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Identification of an Ecto-nucleoside Diphosphokinase and Its Contribution to Interconversion of P2 Receptor Agonists*

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The $P2Y_4$ receptor is selectively activated by UTP. Although addition of neither ATP nor UDP alone increased intracellular Ca^{2+} in 1321N1 human astrocytoma cells stably expressing the $P2Y_4$ receptor, combined addition of these nucleotides resulted in a slowly occurring elevation of Ca^{2+}. The possibility that the stimulatory effect of the combined nucleotides reflected formation of UTP by an extracellular transphosphorylating activity was investigated. Incubation of cells with [^{3}H]UDP or [^{3}H]ADP under conditions in which cellular release of ATP occurred or in the presence of added ATP resulted in rapid formation of the corresponding triphosphates. Transfer of the γ-phosphate from [γ-^{33}P]ATP to nucleoside diphosphates confirmed that the extracellular enzymatic activity was contributed by a nucleoside diphosphokinase. The majority of this activity was associated with the cell surface of 1321N1 cells, suggesting involvement of an ectoenzyme. Both ADP and UDP were effective substrates for transphosphorylation. Since ecto-nucleotidase(s) has been considered previously to be the primary enzyme(s) responsible for metabolism of extracellular nucleotides, the relative rates of hydrolysis of ATP, ADP, UTP, and UDP also were determined for 1321N1 cells. All four nucleotides were hydrolyzed with similar K_m and V_{max} values. Kinetic analyses of the ecto-nucleoside diphosphokinase and ecto-nucleotidase activities indicated that the rate of extracellular transphosphorylation exceeds that of nucleotide hydrolysis by up to 20-fold. Demonstration of the existence of a very active ecto-nucleoside diphosphokinase together with previous observations that stress-induced release of ATP occurs from most cell types indicates that transphosphorylation is physiologically important in the extracellular metabolism of adenine and uridine nucleotides. Since the P2Y receptor class of signaling proteins differs remarkably in their respective specificity for adenine and uridine nucleotides and di- and triphosphates, these results suggest that extracellular interconversion of adenine and uridine nucleotides plays a key role in defining activities in nucleotide-mediated signaling.

The importance of adenine nucleotides as extracellular signaling molecules is well established (1, 2). ATP and/or ADP are released in a regulated fashion from neurons, platelets, and other cells and interact with two major classes of cell surface receptors, the ligand-gated P2X receptors and the G protein-coupled P2Y receptors (3–5). These receptors, which are encoded by at least a dozen different genes, in turn promote an exceptionally broad range of functional responses. Although physiologically important release of uridine nucleotides is less well defined, the identification of at least three P2Y receptors that are selectively activated by low concentrations of UTP or UDP is consistent with an important extracellular signaling role for pyrimidines (5, 6).

Hydrolysis by ecto-nucleotidases provides a mechanism whereby the physiological effects of extracellular nucleotides are terminated (2, 7–9). Degradation of ATP and ADP also apparently serves as a major source of extracellular adenosine, which in turn activates A1, A2, and A3 adenosine receptors (10). The enzymatic species involved in hydrolysis of extracellular nucleotides have not been unambiguously defined, although certain ATP-diphosphohydrolases exhibiting kinetic properties consistent with those of physiologically relevant ecto-nucleotidases have been purified and/or cloned (11, 12). The possibility that other types of ectoenzymes contribute to the metabolism and/or interconversion of extracellular adenine and uridine nucleotides has not been considered extensively.

Nucleoside diphosphokinase (NDPK)[1] catalyzes the transphosphorylation of nucleoside diphosphates utilizing nucleoside triphosphates as the γ-phosphate donor (13). Intracellular NDPK fulfills a crucial role in maintaining the high energy phosphate bond in ATP as part of the citric acid chain. NDPK also has been proposed to play a major role in the cytosolic synthesis of nucleoside triphosphates in addition to ATP and in maintaining a relative balance in the concentrations of nucleoside triphosphates. Human *nm23* genes encode for nucleoside diphosphokinases (14, 15), and an inverse relationship exists between *nm23* expression and metastatic potential (16, 17).

