THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 275, No. 40, Issue of October 6, pp. 31061–31068, 2000
Printed in U.S.A.

Constitutive Release of ATP and Evidence for Major Contribution of Ecto-nucleotide Pyrophosphatase and Nucleoside Diphosphokinase to Extracellular Nucleotide Concentrations*

Received for publication, April 17, 2000, and in revised form, July 24, 2000
Published, JBC Papers in Press, July 25, 2000, DOI 10.1074/jbc.M003255200

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Nucleotides are important extracellular signaling molecules. At least five mammalian P2Y receptors exist that are specifically activated by ATP, UTP, ADP, or UDP. Although the existence of ectoenzymes that metabolize extracellular nucleotides is well established, the relative flux of ATP and UTP through their extracellular metabolic products remains undefined. Therefore, we have studied the kinetics of accumulation and metabolism of endogenous ATP in the extracellular medium of four different cell lines. ATP concentrations reached a maximum immediately after change of medium and decreased thereafter with a single exponential decay ($t½$ ~30–40 min). ATP levels did not fall to zero but attained a base-line concentration that was independent of the medium volume and of the initial ATP concentration. Although the base-line concentration of ATP remained stable for up to 12 h, [γ-^{32}P]ATP added to resting cells as a radiotracer was completely degraded within 120 min, indicating that steady state reflected a basal rate of ATP release balanced by ATP hydrolysis (20–200 fmol × min^{-1} × $cell^{-6}$). High performance liquid chromatography analysis revealed that the γ-phosphate of ATP was rapidly, although transiently, transferred during steady state to species subsequently identified as UTP and GTP, indicating the existence of both ecto-nucleoside diphosphokinase activity and the accumulation of endogenous UDP and GDP. Conversely, addition of [γ-^{32}P]UTP to resting cells resulted in transient formation of [γ-^{32}P]ATP, indicating phosphorylation of endogenous ADP by nucleoside diphosphokinase. The final ^{32}P-products of [γ-^{32}P]ATP metabolism were [^{32}P]orthophosphoric acid and a ^{32}P-labeled species that was further purified and identified as [^{32}P]inorganic pyrophosphate. In C6 cells, the formation of [^{32}P]pyrophosphate from [γ-^{32}P]ATP at steady state exceeded by 3-fold that of [^{32}P]orthophosphate. These results illustrate for the first time a constitutive release of ATP and other nucleotides and reveal the existence of a complex extracellular metabolic pathway for released nucleotides. In addition to the existence of an ecto-ATPase activity, our results suggest a major scavenger role of ecto-ATP pyrophosphatase and a transphosphorylating activity of nucleoside diphosphokinase.

Extracellular nucleotides regulate a broad range of cellular responses such as platelet aggregation, vascular tone, cell proliferation, mucociliary clearance, cardiac and skeletal muscle contraction, and neurotransmission (1–4). The effects of extracellular nucleotides are mediated by two large subfamilies of receptors, the ligand-gated channel P2X receptors ($P2X_{1-7}$) and the G protein-coupled P2Y receptors ($P2Y_{1,\ 2,\ 4,\ 6,\ 11}$) (4–6). ADP, and less potently ATP, activates the $P2Y_1$ receptor, while ATP and UTP are the most potent agonists at the $P2Y_2$ receptor. In addition, ATP selectively activates the $P2Y_{11}$ as well as all P2X receptors. UTP is the selective agonist for the $P2Y_4$ receptor,[1] and UDP acts potently and selectively on the $P2Y_6$ receptor (7–10). The molecular identification and the wide tissue distribution of these nucleotide target proteins confirm that both adenosine and uridine di- and triphosphates subserve important extracellular signaling roles.

ATP is known to be released in a Ca^{2+}-dependent manner from storage compartments in nerve terminals, chromaffin cells, mast cells, and circulating platelets (1, 4, 11). Nucleotide release also occurs from non-excitatory tissues, and an autocrine/paracrine function for extracellular adenine nucleotides has been proposed (2, 3, 14–22). Recent studies indicate that relatively large amounts of ATP and UTP are released by mechanical stimulation (*e.g.* shear stress, hypotonic swelling, or stretch) of epithelial and endothelial cells, smooth muscle, glial cells, fibroblasts, and hepatocytes (14–23), and these nucleotides in the extracellular medium promote a robust activation of P2 receptors (16, 21, 23–25).

Ecto-nucleotidases and other ecto-enzymes metabolize extracellular ATP and UTP (26). However, the relative functional importance of these different enzymatic activities has not been defined for a given cell type, and little understanding is available of how these enzymes work in concert to produce the final pattern of metabolism of nucleotides. This is an important question given the aforementioned different selectivities of activation of P2Y receptors by both di- and triphosphate adenine and uridine nucleotides. As such, we have studied the kinetics of accumulation and metabolism of endogenous ATP in the extracellular medium of several different cell types. Our results illustrate for the first time a "constitutive" release of nucleotide that balances nucleotide hydrolysis and accounts for resting levels of extracellular nucleotides. We establish that the exchange of the γ-phosphate between adenine and uridine nucleotides at steady state markedly exceeds the ecto-ATPase activity. Moreover, our results highlight the importance of a nucleotide pyrophosphatase activity and reveal that this activity approaches or exceeds that of the ecto-ATPase.

* This work was supported by Cystic Fibrosis Foundation Grant Lazaro99G0 and by National Institutes of Health Grants GM38213 and HL34322.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.

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[1] ATP is not an agonist at the human $P2Y_4$ receptor but is a potent agonist at the rat $P2Y_4$ homologue (11, 12).

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MATERIALS AND METHODS

Cell Culture and Incubations—C6 rat glioma, 1321N1 human astrocytoma, and 16HBE14o$^-$ human bronchial epithelial cells were grown in Dulbecco's modified Eagle's medium-H containing 5% fetal bovine serum and antibiotics as described previously (23). ECV-304 human bladder epithelial cells (27) were grown in Medium 199 and 10% fetal bovine serum. All cultures were grown to confluence on 24-well plastic plates (Costar). Prior to assays, the cultures were washed three times and incubated in 0.5 ml of serum- and phenol red-free minimum essential medium (MEM).[2]

Luciferin-Luciferase Assay—Samples of extracellular medium were collected and boiled for 1 min, and the luciferin-luciferase assay was performed using a LB953 AutoLumat luminometer (Berthold GmbH), as described previously (21). Standard curves were performed routinely using molecular biology grade ATP, which was diluted in the same buffer and processed (*e.g.* boiled) in parallel to cell samples. No loss of ATP was observed during sample boiling. The threshold value for ATP detection was 100 pM (5 fmol/sample), and luminescence was linear with ATP concentration to 1000 nM.

