Biochemical Evidence for an Ecto Alkaline Phosphodiesterase I in Human Airways

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Because dinucleotides are signaling molecules that can interact with cell surface receptors and regulate the rate of mucociliary clearance in lungs, we studied their metabolism by using human airway epithelial cells. A membrane-bound enzyme was detected on the mucosal surface of polarized epithelia that metabolized dinucleotides with a broad substrate specificity (diadenosine polyphosphates and diuridine polyphosphates [Up$_n$U], n = 2 to 6). The enzymatic reaction yielded nucleoside monophosphates (NMP) and Np$_{n-1}$ (N = A or U), and was inhibited by nucleoside 5′-triphosphates (α,βmet adenosine triphosphate [ATP] > ATP ≥ uridine triphosphate > guanidine triphosphate > cytidine triphosphate). The apparent Michaelis constant ($K_{m,app}$) and apparent maximal velocity ($V_{max,app}$) for [^{3}H]Up$_4$U were 22 ± 4 μM and 0.24 ± 0.05 nmoles · min^{-1} · cm^{-2}, respectively. Thymidine 5′-monophosphate *p*-nitrophenyl ester and adenosine diphosphate (ADP)-ribose, substrates of ecto alkaline phosphodiesterase I (PDE I) activities, were also hydrolyzed by the apical surface of airway epithelia. ADP-ribose competed with [^{3}H]Up$_4$U, with a K_i of 23 ± 3 μM. The metabolism of ADP-ribose and Ap$_4$A was not affected by inhibitors of cyclic nucleotide phosphodiesterases (3-isobutyl-1-methylxanthine, Ro 20-1724, and 1,3-dipropyl-8-*p*-sulfophenylxanthine), but similarly inhibited by fluoride and *N*-ethylmaleimide. These results suggest that a PDE I is responsible for the hydrolysis of extracellular dinucleotides in human airways. The wide substrate specificity of PDE I suggests that it may be involved in several signaling events on the luminal surface of airway epithelia, including purinoceptor activation and cell surface protein ribosylation.

Diadenosine polyphosphates (Ap$_n$A, n = 2 to 6) are now widely accepted as a novel class of extracellular signaling molecules that exhibit autocrine and paracrine functions through the activation of cell surface receptors (1–3). Activities ascribed to dinucleotides include modulation of platelet aggregation (3) and vascular tone (4, 5). The dinucleotide P^1,P^4-di(adenosine-5′)tetraphosphate (Ap$_4$A) was reported as a full agonist of the P2Y$_2$ receptor (6). In airways, activation of P$_2$Y$_2$ receptors induces Cl^- secretion, increased cilia beating frequency, and mucus secretion, functions important for mucociliary clearance (7).

The degree of phosphorylation of a dinucleotide is a key variable in determining the cellular responses to this class of molecules. In the cardiovascular system, P^1,P^2-di(adenosine-5′)pyrophosphate (Ap$_2$A) and P^1,P^3-di(adenosine-5′)triphosphate (Ap$_3$A) induced vasodilation, whereas Ap$_4$A, P^1,P^5-di(adenosine-5′)pentaphosphate (Ap$_5$A), and P^1,P^6-di(adenosine-5′)hexaphosphate (Ap$_6$A) induced vasoconstriction (8). Dinucleotides also reduced the electrical junction potential of vas deferens smooth muscles (Ap$_5$A > Ap$_4$A > Ap$_3$A > Ap$_2$A). The effects of Ap$_4$A and Ap$_5$A were sensitive to pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid, an antagonist of P$_2$ purinoceptors, whereas those of Ap$_2$A and Ap$_3$A were sensitive to 8-(*p*-sulfophenyl)theophylline (8-pSPT), an antagonist of adenosine purinoceptors (P$_1$) (9). In the guinea pig right atrium, dinucleotides triggered a negative inotropic response (Ap$_2$A ≥ adenosine triphosphate [ATP] ≥ Ap$_4$A = Ap$_3$A = Ap$_5$A). The effects of Ap$_2$A and ATP were sensitive to 8-pSPT, whereas those of Ap$_3$A, Ap$_4$A, and Ap$_5$A were blocked by suramin, a P$_2$ purinoceptor antagonist (10). These studies suggest that, depending on the phosphorylation status, dinucleotides may activate both P$_1$ and P$_2$ purinoceptors. Alternatively, P$_1$ purinoceptor activation may require conversion of the dinucleotide into adenosine by cell surface enzymes.