In contrast to its well established significance in intermediary metabolism, the potential location and function of NDPK as an extracellular enzyme involved in the transfer of terminal phosphates between extracellular nucleotides has not been determined. Therefore, we have tested this possibility using 1321N1 human astrocytoma cells stably expressing the $P2Y_4$ receptor which we show is selectively activated by UTP. Extracellular conversion of UDP to UTP has been measured in the presence of ATP, and $P2Y_4$ receptor-promoted elevation of intracellular Ca^{2+} has been quantitated as a functional measure of this conversion. Accordingly, we have identified an ecto-NDPK activity associated with 1321N1 cells that, in the presence of a γ-phosphate donor, promotes formation of UTP or ATP from their corresponding diphosphate nucleotides. The activity of this enzyme exceeds that of the ecto-nucleotidase

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[1] The abbreviations used are: NDPK, nucleoside diphosphokinase; Ap_5A, P^1,P^5-di(adenosine-5′)-pentaphosphate; DMEM, Dulbecco's modified Eagle's medium; HPLC, high performance liquid chromatography.

activity by 20-fold. Thus, NDPK activity promotes active interchange of γ-phosphates between endogenous adenine and uridine nucleotides on the surface of 1321N1 cells, and this interconversion has significant implications in establishing selectivity of activation of P2Y receptors which differ markedly in their nucleoside diphosphate and triphosphate specificity.

MATERIALS AND METHODS

Cell Culture—Wild type 1321N1 human astrocytoma cells and 1321N1 cells infected with retrovirus harboring the $P2Y_4$ receptor sequence (provided by Dr. R. Nicholas and Dr. J. Schachter) were cultured in DMEM-high glucose (DMEM-H) medium supplemented with 5% fetal bovine serum and antibiotics as described (18). The cells were grown to confluence on 24-well plastic plates (except where indicated otherwise) for nucleotide metabolism studies or on 25-mm glass coverslips previously coated with 0.3 mg/ml vitrogen for calcium measurements.

Measurement of Intracellular Ca^{2+}—$P2Y_4$ receptor-expressing cells were incubated with 3 μM Fura-2/AM for 30 min at 37 °C. After the loading period, the cells were bathed in 0.4 ml of Ringer solution (130 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.3 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES, pH 7.4) and mounted in a microscope chamber. The fluorescence (>450 nm) of 30–50 cells was alternately determined at 340 and 380 nm excitation by a RatioMaster RM-D microscope fluorimetry system (Photon Technology Inc., Monmouth Junction, NJ) at room temperature. A Zeiss Axiovert 35 inverted microscope and Nikon UV-F 100×/1.30 glycerol immersion objective were used. After each experiment, the cells were lysed with 40 μM digitonin, and the background fluorescence was determined by quenching with 4 mM manganese. The background-corrected ratio values (340/380) were calibrated by using the formula originally proposed by Grynkiewicz *et al.* (19). R_{max}, R_{min}, and K_d values were determined by using 1 μM Fura-2 free acid and a series of Ca^{2+} buffers.

Metabolism of Nucleotides by 1321N1 Cells—The cells were washed three times with serum-free DMEM medium and bathed in 0.5 ml of DMEM-H/HEPES, pH 7.4. Incubations were initiated by the addition of drugs and terminated by transferring the medium to a tube containing 50 μl of 50 mM EDTA and subsequently boiled for 1 min. The samples were maintained at −20 °C prior to HPLC analysis.

Conversion of [^{3}H]NDP to [^{3}H]NTP—1321N1 cells were incubated in the presence of ATP and with various concentrations of ADP or UDP and 0.1–0.5 μCi of [^{3}H]ADP or [^{3}H]UDP to provide a range of specific radioactivities of the nucleotides. The conversion of [^{3}H]ADP to [^{3}H]ATP and [^{3}H]UDP to [^{3}H]UTP was determined by HPLC analysis.

Synthesis of [^{3}H]ADP and [^{3}H]UDP—[^{3}H]ADP and [^{3}H]UDP were obtained from their respective ^{3}H-labeled nucleotide triphosphates by a hexokinase-catalyzed reaction as described previously (20). Briefly, 20–50 μCi of either [^{3}H]ATP or [^{3}H]UTP (40–50 Ci/mmol) were incubated with 10 units/ml hexokinase for 30 min at 37 °C in 0.2 ml of DMEM-H/HEPES, pH 7.4. After incubations, the samples were boiled for 1 min to eliminate the hexokinase activity. Full conversion of [^{3}H]NTP to [^{3}H]NDP was confirmed by HPLC.

Transfer of the γ-Phosphate of [γ-^{33}P]ATP to UDP, GDP, CDP, and TDP—1321N1 cells were washed and incubated as above in 0.5 ml of DMEM-H/HEPES medium in the presence of 300 μM [γ-^{33}P]ATP and 100 μM of the various nucleoside diphosphates. The transfer of [γ-^{33}P]ATP was determined by HPLC analysis.

Determination of the Distribution of Extracellular NDPK Activity—Two sets of confluent 1321N1 cells grown on 24-well plates were washed three times and bathed in 0.5 ml of DMEM-H/HEPES. After 1 min at 37 °C the medium from one set of cells was collected, rapidly centrifuged to remove any detached cells, and transferred to a tube containing 0.5 μCi of [^{3}H]ADP (50 nmol) and ATP (150 nmol). [^{3}H]ADP and ATP also were added to the second set of cells, and the NDPK activity of the medium and cells was assayed for 1 min. Total cellular activity of NDPK was also determined. Cells from 4 wells cultured in a separate 24-well plate were trypsinized, washed twice, resuspended in 2 ml of 50 mM ice-cold Tris, pH 7.2, and sonicated. NDPK activity was assayed in a 1:30 dilution of the cell sonicate.