Enzymatic Synthesis of ^{32}P-Labeled Inorganic Pyrophosphate, [γ-^{32}P]UTP, and [γ-^{32}P]GTP—^{32}P-Labeled inorganic pyrophosphate ($^{32}PP_i$) was obtained by incubating 1 μCi of [γ-^{32}P]ATP (100 μM) with 2 units/ml commercial nucleotide pyrophosphatase at 30 °C for 5 min. The quantitative conversion of [γ-^{32}P]ATP to $^{32}PP_i$ plus AMP was monitored by HPLC. [γ-^{32}P]UTP and [γ-^{32}P]GTP were obtained by incubating 3 μCi of [γ-^{32}P]ATP (specific activity >3000 Ci/mmol) in the presence of 0.1 units/ml bovine liver nucleoside diphosphokinase (NDPK) and 100 nM UDP or GDP, respectively, for 5 min at 30 °C (28).

Quantification of Nucleotides by HPLC—Except where indicated otherwise, species were separated by HPLC (Shimadzu) via a Dynamax C18 column (Varian) as described previously (21). The ion pairing mobile phase consisted of 8 mM tetrabutylammonium hydrogen sulfate (TBAHS), 17 mM KH_2PO_4, pH 5.3 (solvent A), or 8 mM TBAHS, 100 mM KH_2PO_4, pH 5.3, and 10% methanol (solvent B). The mobile phase developed at 1 ml/min from 0 to 4 min in 100% solvent A, and from 4 to 30 min in 100% solvent B. The elution times of authentic nucleotides were, ATP, 23.30 min; GTP, 18.20 min; UTP, 18.15 min; CTP, 16.5 min; ADP, 14.2 min, and AMP, 6.0 min. In some experiments (Fig. 7*F*), an anion exchange system with a 3021C4.6 ion chromatography column (Vydac, Hesperia, CA) was used, and the mobile phase (2 ml/min) developed in 25 mM NaH_2PO_4, pH 2.8 (solvent A), for 2 min followed by a linear gradient to 125 mM NH_2PO_4, pH 2.9 (solvent B), from 2 to 10 min. 100% solvent B was maintained from 10 to 30 min. The elution times for GTP, ATP, and UTP were 24.7, 20.3, and 16.8 min, respectively. Absorbance at λ = 260 nm was monitored on-line with an SPD-10A UV detector (Shimadzu, Japan), and radioactivity was measured on-line with a 500TR Radiomatic analyzer (Packard, Australia), as described previously (24). Full recovery of injected ^{32}P counts was routinely verified during HPLC runs.

Reagents—ATP, UTP, GTP, and CTP were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). UDP, ADP, GDP, CDP, 3′,5′-cyclic AMP, and AMPPNP were from Roche Molecular Biochemicals. AMPPCP and α,β-metATP were from RBI (Natick, MA). Bovine liver UDP-glucose pyrophosphorylase, yeast inorganic pyrophosphatase, *Crotalus adamateus* nucleotide pyrophosphatase, yeast nucleoside diphosphokinase, firefly luciferase, luciferin, Ap_4A, Ap_5A, ADP-glucose, ADP-ribose, and AMPPCP were obtained from Sigma. [γ-^{33}P]ATP (>3000 Ci/mmol) was from Amersham Pharmacia Biotech.

RESULTS

Extracellular Accumulation of ATP—The kinetics of extracellular accumulation of endogenous ATP and its metabolism were examined with 16HBE14o$^-$ human bronchial epithelial cells, ECV-304 human bladder epithelial cells, C6 rat glioma cells, and 1321N1 human astrocytoma cells. The growth medium bathing confluent cultures was replaced with 0.5 ml of MEM, and extracellular ATP levels were measured by the luciferin-luciferase assay over a 12-h interval. Consistent with ATP release secondary to mechanical stimulation of the cells (21, 23–25), relatively large concentrations of ATP were detected immediately after a change of medium, and these levels decreased steadily with $t_{1/2}$ values ~30–40 min (Fig. 1 and Table I). Extracellular levels of ATP did not fall to zero but attained a base line in the low nanomolar range within 3 h and remained unchanged for up to 12 h (Fig. 1 and Table I).

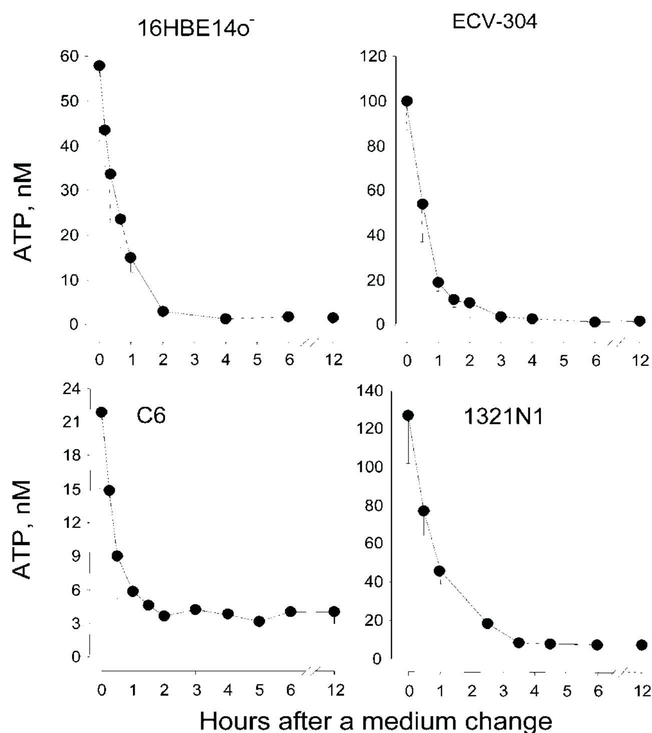

FIG. 1. **Decay of released ATP.** Confluent 16HBE14o$^-$, ECV-304, C6, and 1321N1 cells grown in 24-well plates were rinsed twice and incubated in 0.5 ml of serum- and phenol red-free MEM. The medium was removed at the indicated times, and ATP concentration was determined by the luciferase assay as described under "Materials and Methods." The results indicate the mean ± S.D. from at least six experiments performed with quadruplicate samples.