The metabolism of extracellular dinucleotides has been reported at the surface of vascular endothelial cells (11–13), chromaffin cells (14, 15), and synaptosomes of the *Torpedo* electric organ (16). These enzyme activities catalyze the asymmetrical cleavage of Np$_n$N into nucleoside monophosphates (NMP) and Np$_{n-1}$ (N = A, U [uridine], G [guanosine]; n = 2 to 6) in the presence of divalent cations, with Michaelis constant (K$_m$) values of 0.5 to 20 μM. Recent studies suggest that these ectoenzymes may belong to a family of glycoproteins designated as ecto alkaline phosphodiesterase I (PDE I). Several PDE I isoforms have been cloned and detected in mammalian tissues, including the brain, heart, intestines, liver, lungs, and vascular system (17–25). These proteins play important roles under normal and pathologic conditions, such as myelin sheath formation in the brain (21) and initiation of tumor metastasis (23). They cleave phosphodiester bonds of nucleotides like thymidine 5′-monophosphate *p*-nitrophenyl ester (TMP-pnp), as well as pyrophosphate bonds of nucleotides like nicotinamide adenine dinucleotide (NAD), ATP, adenosine diphosphate (ADP), and nucleoside diphos-

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Abbreviations: adenosine diphosphate, ADP; adenosine monophosphate, AMP; P^1,P^2-di(adenosine-5′)pyrophosphate, Ap$_2$A; P^1,P^3-di(adenosine-5′)triphosphate, Ap$_3$A; P^1,P^4-di(adenosine-5′)tetraphosphate, Ap$_4$A; P^1,P^5-di(adenosine-5′)pentaphosphate, Ap$_5$A; P^1,P^6-di(adenosine-5′)hexaphosphate, Ap$_6$A; adenosine triphosphate, ATP; ADP-ribosyl transferase, ART; cyclic AMP, cAMP; cyclic guanosine monophosphate, cGMP; cytidine triphosphate, CTP; 1,3-dipropyl-8-p-sulfophenylxanthine, DPSPX; guanidine triphosphate, GTP; high performance liquid chromatography, HPLC; 3-isobutyl-1-methylxanthine, IBMX; Michaelis constant, K$_m$; inhibition constant, K$_i$; Krebs buffer, KRB; 8-methoxymethyl-3-isobutyl-1-methylxanthine, mmIBMX; nicotinamide adenine dinucleotide, NAD; *N*-ethylmaleimide, NEM; phosphodiesterase I, PDE I; tetrabutyl ammonium hydrogen sulfate, TBASH; thymidine 5′-monophosphate *p*-nitrophenyl ester, TMP-pnp; 8-(*p*-sulfophenyl)theophylline, 8-pSPT; uridine diphosphate, UDP; uridine monophosphate, UMP; uridine triphosphate, UTP, P^1,P^4-di(uridine-5′)tetraphosphate, Up$_4$U.

phate sugars (uridine diphosphate glucose and ADP-ribose) (17, 18, 24–27). Dinucleotides were reported as substrates for PDE I purified from human plasma (28), rat liver (29), and rat C6 glioma (30). In contrast, these purified PDE I showed little or no activity towards RNA, adenosine monophosphate (AMP), or cyclic AMP (cAMP) (24, 26, 28, 29). In this study, we report for the first time the metabolism of extracellular dinucleotides on the apical membrane of human airway epithelial cells. We also provide biochemical evidence that the enzyme that mediates this function is a PDE I.

Materials and Methods

Cell Culture

Well-differentiated cultures from passage 1 human airway epithelial cells were grown as previously described (31). In brief, nasal and bronchial cells were harvested from resected surgical specimens (32). Primary cells were isolated by protease digestion and plated on a collagen-coated tissue culture dish (5 to 10 d) in LHC9 medium (33) containing 25 ng/ml epidermal growth factor (EGF), 50 nM retinoic acid, 40 μg/ml gentamicin, 0.5 mg/ml bovine serum albumin, 0.8% bovine pituitary extract, 50 U/ml penicillin, 50 μg/μl streptomycin, and 0.125 mg/ml amphotericin, termed bronchial epithelial growth medium (BEGM). The cells were trypsinized and subpassaged on porous Transwell Col filters (diameters: well, 24 mm; pore, 0.45 μM) in an air–liquid interface (ALI) medium. ALI was similar to BEGM, except for a 50:50 mixture of LHC basal and Dulbecco's modified Eagle's medium-high glucose as the base, amphotericin and gentamicin were omitted, and EGF concentration was reduced to 0.5 ng/ml. Enzyme assays were carried out 4 to 5 wk after the cells reached confluence. The cultures were composed mainly of ciliated cells (> 90%) and exhibited a transepithelial electrical resistance of at least 300 Ω/cm^2. Lactate dehydrogenase activity was employed as a test of cellular integrity.

Dinucleotide Hydrolase Assays

The cell surfaces were rinsed three times with Krebs buffer (KRB [in mM]): 140 Na^+, 120 Cl^-, 5.2 K^+, 25 HCO_3^-, 2.4 HPO_4^-, 1.3 Ca^{2+}, 1.3 Mg^{2+}, 5.2 glucose, and 25 *N*-2-hydroxyethylpiperazine-*N′*-ethane sulfonic acid (Hepes) (pH 7.4); and then pre-incubated 30 min in KRB (0.35 ml apical/2 ml basolateral) at 37°C (5% CO_2/95% O_2). The enzyme reaction was initiated by nucleotide addition (0.1 mM, unless stated otherwise) and stopped by transferring 25-μl aliquots to tubes containing 0.3 ml ice-cold water. The samples were boiled for 3 min, filtered, and analyzed by reversed-phase, paired-ion high performance liquid chromatography (HPLC). For the determination of optimum pH, Hepes was used to buffer solutions at pH 6.5 to 8.0, Tricine for solutions at pH 8.0 and 8.5, and 2-[*N*-cyclohexylamino]ethanesulfonic acid (CHES) for solutions at pH 8.5 and 9.0. In these buffers, bicarbonate was omitted for a better control of pH.

