelial cell ADP metabolism was inhibited by AP₅A, suggesting that AK significantly contributes to the metabolism of this nucleotide at this site. NDPK, on the other hand, will primarily serve to distribute phosphate groups among available nucleotide species.

In human nasal lavage samples, however, a very different profile of nucleotidase activities was observed. NDPK and AK activities were much greater than that of the E-NTPase (10) (~ 30 -fold and 6-fold, respectively) in nasal lavages collected under unstimulated conditions. After stimulating glandular secretion with methacholine, all three enzyme activities significantly increased, although NDPK activity continued to dominate (NDPK $>$ AK $\approx$ NTPase). In contrast to the pattern seen on superficial epithelial cells, ADP metabolism was entirely sensitive to AP₅A. This result suggests that AK is essential for ADP metabolism in SMG secretions and that the secreted E-NTPase strongly prefers NTP species over NDPs.

The relative importance of secreted versus cell-attached nucleotidases in NTP metabolism on airway surfaces *in vivo* is difficult to assess because the relative contribution of submucosal glands and superficial epithelia to airway surface liquid volume/composition is generally unknown and likely to be extremely variable. In lung regions that are heavily populated with submucosal glands (proximal airways), especially under conditions that increase the rate of SMG secretion (*see below*), extracellular nucleotide metabolism may be profoundly affected, or even dominated, by nucleotidases contained in SMG secretions. In these regions, the secreted NTPase will contribute to NTP metabolism, whereas AK is expected to prevent the buildup of ADP when coupled to the actions of this NTPase. The importance of NDPK in these systems relies on its ability to transfer phosphate groups between different nucleotide species and thus potentially alter the pattern of nucleotide receptor activation (15, 16).

The finding that nucleotide-metabolizing enzymes are secreted from submucosal glands provides new insights into the regulatory processes that limit airway nucleotide receptor activation. For example, one may speculate that gland secretions provide a reserve capacity for nucleotide metabolism. In the setting of airway inflammation/irritants, where nucleotide levels are expected to be elevated due to cellular breakdown, stimulated gland secretion may provide a means to regulate ATP and ADP levels through the combined actions of the secreted NTPase (10) and AK. These activities, in turn, may prevent prolonged nucleotide receptor stimulation and/or desensitization. Whether diseases characterized by pathologic gland hypersecretion, e.g., chronic bronchitis, have reduced nucleotide levels *in vivo* is worth exploring given the potential ramifications of

reduced ATP levels on mucociliary clearance. The impact that secreted nucleotidases have on nucleotide-based therapies for lung diseases will also need to be considered.

The finding of secreted nucleotidases in a biologic system is not a unique phenomenon. In other tissues with exocrine glands, such as oviduct (29, 30) and prostate (31, 32), the secretion of an ATPase has previously been observed. In these tissues, enzyme secretion is mediated via "shedding" of vesicular material (33). In addition, release of an ATPase from nerve terminals upon electric stimulation has been described (34). It is possible, therefore, that the secretion of nucleotidases from nasal submucosal glands may occur either via microvesicular shedding from epithelial cell surfaces or through a classic exocytotic pathway. The mechanism underlying the release of surface-associated nucleotidases in our cultured epithelial preparations is also unclear. A similar phenomenon, including the release of both an ATPase and 5'-nucleotidase, has been observed in endothelial cells in response to shear stress (35). Our data demonstrate for the first time, however, that nucleotidase secretion can be mediated by receptor activation. This finding raises the question whether other tissues that respond to cholinergic stimulation may release nucleotidases in a similar fashion.

The identification of ecto-NDPK and ecto-AK as potentially important determinants of extracellular nucleotide concentrations furthers our knowledge of the regulatory processes that influence nucleotide-mediated signaling in airways. Future studies focusing on molecular identification of specific ecto-nucleotidases/ecto-kinases in airways, the biochemic characterization of the integrated system at physiologic substrate concentrations, and of cell biologic processes that determine cell surface enzyme expression and/or secretion should greatly improve our understanding of this complex enzyme system.

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