The dependence of the ATP concentration at base line on both the volume of the extracellular medium and the magnitude of the initial concentration of ATP was determined. The concentration of ATP 1 min subsequent to a wash was 132 ± 19 nM (n = 6) for ECV-304 cells incubated in 0.5 ml of bathing solution. Addition of 1 ml of mineral oil to the post-wash aqueous medium of these cells resulted in a slight increase of the ATP concentration within 1 min (152 ± 25 nM, n = 6), possibly reflecting additional ATP release due to the higher hydrostatic pressure and/or medium stirring. Addition of 1 ml of aqueous medium instead of mineral oil (*i.e.* the final volume of aqueous medium was 1.5 ml) resulted in a decrease in ATP concentration to 46 ± 7 nM (measured at 1 min, n = 6), consistent with the 1:3 dilution of the initial solution. ATP concentrations further decayed to base-line levels that were similar in non-diluted (0.5 ml of medium: 3.2 ± 1 nM, n = 6) and 1:3 diluted samples (1.5 ml of medium: 2.6 ± 0.4 nM, n = 6). In the four cell lines, addition of exogenous ATP (30 and 300 nM) to resting cells incubated in 0.5 ml of MEM resulted with time in base-line ATP concentrations that were indistinguishable from base-line values of control cells (shown in Table I). The $t_{1/2}$ values for hydrolysis of exogenous ATP (data not shown) at both 30 and 300 nM were identical to the $t_{1/2}$ values for endogenous ATP shown in Table I. These results indicate that extracellular ATP concentrations attain a constant value that is independent of

[2] The abbreviations used are: MEM, minimal essential medium; PP_i, inorganic pyrophosphate; AMPPNP, adenylyl imidodiphosphate; AMP-PCP, β,γ-methylene ATP; α,β-metATP, α,β-methylene ATP; NDPK, nucleoside diphosphokinase; Ap_4A, P^1–P^4-di(adenosine-5′) tetraphosphate; Ap_5A, P^1–P^5-di(adenosine-5′) tetraphosphate; HPLC, high performance liquid chromatography; TBAHS, tetrabutylethyl ammonium sulfate; E-NPP, ecto-nucleotide pyrophosphatase.

TABLE I
ATP decay to steady state

The $t_{1/2}$ values and first order rate constants (k) of ATP decay were obtained from the experiments described in Fig. 1 using a non-linear regression fit for single exponential decay (SigmaPlot, Jandel Corp.). Baseline ATP concentration ($[ATP]_{bl}$) is expressed as the mean ± S.D. from 4-h incubation samples obtained from three experiments performed in quadruplicate. The hypothetical rate of ATP hydrolysis at steady state ($\nu_{hydrolysis}$) was calculated as $\nu = k\ [ATP]_{bl}$.

	$t_{1/2}$	k	$[ATP]_{bl}$	$\nu_{hydrolysis}$ per 10^6 cells	$\nu_{hydrolysis}$ per cm^{-2}
	min	min^{-1}	*nM*	*fmol/min*	*fmol/min*
16HBE14o⁻	29	0.023	1.9 ± 0.4	145	17.3
ECV-304	29	0.023	2.4 ± 0.6	204	20.1
C6	32	0.022	1.1 ± 0.2	24	9.4
1321N1	41	0.017	4.9 ± 0.9	166	32.6

the volume of the bathing medium and of the magnitude of the initial ATP concentration.

ATP Release at Steady State—We further examined the capacity of 16HBE14o⁻, ECV-304, C6, and 1321N1 cells to metabolize exogenous [γ-^{32}P]ATP added after ATP concentrations stabilized. Cells were maintained undisturbed for at least 3 h, [γ-^{32}P]ATP then was added at high specific radioactivity, and both the ATP concentration and [γ-^{32}P]ATP were quantified by the luciferin-luciferase assay and HPLC analysis, respectively. Although levels of ATP mass remained unchanged, [γ-^{32}P]ATP was near completely metabolized within 120 min (Fig. 2). Thus, the development of a base-line ATP concentration reflects a steady state where hydrolysis is balanced by basal ("constitutive") release of ATP. Consequently, resting cells release ATP at a rate that can be quantitatively deduced from the rate of ATP hydrolysis at steady state.

The simplest approach to determine the rate of ATP release at steady state would be to measure directly the initial rate of [γ-^{32}P]ATP decay or, alternatively, the rate of accumulation of [^{32}P]orthophosphoric acid ($^{32}P_i$). However, disappearance of [γ-^{32}P]ATP does not necessarily represent hydrolysis since occurrence of enzyme-catalyzed exchange of the γ-phosphate between ATP and co-released nucleotides (without involving $^{32}P_i$ release) may substantially affect the first segment of the [γ-^{32}P]ATP decay curve. Moreover, $^{32}P_i$ was not the sole end-product of [γ-^{32}P]ATP metabolism (see below). The rates of ATP hydrolysis at steady state were deduced by kinetic analysis calculating the first order rate constant k of ATP decay ($k = 0.693/t_{1/2}$, Table I) and using the ATP concentrations measured at steady state ($\nu_{hydrolysis} = k\ [ATP]$). The $\nu_{hydrolysis}$ values obtained ranged from ~20 to 200 fmol/min × 10^{-6} cells within the four cell lines studied (Table I).

Metabolism of [γ-^{32}P]ATP—HPLC analysis of the products of [γ-^{32}P]ATP metabolism (Fig. 3) revealed that, in addition to $^{32}P_i$, two other ^{32}P-species appeared in the extracellular medium, with retention times of 8 and 18 min (hereafter referred to as ^{32}P-8 and ^{32}P-18, respectively). The ^{32}P-18 species arose soon after addition of [γ-^{32}P]ATP and subsequently decreased in parallel with the decrease of [γ-^{32}P]ATP (Fig. 4). The ^{32}P-8 species accumulated more slowly but steadily in the medium of 16HBE14o⁻ cells, ECV-304 cells, and 1321N1 cells. Accumulation of ^{32}P-8 was greater than accumulation of $^{32}P_i$ in the medium of C6 rat glioma cells (Figs. 3 and 4).

The metabolism of [γ-^{32}P]ATP in the medium bathing C6 cells was compared with that observed with conditioned medium obtained from these cells (Fig. 5). The formation of ^{32}P-8 and $^{32}P_i$ was dependent on the presence of cells. In contrast, the ^{32}P-18 species was generated by adding [γ-^{32}P]ATP to the cell-free conditioned medium (Fig. 5). Similar results were observed with ECV-304 and 16HBE14o⁻ cells (data not shown).