Enzyme Kinetics and Competition Assays

The kinetic parameters of dinucleotide metabolism by human bronchial epithelial cells were measured with [^{3}H]Up_4U (P^1,P^4-di[uridane-5′]tetraphosphate). A diuridine nucleotide was chosen over a diadenosine nucleotide because of the availability of the tritiated form [^{3}H]Up_4U, which was generously provided by Inspire Inc. (Durham, NC). Competition assays were conducted with ADP-ribose and Up_4U, as substrates for PDE I and Ap_nA hydrolase activities, respectively. The choice of ADP-ribose over TMP-pnp reflected its higher specificity for PDE I, as well as the convenience of monitoring the reaction by HPLC. Under these conditions, the metabolites of the two substrates could be distinguished on the HPLC tracings. The reactions were initiated by the addition of the two substrates previously mixed. All kinetic and competition assays were performed as stated previously for the dinucleotide hydrolase assays and in the presence of 5 U of commercial alkaline phosphatase to avoid enzyme inhibition by products of the reaction. We verified that dinucleotides and ADP-ribose were not substrates of alkaline phosphatase.

PDE I Assays

PDE I activity was measured using a modification of the method of Kelly and Butler (34). The epithelial surfaces were rinsed three times with KRB and then pre-incubated 30 min at 37°C with the same buffer (0.35 ml apical/2 ml basolateral). The enzyme reaction was initiated with 1 mM TMP-pnp and stopped by transferring an aliquot of 250 μl into a tube containing 25 μl of NaOH 10 mM. Absorbance was read at 405 nm, and the enzyme activity was quantified with a standard curve for *p*-nitrophenol. PDE I activity was also measured with ADP-ribose under the conditions mentioned previously for the dinucleotide hydrolase assays.

HPLC Analysis

The separation system consisted in 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 15 min, buffer B (100 mM KH_2PO_4, 8 mM TBASH, and 10% MeOH, pH 5.3) from 15 to 50 min, and buffer A from 50 to 60 min. Absorbance was monitored at 254 nm with an on-line model 490 multiwavelength detector (Shimadzu Scientific Instruments Inc., Norcross, GA), and radioactivity was determined on-line with a Flo-One Radiomatic β detector (Packard, Canberra, Australia), as described previously (6).

Statistics

All enzyme assays were performed on cultures of differentiated nasal or bronchial epithelial cells obtained from at least three different donors ($n \geq 3$). Rates of hydrolysis were calculated from the decrease in the amount of substrate monitored by HPLC and presented as nmoles/min · cm^2 of surface area. These values were expressed as means ± standard error of the mean (SEM). Unpaired Student's *t* tests were used to assess the significance between means. Paired *t* tests were used when comparing hydrolysis rates measured on the apical and basolateral sides of the same epithelium. All linear regressions, curve fits, and data transformations were performed with PC computer programs Origin and Sigma plot.

Materials

ADP-ribose, alpha,beta methylene ATP (α,βmetATP), Ap_2A, Ap_3A, Ap_4A, Ap_5A, Ap_4, 3′,5′-cyclic guanosine monophosphate, 3′5′cAMP, 1,3-dipropyl-8-*p*-sulfophenylxanthine (DPSPX), 3-isobutyl-1-methylxanthine (IBMX), 8-methoxymethyl-3-isobutyl-1-methylxanthine (mmIBMX), NAD, TMP-pnp, *N*-ethylmaleimide (NEM), Ro 20-1724, sodium fluoride, and TBASH were obtained from Sigma (St. Louis, MO). Mononucleotides and calf intestine alkaline phosphatase were purchased from Boehringer (Mannheim, Germany). Tritium-labeled Up_4U (1 mCi/mmol) and Up_nU (n = 2 to 6) were generously provided by Inspire, Inc. All other reagents were of analytical grade.

Results

Distribution of Dinucleotide Hydrolase Activity in Human Airways

The metabolism of extracellular dinucleotides was first detected upon addition of 0.1 mM Ap_4A to the apical surface

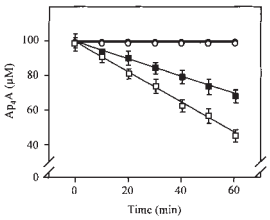

Figure 1. Distribution of dinucleotide hydrolase activity in human airways. Ap_4A hydrolysis was measured on the apical (*squares*) and basolateral (*circles*) surfaces of human nasal (*closed symbols*) and bronchial (*open symbols*) epithelial cells. Assays were conducted in KRB (pH 7.4) with 0.1 mM Ap_4A, and aliquots of 25 μl were collected over 60 min to be analyzed by HPLC. No hydrolytic activity was detected on basolateral surfaces. On apical surfaces, Ap_4A hydrolysis rates were significantly higher with bronchial cells than with nasal cells (paired *t* test: $t = 3.5$, $n = 4$).