The ^{32}P-18 species, which co-eluted with authentic standards of GTP and UTP (Fig. 6*A*), was sensitive (as was [γ-^{32}P]ATP) to acid phosphatase, but the ^{32}P-8 species was not (Fig. 6*B*). The ^{32}P-8 species, which co-eluted with authentic $^{32}PP_i$, was hydrolyzed by inorganic pyrophosphatase, but ^{32}P-18 and [γ-^{32}P]ATP were not (Fig. 6*C*). Incubation of the ^{32}P-species containing-samples with *C. adamateus* nucleotide-pyrophosphatase (EC 3.6.1.9) resulted in conversion of both ^{32}P-18 and [γ-^{32}P]ATP into a ^{32}P-species that co-eluted with ^{32}P-8 (Fig. 6*D*). Therefore, ^{32}P-18 was tentatively identified as [^{32}P]UTP/[^{32}P]GTP, and ^{32}P-8 as $^{32}PP_i$. These results suggest the presence of extracellular nucleoside diphosphokinase (NDPK) and ecto-nucleotide pyrophosphatase (E-NPP) activities.

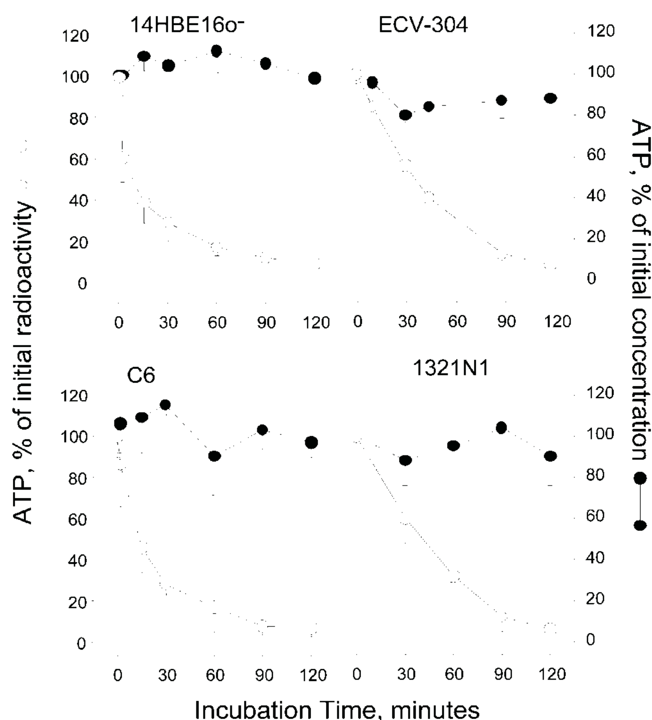

FIG. 2. **Stable ATP concentrations co-exist with [γ-^{32}P]ATP hydrolysis.** Cells were rinsed and pre-incubated for 3 h in 0.5 ml of MEM. [γ-^{32}P]ATP (0.1 μCi) was added to the medium, and samples were collected at the indicated times. The ATP concentration was measured by the luciferase assay and the ^{32}P-species quantified by HPLC. The results indicate the mean value from at least three experiments performed with triplicate (luciferase assay) or duplicate samples (HPLC).

Nucleoside Diphosphokinase—We previously reported the existence of an ecto-NDPK activity in 1321N1 human astrocytoma cells (29). Unambiguous proof for NDPK-mediated formation of the ^{32}P-18 species by 16HBE14o⁻, ECV-304, and C6 cells was obtained by three approaches. First, addition of a molar excess of UDP (100 μM) to cells that were pre-incubated with [γ-^{32}P]ATP for 30 min resulted in further conversion of [^{32}P]ATP to [^{32}P]UTP within 5 min (with no visible changes in the earlier ^{32}P-species; Fig. 7*B*). Similarly, addition of 100 μM CDP resulted in a shift of the radioactivity from both [γ-^{32}P]ATP and ^{32}P-18 to [γ-^{32}P]CTP (Fig. 7*C*), and addition of 100 μM ADP resulted in reversion of ^{32}P-18 back to [γ-^{32}P]ATP (data not shown). Second, cell-free conditioned medium was deproteinized with trichloroacetic acid, trichloroacetic acid was removed by ethyl ether extraction, and the solutions neutralized (pH 7.4) and incubated for 10 min with [γ-^{32}P]ATP (3 nM) in the absence or presence of 0.05 units/ml bovine liver NDPK. No changes in [γ-^{32}P] ATP were observed in the absence of NDPK, confirming that no endogenous activity was present after the trichloroacetic acid extraction. Inclusion of NDPK during incubation resulted in partial conversion of [γ-^{32}P]ATP to ^{32}P-18 (Fig. 7*D*). Thus, the endogenous transphosphorylat-

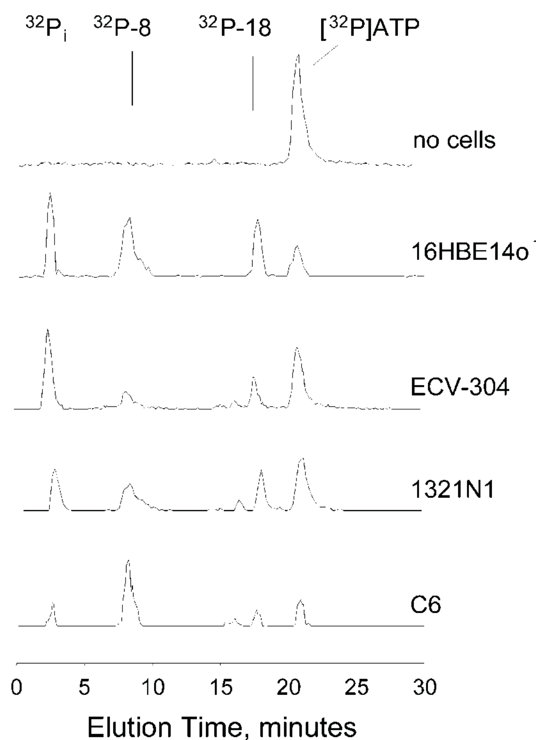

FIG. 3. **Metabolism of extracellular [γ-^{32}P]ATP.** Cells were rinsed and pre-incubated as described in the legend of Fig. 2. The HPLC (C18 column) tracings are representative of at least three experiments where [γ-^{32}P]ATP was incubated for 30 min in 0.5 ml of MEM in the absence of cells (*top panel*) or the presence of the indicated cells.

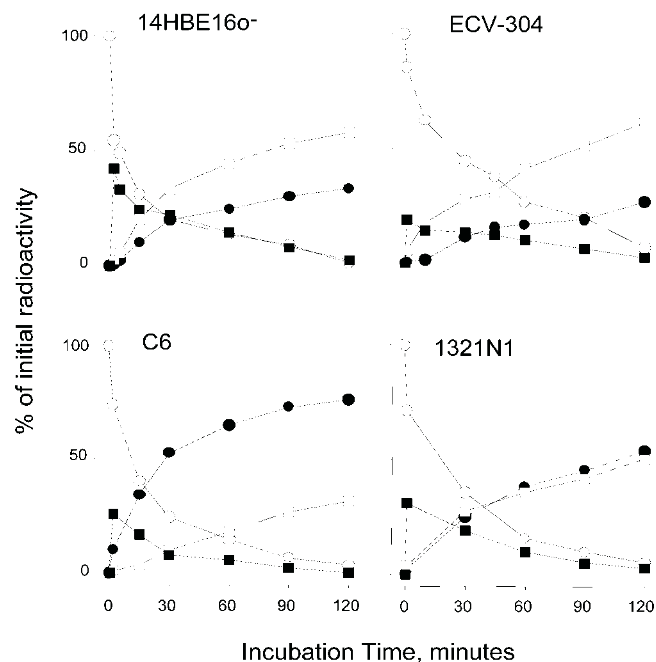

FIG. 4. **Time course of extracellular [γ-^{32}P]ATP metabolism.** [γ-^{32}P]ATP was added to resting cells for the indicated times and the resulting ^{32}P-species analyzed by HPLC. The data represent the mean value from a single experiment with duplicate samples differing by less than 20% from each other. Similar results were obtained in at least three experiments performed under similar conditions. *Open circle*, [γ-^{32}P]ATP; *open square*, $^{32}P_i$; *closed square*, ^{32}P-18; *closed circle*, ^{32}P-8.

ing activity that promotes formation of the ^{32}P-18 species in the cell-bathing medium could be substituted by exogenous NDPK added to the deproteinized solutions. Third, the ^{32}P-18 species that co-elute as UTP and GTP on the C18 column (Fig. 7*E*)

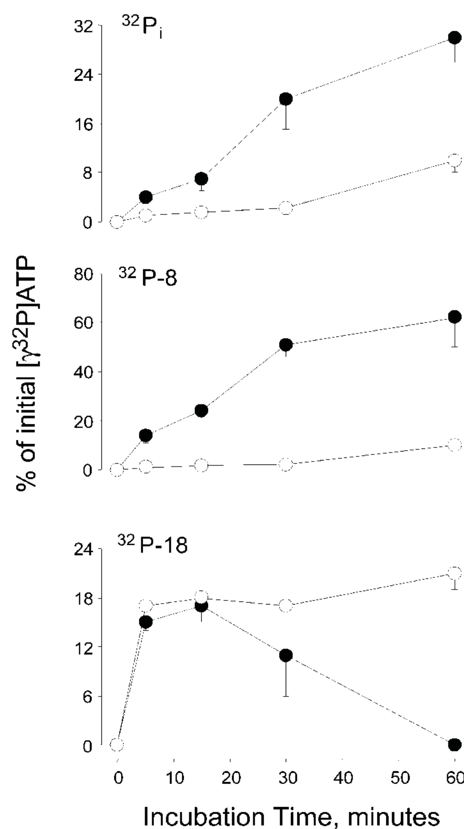

FIG. 5. **Metabolism of [γ-^{32}P]ATP by C6 cells and cell-free conditioned medium.** [γ-^{32}P]ATP was added to either resting C6 cells (*closed circle*) or conditioned medium obtained from resting C6 cells (*open circle*), and the resulting ^{32}P-species were separated by HPLC. The data represent the mean ± S.D. from one experiment performed with triplicate samples. The results are representative of three independent experiments performed under similar conditions.

were resolved as [^{32}P]UTP (~45%) and [^{32}P]GTP (55%), respectively, by an ion chromatography column (Fig. 7*F*).

These findings indicate that endogenous GDP and UDP accumulate in the extracellular medium and serve as acceptor substrates of NDPK. This notion also applies for ADP, as is shown below.

Nucleotide Pyrophosphatase—The ^{32}P-8 species was purified from C6 cell medium by HPLC (Fig. 8, *A* and *B*) and positively identified as $^{32}PP_i$ by incubating the purified fraction with 2 units/ml UDP-glucose pyrophosphorylase and a molar excess of UDP-glucose. Consistent with the reaction: PP_i + UDP-glucose → UTP + glucose-1P (23), full conversion of the ^{32}P-8 species into [^{32}P]UTP was achieved under these conditions (Fig. 8*C*).

Since different ratios of extracellular $^{32}PP_i$ *versus* $^{32}P_i$ were observed with the different cell types studied, the possibility that an extracellular inorganic pyrophosphatase activity was differentially expressed in a cell-specific manner was examined. C6, 16HBE14o$^-$, and ECV-304 cells were incubated in the presence of $^{32}PP_i$, and the resulting species accumulating in the medium were analyzed by HPLC. Surprisingly, $^{32}PP_i$ remained unchanged for up to 120 min after its addition (Fig. 8), indicating that inorganic pyrophosphatase is not expressed on the surface of these cells. These results suggest that the relative ratio of accumulation of PP_i over P_i is determined exclusively by the relative activities of E-NPP *versus* ecto-ATPase.

$^{32}PP_i$ also was the major end product of [γ -^{32}P]UTP on C6 cells (Fig. 9). Interestingly, the terminal phosphate of UTP was transiently (although not completely) transferred to ATP (Fig. 9), consistent with phosphorylation of endogenous ADP by endogenous NDPK. Since formation of $^{32}PP_i$ further proceeded

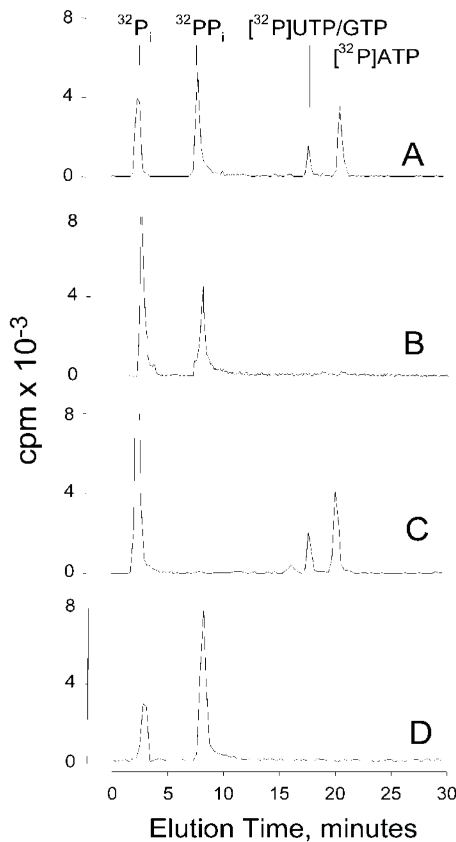

FIG. 6. **Enzymatic identification of the ^{32}P-8 and ^{32}P-18 species from C6 cells.** [γ-^{32}P]ATP was added to resting C6 cells for 30 min. The medium was removed and incubated for 5 min in the absence (*A*) or in the presence of 1 unit/ml acid phosphatase (*B*), inorganic pyrophosphatase (*C*), or nucleotide pyrophosphatase from *C. adamateus* (*D*). The resulting ^{32}P-species were separated by HPLC. The elution time of authentic standards of ^{32}P$_i$, ^{32}PP$_i$, [γ-^{32}P]ATP, [γ-^{32}P]UTP, and [γ-^{32}P]GTP are indicated at the *top* of *panel A*. The tracings are representative of at least three independent experiments performed in duplicates.