of human airway epithelial cells (Figure 1). The enzyme activity was higher on bronchial (0.12 ± 0.01 nmoles $\cdot$ $min^{-1} \cdot cm^{-2}$) than on nasal (0.07 ± 0.01 nmoles $\cdot$ $min^{-1} \cdot cm^{-2}$) cells. In contrast, Ap_4A was not hydrolyzed by the basolateral surfaces of either cell type over a 60-min incubation. The protein responsible for Ap_4A hydrolysis in human airways is an ectoenzyme for the following reasons: (*1*) the substrate was hydrolyzed by intact cells and the products were released in the extracellular milieu; (*2*) the amount of substrate hydrolyzed over 60 min followed a linear relationship over time, making it unlikely that a significant amount of the substrate had been hydrolyzed after entering the cells; and (*3*) the rate of Ap_4A hydrolysis measured in buffer, collected after 60 min of incubation on the cells, corresponded to less than 4% of the total cell surface enzyme activity (data not shown). Thus, human airway epithelial cells possess a membrane-bound ecto dinucleotide hydrolase activity located on the apical surface.

Substrate Specificity and Mode of Action

The metabolism of Ap_nA on human bronchial epithelial cells was examined by HPLC. Representative chromatographic profile sets obtained for Ap_3A, Ap_4A, and Ap_5A after 0, 20, and 40 min of incubation on the cells are shown in Figure 2. The major products of Ap_3A hydrolysis were ADP and adenosine. The mass of AMP remained very small throughout the incubation period, probably due to the high rate of degradation by an ecto-5′-nucleotidase. In the case of Ap_4A, the major peaks were ADP and adenosine, with small peaks of AMP and ATP. The presence of ATP suggests that the enzyme cleaved Ap_4A into ATP and AMP, and not into two ADPs. Finally, the metabolism of Ap_5A led to the accumulation of Ap_4, and smaller amounts of ATP, ADP, AMP, and adenosine. Altogether, these results demonstrate that the ectoenzyme proceeds by an asymmetrical mode of cleavage, with AMP and Ap_{n-1} as products of the reaction.

Human airway epithelial cells hydrolyzed both diadenosine and diuridine polyphosphates bearing two to six phosphate groups (Figures 3A and 3B). Diadenosine polyphosphates were hydrolyzed on average 20% more rapidly than diuridine polyphosphates, when values were compared for dinucleotides containing the same number of phosphates. In addition, the rates of hydrolysis were inversely related to the length of the phosphate chain.

We conducted a series of experiments to investigate whether the relationship between phosphate chain length and hydrolysis rates could be explained by product inhibition. First, addition of commercial alkaline phosphatase (5 U) to efficiently remove any nucleotide formed during the reaction accelerated the metabolism of $[^3H]Up_4U$ (Figure 3C). We verified that alkaline phosphatase had no hydrolytic activity toward dinucleotides *in vitro* (data not

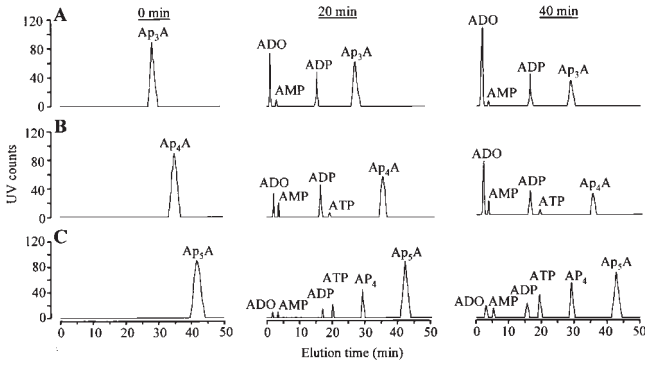

Figure 2. HPLC profiles of the time-dependent hydrolysis of (*A*) Ap_3A, (*B*) Ap_4A, and (*C*) Ap_5A by human bronchial epithelial cells. Assays were conducted in KRB (pH 7.4) with 0.1 mM substrates. Aliquots (25 μl) of apical buffer were collected after 0, 20, and 40 min of incubation, and analyzed by HPLC. The results are representative of three to four independent experiments.