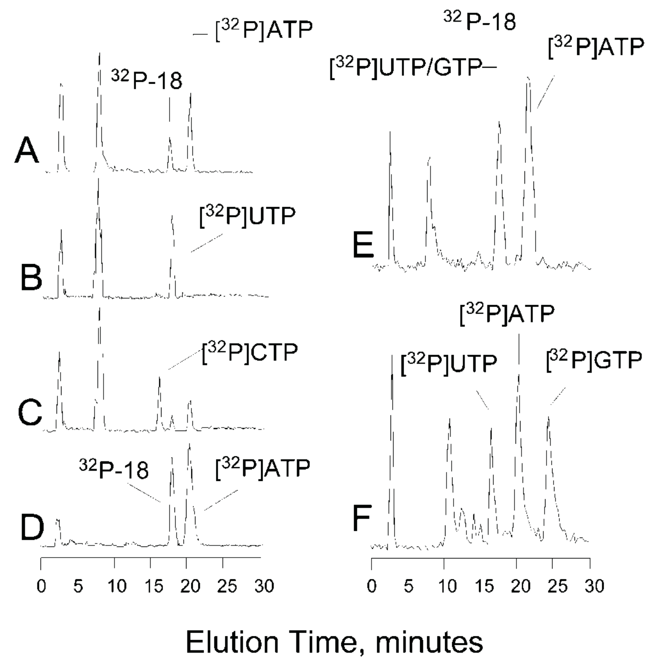

FIG. 7. **NDPK promotes the formation of [γ-^{32}P]UTP and [γ-^{32}P]GTP.** Resting C6 cells were incubated for 30 min with [γ-^{32}P]ATP (*A*), after which 100 μM UDP (*B*) or CDP (*C*) were added for 5 min. Conditioned medium from C6 cells was deproteinized with trichloroacetic acid and subsequently incubated for 30 min with 0.1 μCi of [γ-^{32}P]ATP and 0.1 units/ml nucleoside diphosphokinase (*D*). The HPLC tracings are representative of at least two experiments performed in duplicate. In a separate experiment, resting C6 cells were incubated for 10 min with [γ-^{32}P]ATP and the resulting species separated by HPLC via a C18 (*E*) or an ion exchange column (*F*). The data are representative of two independent experiments. The elution times of ^{32}P-labeled nucleotide standards are indicated. Similar results were obtained with 16HBE14o$^-$ and ECV-304 cells (data not shown).

after 20 min while [^{32}P]ATP was no longer detected (Fig. 9), we conclude that UTP is itself an efficient substrate for the formation of pyrophosphate.

Ecto-nucleotide pyrophosphatases hydrolyze dinucleoside polyphosphates and nucleotide sugars, but also generate free pyrophosphate (and AMP) from ATP (26). Therefore, these enzymes are logical candidates for the ATP pyrophosphatase activity described in this study. Although we have not measured the direct conversion of ATP to AMP as proof of principle for E-NPP activity, conversion of [γ-^{32}P]ATP to ^{32}PP$_i$ was effectively prevented by the E-NPP inhibitor α,β-metATP (Fig. 10) and was delayed by Ap$_4$A, Ap$_5$A, UDP-glucose, ADP-glucose, ADP-ribose, AMPPCP, and AMPPNP (Fig. 10), all of which are known substrates of the E-NPP family.

The kinetic constants for the ATP-dependent accumulation of ^{32}PP$_i$ and ^{32}P$_i$ on C6 cells were calculated (Table II). Although V_{max} values for PP$_i$ formation were about one half of those for P$_i$, the PP$_i$-generating activity exhibited a $K_{m(\mathrm{app})}$ for ATP that was approximately 6 times lower than the $K_{m(\mathrm{app})}$ of the ecto-ATPase activity. Thus, at steady state where [ATP] $\ll K_m$, the formation of PP$_i$ on C6 cells exceeds by a factor of 3 that of P$_i$ (determined by the V_{max}/K_m ratios) suggesting that E-PPN activity is the major regulator of the extracellular levels of nucleotides that are released by resting cells. This conclusion also is consistent with the results described earlier (Fig. 3), which illustrated that accumulation of ^{32}PP$_i$ at steady state on resting C6 cells was 3–4 times higher than accumulation of ^{32}P$_i$. The V_{max}/K_m values for the overall ATP hydrolysis reaction on C6 cells (~0.03 min^{-1}; Table II) closely approached the first order rate constant value (k = 0.02 min^{-1}) calculated from the $t_{1/2}$ values, as detailed in Fig. 1 and Table I.

DISCUSSION

In the present study, we demonstrated that basal (or "constitutive") release of ATP is a characteristic of resting cells, and we have defined conditions that allow quantification of the basal rate of ATP release. Both mechanically released ATP and exogenous ATP decay to a steady state where the magnitude of nucleotide hydrolysis and basal release are balanced. At steady state, extracellular ATP concentrations are independent of both the volume of the extracellular solution and the initial ATP input. Thus, ATP concentrations in the extracellular medium of resting cells are an intrinsic attribute of the tissue and reflect a balance between the capacity of the cells to release ATP "constitutively," and the efficiency of the ATP-hydrolyzing ecto-enzymes.