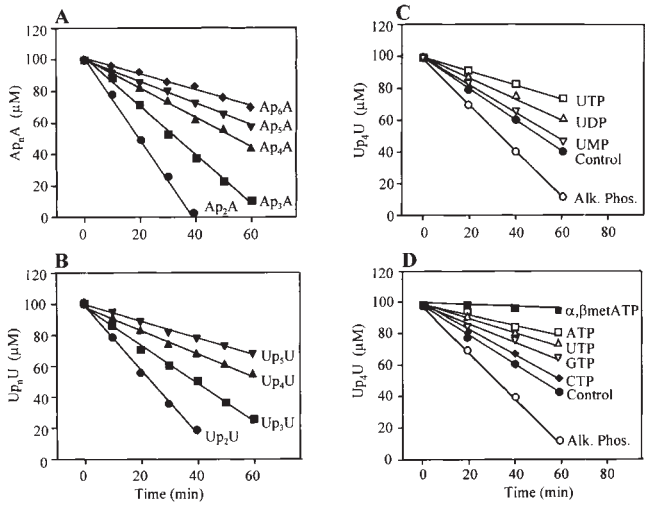

Figure 3. Substrate specificity of the dinucleotide hydrolase activity on human bronchial epithelial cells. Rates of hydrolysis for (*A*) Ap_nA and (*B*) Up_nU increased significantly with the number of phosphates ($t = 2.4$ to 3.9, $P < 0.05$) and were $18 \pm 3\%$ higher for Ap_nA over Up_nU of equal number of phosphate groups ($t = 2.2$ to 3.4, $P < 0.05$, $n = 5$ to 7). Assays were conducted as described in MATERIALS AND METHODS. (*C*, *D*) Inhibition of dinucleotide hydrolysis by mononucleotides. Assays were conducted with 0.1 mM $[^3H]Up_4U$ (0.1 μCi) and (*C*) 0.1 mM UTP, UDP, UMP, or alkaline phosphatase (Alk. Phos., 5 U), and (*D*) 0.1 mM α,βmetATP, ATP, UTP, GTP, CTP, or alkaline phosphatase (Alk. Phos., 5 U). Student's *t* tests: UTP > UDP > UMP, and α,βmetATP > ATP ⩾ UTP > GTP > CTP ($t = 3.3$ to 4.7, $P < 0.05$; $n = 5$ to 6; SEM < 5% of the mean).

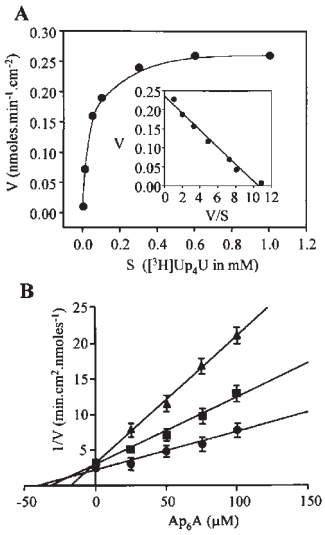

Figure 4. Kinetic parameters of the human airway dinucleotide hydrolase activity. (*A*) Michaelis-Menten relationship for [^{3}H]Up_4U hydrolysis. The reaction was initiated by the addition of 0.001 to 1 mM [^{3}H]Up_4U (0.1 μCi) in KRB buffer (pH 7.4) in the presence of 5 U of commercial calf intestine alkaline phosphatase to prevent inhibition by reaction products. Aliquots (10 μl) of apical buffer were collected after incubation periods that limited substrate hydrolysis to less than 10% and were analyzed by HPLC. *Insert:* Woolf-Augustinson Hoftsee plot of the saturation curve (r = 0.99). Calculated $K_{m,app}$, and $V_{max,app}$ values are 22 ± 4 μM and 0.24 ± 0.05 nmoles · min^{-1} · cm^{-2}, respectively (n = 6; SEM < 5% of the mean). (*B*) Dixon plot for the competitive inhibition of [^{3}H]Up_4U hydrolysis by Ap_6A. The reaction was started by addition of the two nucleotides previously mixed together. Aliquots (10 μl) of apical buffer were collected over 60 min and analyzed by HPLC. The correlation coefficients (r) were 0.99, 0.97, and 0.99 for 0 (*closed circles*), 40 (*closed squares*), and 100 μM (*closed triangles*) [^{3}H]Up_4U, respectively (n = 5, SEM < 7% of the mean).

shown). Second, addition of 0.1 mM uridine nucleotides decreased the rate of 0.1 mM [^{3}H]Up_4U hydrolysis in the order of potency: uridine triphosphate (UTP) > uridine diphosphate (UDP) > uridine monophosphate (UMP) (Figure 3C). These results show that the rate of hydrolysis of dinucleotides was inversely related to the degree of phosphorylation of the mononucleotides. Third, we tested whether other nucleoside triphosphates could affect the rate of [^{3}H]Up_4U hydrolysis. All nucleotides slowed [^{3}H]Up_4U hydrolysis in the order of potency: α,βmetATP > ATP ⩾ UTP > guanidine triphosphate (GTP) > cytidine triphosphate (CTP) (Figure 3D). Together, these results demonstrate that the rate of dinucleotide hydrolysis was limited by product inhibition. Consequently, most endogenous nucleotides would be expected to modulate the metabolism of extracellular dinucleotides on airway epithelia.

Kinetic Analysis of Dinucleotide Metabolism

The kinetic properties of the ectodinucleotide hydrolase activity were examined on human bronchial epithelial cells. The metabolism of [^{3}H]Up_4U followed simple Michaelis-Menten kinetics, and saturation was reached with 1 mM substrate (Figure 4A). Regression analysis of a Woolf-Augustinson Hoftsee transformation provided an apparent Michaelis constant ($K_{m,app}$) of 22 ± 4 μM and an apparent maximal velocity ($V_{max,app}$) of 0.24 ± 0.05 nmoles · min^{-1} · cm^{-2} (Figure 4A, *insert*). The hydrolysis of [^{3}H]Up_4U was inhibited by Ap_2A and Ap_6A, with inhibition constant (K_i) values of 26 ± 3 and 19 ± 2 μM, respectively. The competitive pattern of inhibition is illustrated by the Dixon plot obtained with Ap_6A (Figure 4B). Because these values are close to the $K_{m,app}$ calculated for [^{3}H]Up_4U, they can be considered as indirect but accurate estimations of $K_{m,app}$ for Ap_2A and Ap_6A. Together, these results suggest that a single enzyme would be responsible for the metabolism of diadenosine and diuridine polyphosphates on human airway epithelial cells.

Effects of Cations and pH on Ap_4A Hydrolysis

The metabolism of extracellular dinucleotides by human airway epithelial cells requires the presence of divalent cations. Ap_4A hydrolysis measured in KRB containing 1.3 mM Ca^{2+} and 1.3 mM Mg^{2+} was completely abolished by the addition of 5 mM ethylenediaminetetraacetic acid (EDTA). Both Mg^{2+} and Ca^{2+} were enzyme activators, with half-effective concentration (EC_{50}) values of 0.8 and

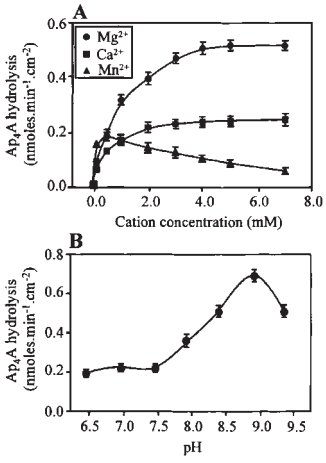

Figure 5. Effects of cations and pH on dinucleotide hydrolysis by human bronchial epithelial cells. (*A*) The effects of cations on the enzyme activity was assayed with 0.1 mM Ap_4A and KRB buffer (pH 7.4) containing various concentrations of Ca^{2+}, Mg^{2+}, or Mn^{2+}. The zero value was obtained in the presence of 5 mM EDTA and standard KRB containing 1.3 mM Ca^{2+} and 1.3 mM Mg^{2+}. (*B*) For the pH curve, different buffers were used to control pH over 6.5 to 9.5 (*see* MATERIALS AND METHODS). In both sets of experiments, the reaction was started with Ap_4A, and aliquots (25 μl) of apical buffer were collected over 60 min to be analyzed by HPLC (n = 4 to 5; SEM < 5% of the mean).

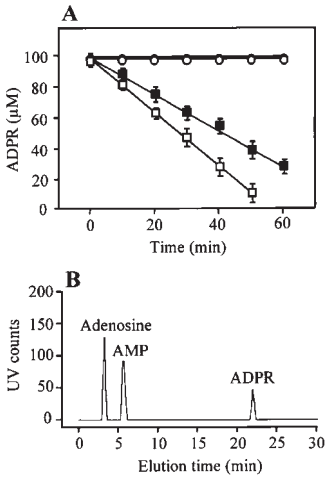

Figure 6. Metabolism of ADP-ribose in human airways. (*A*) ADP-ribose (ADPR) hydrolysis measured on the apical (*squares*) and basolateral (*circles*) surfaces of human nasal (*closed symbols*) and bronchial (*open symbols*) epithelial cells. Assays were conducted in KRB (pH 7.4) with 0.1 mM ADP-ribose, and buffer aliquots (25 μl) were collected over 60 min to be analyzed by HPLC. ADPR hydrolysis was significantly faster on bronchial than on nasal cells, with average values of 0.14 ± 0.01 and 0.04 ± 0.01 nmoles · min^{-1} · cm^{-2}, respectively (paired Student's t test: t = 4.5, n = 5). (*B*) Typical HPLC profile obtained for ADPR hydrolysis by the apical membrane of bronchial cells after 40 min of incubation. The metabolism of ADPR was monitored by the production of AMP and adenosine because ribose-5-phosphate does not absorb in ultraviolet.

0.4 mM, respectively (Figure 5A). With Mn^{2+}, a sharp peak of activity was reached at about 0.5 mM, whereas higher concentrations produced lower rates of hydrolysis. The effect of pH on Ap_4A hydrolysis was examined in the range 6.5 to 9.5 (Figure 5B). The ectoenzyme reached maximum activity at a pH of 9.0. A threefold increase in enzyme activity was observed by raising the pH from 7.5 to 9.0.

Presence of a PDE I on Human Airway Epithelium

Human bronchial epithelial cells hydrolyzed two substrates of PDE I, TMP-pnp (1 mM; 0.07 ± 0.01 nmoles · min^{-1} · cm^{-2}) and ADP-ribose (1 mM; 0.32 ± 0.03 nmoles · min^{-1} · cm^{-2}). The metabolism of ADP-ribose was restricted to the apical surface, and the enzyme activity was higher on bronchial than on nasal cells (Figure 6A). ADP-ribose hydrolysis led to the production of AMP and ribose-5-phosphate (35). Whereas the latter cannot be detected by ultraviolet absorbance on the HPLC, we monitored the accumulation of AMP and the conversion of the monophosphate into adenosine (Figure 6B). These results support the presence of a PDE I activity on airway epithelia that shares the same distribution and asymmetrical mode of cleavage with the dinucleotide hydrolase activity.

We therefore performed competition studies to investigate whether the same enzyme could be responsible for the metabolism of dinucleotides and ADP-ribose. The rate of ADP-ribose hydrolysis was reduced by increasing concentrations of Up_4U and vice versa (Figures 7A and 7B). The highest concentration (1 mM) of Up_4U (Figure 7A) or ADP-ribose (Figure 7B) almost completely blocked the reaction (> 95%), which suggests that a single enzyme hydrolyzed dinucleotides and ADP-ribose. Dixon plot analysis confirmed that ADP-ribose acted as a competitive inhibitor of Up_4U hydrolysis, with a K_i of 23 ± 3 μM (Figure 7C).