The basal rate of ATP release was determined based on bulk ATP concentrations. However, ATP concentrations measured in the entire medium are unlikely to accurately reflect the concentration at the cell surface. ATP and UTP levels observed in the medium bathing resting 1321N1 cells (Table I and Refs. 23 and 25) are below threshold values for stimulation of P2Y receptors (24, 25). However, addition of apyrase or hexokinase to resting P2Y$_2$ or P2Y$_4$ receptor-expressing 1321N1 cells resulted in significant reduction of the basal accumulation of inositol phosphates (21, 24, 25), suggesting that local nucleotide concentrations were high enough to confer basal activity to these receptors. These results indicate that quantification of

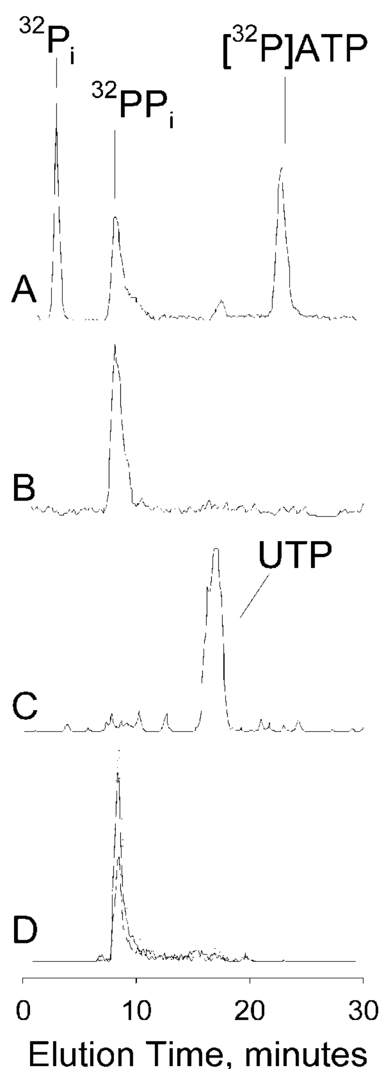

FIG. 8. **Identification of $^{32}PP_i$.** C6 cells were incubated for 30 min in the presence of 1 μCi of [γ-^{32}P]ATP in 0.5 ml of MEM (*A*). The ^{32}P-8 species was purified and further incubated for 1 h with 1 mM UDP-glucose in the absence (*B*) or in the presence (*C*) of 2 units/ml UDP-glucose pyrophosphorylase (21). The formation of [^{32}P]UTP was monitored by HPLC. The completeness of the reaction was confirmed in a parallel incubation where authentic $^{32}PP_i$ was quantitatively converted to [^{32}P]UTP. *D*, HPLC tracings corresponding to a 2-h incubation of C6 (●●●), 16HBE14o$^-$ (- - -), or ECV-304 (. . .) cells with 0.5 μCi of $^{32}PP_i$.

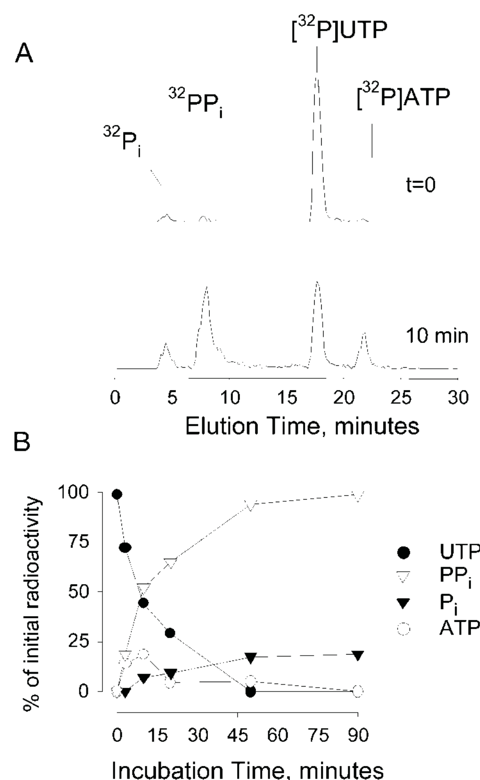

FIG. 9. **Metabolism of [γ-^{32}P]UTP on C6 cells.** C6 cells were incubated in 0.5 ml of MEM containing 0.1 μCi of [γ-^{32}P]UTP. *A*, HPLC tracings representative of $t = 0$ and 10-min incubation periods. *B*, time course of ^{32}P-species accumulation. The data represent the mean value of two experiments performed with duplicate samples that differed by less than 20% from each other.

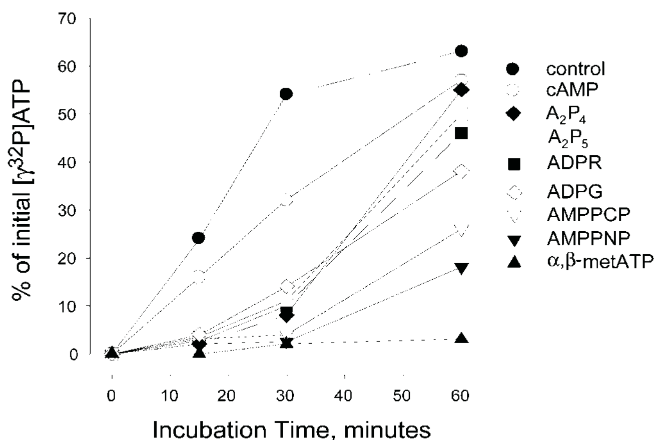

FIG. 10. **Effect of nucleotide analogues on the formation of $^{32}PP_i$.** Resting C6 cells were incubated for the times indicated in the presence of 100 μM indicated nucleotide and 0.1 μCi of [γ-^{32}P]ATP (specific activity >3000 Ci/mmol), and the resulting ^{32}PPi was quantified by HPLC. The results are expressed as the percentage of radioactivity accumulated as $^{32}PP_i$ relative to initial [γ-^{32}P]ATP. The data represent the mean value from two different experiments performed with duplicate samples that differed by less than 10% from each other.

ATP and UTP in the bulk medium likely reflects a fraction of the actual nucleotide mass that accumulates transiently in the vicinity of the P2Y receptors. Consistent with this notion, using an *in situ* assay for ATP whereby cell surface-bound luciferase acts as a bioluminescent sensor for ATP, Dubyak and colleagues (30) recently reported that ATP concentrations in the bulk medium of thrombin-stimulated platelets underestimate by at least 1 order of magnitude the ATP concentration near the cell surface.

"Constitutive" release of ATP may be physiologically relevant particularly in tissues such as human airways, which are covered by a thin layer (~1 μl/cm (Ref. 2)) of surface liquid (31). The $P2Y_2$ receptor of airways controls several components of mucociliary clearance by promoting Ca^{2+}-dependent Cl^- secretion, mucin secretion, and ciliary beating (31–35). Based on the observed rate of ATP release from resting 16HBE14o$^-$ cells (Table I), accumulation of ATP in the periciliar airway surface liquid would proceed at a rate of ~20 nM/min, and ATP accumulation would rapidly exceed EC_{50} values for $P2Y_2$ receptor activation if substantial metabolism of nucleotide did not occur. We speculate that basal release of ATP provides a mechanism of activation of the $P2Y_2$ receptor that controls physiological functions in "resting" airways.

We reported recently that nucleotide release by mechanically stimulated cells, which represented 0.5–10% of the intracellular pool (21, 23–25), was not associated with cell damage (assayed by LDH activity, ^{51}Cr release, or with the fluorescent probe calcein (21, 23–25)). Resting cells release one millionth of the total cellular ATP (23) per minute, a value far below the

TABLE II
Michaelis-Menten constants for P_i and PP_i formation during ATP hydrolysis on C6 cells

C6 cells were incubated at various times with increasing concentrations of ATP (1–2000 μM) in the presence of 0.1 μCi of [γ-^{32}P]ATP. The formation of $^{32}P_i$ and $^{32}PP_i$ was quantified by HPLC. The kinetic constants were deduced using Hanes-Woolf plots.

	K_m	V_{max}	V_{max}/K_m
	nmol ml^{-1}	*nmol ml^{-1} min^{-1}*	*min^{-1}*
P_i	86	0.754	0.009
PP_i	13	0.324	0.025

sensitivity of available assays for cell lysis. Thus, our data do not rule out the possibility that ongoing irreversible membrane damage of a very small number of cells may contribute to the observed rates of ATP release. However, increasing evidence suggests that release of cytosolic ATP is a cell-regulated process (4, 16, 22, 36, 37), which may involve the action of glibenclamide-sensitive K^+ channels (16), 5-nitro-2-(3phenylpropylamino)benzoic acid-sensitive Cl^- channels (22, 37), and/or phosphatidylinositol 1,4,5-trisphosphate kinase (36). It has been speculated that ATP release occurs via members of the ABC transporter family of proteins such as the cystic fibrosis transmembrane regulator (38, 39), but this notion was not sustained by a number of studies including experiments with cystic fibrosis transmembrane regulator-deficient cells in experiments where cystic fibrosis transmembrane regulator was overexpressed in cells where the $P2Y_2$ receptor acted as a biosensor for ATP/UTP release (22, 27, 37, 40). Although the mechanism whereby non-excitatory cells release nucleotides remains unclear, basal ATP release occurs at negligible rates compared with cellular ATP turnover and, thus, may provide a "purinergic tone" to non-excitatory tissues without compromising the energetic balance of the cells.

The present study also illustrates the complexity of the cellular pathways involved in the regulation of extracellular nucleotide levels. Our results indicate that an extracellular NDPK activity is responsible for the continuous and reversible interchange of γ-phosphate within endogenous nucleotides. This NDPK activity is so large that transfer of [γ-^{32}P]phosphate from ATP to both GTP and UTP was detected in all cell types prior to measurable accumulation of $^{32}PP_i$ or $^{32}P_i$. These data are consistent with our previous kinetic analysis of NDPK and nucleotidase activities on 1321N1 cells (29), which indicated that NDPK activity exceeded by a factor of 20 that of the ecto-nucleotidase (29). To our knowledge, this is the first observation of ecto-NDPK activity that plays an active role in the forward and reverse transfer of the terminal phosphate of endogenous ATP to other endogenous nucleotides, *e.g.* UDP. Our results are not only consistent with our previous observation of extracellular accumulation of UTP (22, 25) but also indicate that UDP, ADP, and GDP accumulate in amounts comparable to those of triphosphonucleotides. Recently, we have observed that ADP concentrations in the medium of resting C6, 16HBE14o$^-$, ECV-304, and 1321N1 cells were at least 5 times higher than ATP concentrations at steady state (41). Although the present study cannot rule out the possibility that ADP and other nucleoside diphosphates are co-released with NTPs, our previous observations with [^{3}H]adenine-labeled 1321N1 astrocytoma and human nasal epithelial cells indicated that accumulation of extracellular [^{3}H]ADP is secondary to [^{3}H]ATP release and hydrolysis (21, 24). Further, studies of [^{3}H]ADP metabolism on the cells used in this study showed gradual conversion to [^{3}H]AMP and [^{3}H]ADO without measurable formation of [^{3}H]ATP, indicating that synthesis of ATP via an ecto-adenylate kinase activity did not occur.[3] Additionally, absence of adenylate kinase activity during steady state measurements was supported by the fact that no formation of [^{32}P]ADP was detected following the addition of [^{32}P]ATP to resting cells (Figs. 3 and 4). The NDPK activity not only establishes a constant ratio between the triphosphate nucleotide signaling molecules, but also provides an extracellular ATP regenerating system under conditions where the high energy phosphate bond of ATP is selectively consumed, *e.g.* during phosphorylation of extracellular proteins (4).

Our data indicate two extracellular pathways for ATP hydrolysis at steady state. Accumulation of orthophosphate along with the higher levels of extracellular nucleoside diphosphates relative to triphosphates (as discussed above) suggests an ecto-nucleotidase activity with preference for NTPs over NDPs, *i.e.* CD39-L1. However, $^{32}PP_i$ is the major end product of [γ-^{32}P]ATP hydrolysis in C6 cells, and it accumulates significantly on ECV-304, 16HBE14o$^-$, and 1321N1 cells. Since the observed K_m value for PP_i formation on C6 cells was ~6 times smaller than K_m values for P_i formation (Table II), we speculate that the PP_i-generating activity (likely E-NPP) might be important for controlling the net NTP concentrations while the CD39-L1 activity determines the relative amounts of NTP *versus* NDP at steady state. A recent study suggested that PC-1 hydrolyzes ATP on C6 cells (42). Whether this E-PPN is responsible for the ATP/UTP pyrophosphatase activity described in the present study and whether additional mechanisms are involved in the formation of PPi remain to be determined.

The biological significance of extracellular ATP and UTP has been supported in recent years by direct demonstration of their release and by pharmacological studies of P2 receptors that are potently stimulated by these nucleotides. However, a subset of the cloned P2Y receptors, *i.e.* $P2Y_1$ and $P2Y_6$ receptors, and a yet to be cloned G_i/adenylyl cyclase-coupled P2Y receptor are specifically activated by nucleoside diphosphates. The concerted action of ecto-ATPases, which convert NTP to NDP, and ecto-NTP pyrophosphatases, which hydrolyze NTP to AMP bypassing the formation of NDP, offers a control mechanism for the autocrine activation of $P2Y_1$ and $P2Y_6$ receptors. Our data support the idea that nucleotide release, accumulation, and interconversion are integrated components of resting cells.

Acknowledgments—We are indebted to Catharina van Heusden and Todd Listwa for technical assistance and to Jose L. Boyer for helpful discussions.

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