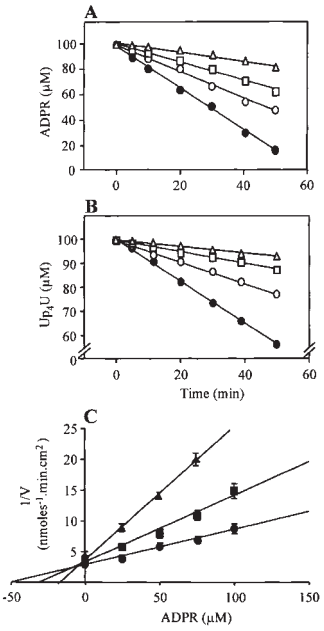

Figure 7. Competitive inhibition of ADP-ribose and Up_4U hydrolysis. (*A*) Time course of the hydrolysis of ADP-ribose (ADPR) 0.1 mM in the presence of [^{3}H]Up_4U (0 [*closed circles*], 0.1 [*open circles*], 0.5 [*open squares*], or 1.0 [*open triangles*]; 0.1 μCi). The reaction was initiated by addition of the two nucleotides previously mixed in the presence of 5 U of commercial calf intestine alkaline phosphatase to prevent feedback inhibition from reaction products. Aliquots of 10 μl were collected over 60 min and analyzed by HPLC. (*B*) Reciprocal experiment with [^{3}H]Up_4U 0.1 mM and ADPR 0 (*closed circles*), 0.1 (*open circles*), 0.5 (*open squares*), or 1.0 mM (*open triangles*). SEM were omitted for clarity (n = 4 to 6; SEM < 5% of the mean). (*C*) Dixon plot for the competitive inhibition of [^{3}H]Up_4U hydrolysis by ADP-ribose. Correlation coefficients for the linear regressions are 0.95, 0.98, and 0.99 for [^{3}H]Up_4U 10 (*closed circles*), 50 (*closed squares*), and 100 μM (*closed triangles*), respectively (n = 4, SEM < 10% of the mean).

Table 1 compares the effects of selected inhibitors on the metabolism of ADP-ribose and Ap_4A. The two enzyme reactions were similarly inhibited by sodium fluoride (EC_{50} ≈ 0.9 to 1.0 mM) and NEM (EC_{50} ≈ 5 mM). Ap_4A and ADP-ribose hydrolysis were not affected by inhibitors of cyclic nucleotide phosphodiesterases (DPSPX, IBMX, and Ro 20-1724). In addition, cAMP and cGMP were not hydrolyzed by nasal or bronchial epithelial cells (data not shown), which rules out the involvement of an extracellular cyclic nucleotide phosphodiesterase in the hydrolysis of ADP-ribose or dinucleotides. Together, these results suggest that a PDE I was responsible for the metabolism of extracellular diadenosine and diuridine polyphosphates on the apical membrane of human airway epithelial cells. Messenger RNA for two PDE I (PC-1 and PD-1β) was detected in human nasal epithelial cells by reverse transcriptase/polymerase chain reaction (data not shown).

Discussion

The initial focus of this study was to investigate the metabolism of putative regulators of P_2Y_2 receptors, dinucleotides, by human airway epithelia. Nasal and bronchial epithelial cells exhibited an ectoenzyme activity that was restricted to the luminal surface, and that effected an asymmetrical cleavage of dinucleotides (Np_nN; N = A or U) into NMP and Np_{n-1}. The mononucleotides derived from the hydrolysis of Np_2N, Np_3N, and Np_4N were efficiently dephosphorylated into adenosine or uridine by other ectonucleotidases previously reported on the luminal surface of airway epithelial cells (36–38). In contrast, the metabolism of longer dinucleotides (Ap_5A and Ap_6A) led to the accumulation of Ap_4 and Ap_5, suggesting that highly phosphorylated nucleotides were poor substrates for ectonucleotidases on airway epithelial cells. Similar findings have been reported for endothelial cells (11–13). In contrast, Ap_nA hydrolysis on chromaffin cells led to the accumulation of AMP, suggesting that the ecto-5′-nucleotidase activity was a limiting step in the production of adenosine (14, 15).

The dinucleotide hydrolase activity we measured on airway epithelia shares several properties with ecto Ap_nA hydrolases reported on endothelial cells (11–13), chromaffin cells (14, 15), and *Torpedo* synaptosomes (16): an asymmetrical mode of cleavage, a broad substrate specificity,

TABLE 1

Effects of inhibitors on Ap_4A and ADP-ribose hydrolysis by human bronchial epithelial cells

Compounds	Ap_4A	ADP-ribose
Sodium fluoride	54 ± 3	50 ± 2
NEM	49 ± 5	44 ± 5
IBMX	104 ± 3	102 ± 3
mmIBMX	100 ± 6	94 ± 4
Ro 20-1724	91 ± 5	101 ± 3
DPSPX	101 ± 3	105 ± 5

Assays were conducted with 0.1 mM substrate and 1 mM inhibitor, except for NEM (5 mM). Values are expressed as percent from rates of Ap_4A or ADP-ribose hydrolysis without inhibitors (n = 4 to 7; SEM < 5% of the mean).

and inhibition by ATP and ATP analogues. We also showed that the metabolism of extracellular dinucleotides on airway epithelia was inhibited by all nucleoside triphosphates tested (α,βmetATP > ATP > UTP > GTP > CTP). Moreover, Up_4U hydrolysis was inhibited by uridine nucleotides in the order of potency: UTP > UDP > UMP. Therefore, the rate of dinucleotide hydrolysis was inversely related to the degree of phosphorylation of the products of the reaction. Altogether, these results suggest that the metabolism of extracellular dinucleotides *in vivo* could be modulated by endogenously released or produced extracellular nucleotides.

The reported dinucleotide hydrolase activities can be distinguished on the basis of their substrate affinity and ion sensitivity. The substrate affinity of the airway dinucleotide hydrolase falls within the range reported for the porcine and bovine aorta endothelial enzymes, with K_m values of 20 μM for Ap_3A (11) and 10 μM for Ap_4A (12), respectively. The ecto Ap_nA hydrolases of adrenomedullary endothelial cells (13), chromaffin cells (14, 15), and *Torpedo* presynaptic membranes (16) exhibit higher affinities, with an overall K_m/K_i range of 0.5 to 7 μM for Ap_2A, Ap_3A, Ap_4A, Ap_5A, and Ap_6A. We also observed that the airway dinucleotide hydrolase activity required millimolar concentrations of divalent cations ($Mg^{2+} > Ca^{2+} > Mn^{2+}$) and was inhibited by fluoride (IC_{50} of 1 mM). Whereas these ionic properties correspond to those of the *Torpedo* Ap_nA hydrolase (16), the endothelial ecto Ap_nA hydrolases were inhibited by Ca^{2+} (12, 13), and the chromaffin ecto Ap_nA hydrolase was not affected by fluoride (15). Such diversity in biochemical properties supports the existence of different dinucleotide hydrolase isoforms or enzymes.

We therefore performed a series of experiments that appeared to identify the ectoenzyme responsible for dinucleotide metabolism on human airways as a PDE I. First, PDE I purified from human plasma (28), rat liver (29), small intestine, and testis (18) was shown to hydrolyze NAD, ADP-ribose, ATP, UTP, TMP-pnp, and dinucleotides, but not cyclic nucleotides. Human bronchial epithelia exhibited a similar pattern, hydrolyzing dinucleotides, TMP-pnp, and ADP-ribose, but not cAMP or cGMP. Second, the substrate affinity of the purified liver PDE I for dinucleotides falls within the range we report for the airway dinucleotide hydrolase, with K_m values of 8 to 22 μM for Ap_2A, Ap_3A, and Ap_4A (29). Third, as we observed on airway epithelia, the rate of dinucleotide hydrolysis by PDE I was inversely related to the length of the phosphate chain, and the reaction was inhibited by α,βmetATP (18). Finally, human plasma (28) and rat liver (29) PDE I exhibited a pH optimum (8.5 to 9.0) in the same range as the airway enzyme.

These comparisons strongly suggest the presence of a PDE I on airway epithelial cells. However, because our studies were conducted on intact cells, the possibility remained that two distinct enzymes could be responsible for the metabolism of dinucleotides and ADP-ribose, an ecto Ap_nA hydrolase and a PDE I, respectively. To address this possibility, we tested whether the two substrates competed for the same catalytic site. Simultaneous addition of Up_4U and ADP-ribose on the apical membrane of human bronchial epithelia reduced both rates of hydrolysis. The most compelling evidence of the presence of a single enzyme was the nearly complete inhibition (> 95%) of 0.1 mM Up_4U hydrolysis by 1 mM ADP-ribose and vice versa. Enzyme kinetic analyses indicated that ADP-ribose acted as a competitive inhibitor for Up_4U hydrolysis, with a K_i of 23 μM. Together, these results demonstrate that the ecto-dinucleotide hydrolase activity we characterized on human airway epithelia is a PDE I.

In summary, we report for the first time the metabolism of extracellular dinucleotides on the luminal surface of human airway epithelia. We also provide biochemical evidence that this enzyme belongs to the PDE I family, as reported recently for the chromaffin cell ecto dinucleotide hydrolase (39). The wide substrate specificity of PDE I suggests that the enzyme may be involved in other signaling events, in addition to regulating concentrations of purinoceptor agonists. For instance, PDE I could be involved in the ADP-ribosylation of cell surface proteins (40). ADP-ribosyl transferases (ART) use extracellular NAD^+ to transfer ADP-ribose to arginine residues. Removal of AMP from the bound ADP-ribose by PDE I renders the protein unavailable for further ribosylation. Reported extracellular ribosylation targets include the lymphocyte-associated molecule-1 (41) and the extracellular domain of integrin α7β1 (42). Three members of the ART family were recently localized on human bronchial epithelial cells: ART1, ART3, and ART4 (43). Given that airway epithelia play an active role in inflammatory processes by recruiting and interacting with leukocytes and macrophages, this ART–PDE I enzyme system could be involved in the regulation of immune responses via cell-to-cell and cell–matrix interactions.

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