Measurement of Ecto-nucleotidase Activity—1321N1 cells were washed and incubated in 0.5 ml of DMEM-H/HEPES in the presence of 0.5 μCi and the indicated concentrations of [^{3}H]ADP, [^{3}H]UDP, [^{3}H]ATP, or [^{3}H]UTP. Since preliminary experiments indicated that 1321N1 cells avidly transport adenosine but not uridine, studies of [^{3}H]ADP and [^{3}H]ATP hydrolysis were carried out in the presence of the nucleoside transport inhibitor dipyridamole (100 μM). Dipyridamole did not affect the rate of hydrolysis of ATP or ADP by 1321N1 cells (data not shown).

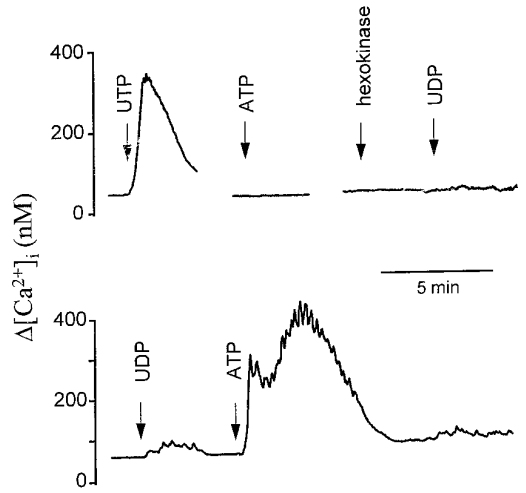

FIG. 1. **Effects of UDP and ATP on $[Ca^{2+}]_i$ in $P2Y_4$ receptor-expressing 1321N1 cells.** Changes in $[Ca^{2+}]_i$ were measured in Fura-2 preloaded cells in response to 1 μM UTP or 10 μM ATP or in response to 1 μM UDP added subsequent to the addition of 1 unit/ml hexokinase (*upper panel*) or the sequential addition of 1 μM UDP and 10 μM ATP (*bottom panel*). Stock solutions of UDP (1 mM) were made UTP-free by preincubation with 10 units/ml hexokinase followed by boiling for 1 min as previously reported (20). The results are representative of at least three different experiments.

HPLC Separation of Nucleotides—Nucleotides were separated and quantified by HPLC (Shimadzu) via a Hypersil-SAX column (Bodman, Aston, PA) using a 30-min linear gradient developed from 5 mM $NH_4H_2PO_4$, pH 2.8, to 0.75 M $NH_4H_2PO_4$, pH 3.7. Absorbance at 264 nm was monitored with an SPD-10A UV detector (Shimadzu), and radioactivity was determined on-line with a Flo-One Radiomatic β detector (Packard, Canberra, Australia) as described previously (21).

Reagents—All nucleoside triphosphates were purchased from Pharmacia (Uppsala, Sweden). Hexokinase, ouabain, tetramisol, Ap_5A, ADP, UDP, GDP, and CDP were from Boehringer Mannheim. Dipyridamole and TDP were from Sigma. Vitrogen was from Collagen Corp., Palo Alto, CA. Fura-2/AM and Ca^{2+} buffer were from Molecular Probes (Eugene, OR). [^{3}H]ATP (40 Ci/mmol), [^{3}H]UTP (50 Ci/mmol), and [γ-^{33}P]ATP (3000 Ci/mmol) were from Amersham.

RESULTS

Our initial evidence that extracellular NDPK activity is associated with 1321N1 human astrocytoma cells emanated from studies of the cloned $P2Y_4$ receptor stably expressed in these cells. We (18) and others (22, 23) originally reported that, although much less potent than UTP (EC_{50} = 0.4 μM), ATP (EC_{50} = 30 μM) nonetheless was an agonist in 15–20-min assays of inositol phosphate accumulation in $P2Y_4$ receptor-expressing 1321N1 cells. However, contrasting results were obtained in studies of Ca^{2+} mobilization by Nguyen *et al.* (24), and we have confirmed their results here. Addition of 1 μM UTP to Fura-2-loaded $P2Y_4$ receptor-expressing 1321N1 cells resulted in rapid mobilization of intracellular Ca^{2+} (Fig. 1, *upper tracing*). In contrast, neither 1 μM UDP (in the presence of hexokinase) nor 10 μM ATP elevated intracellular Ca^{2+} levels. However, a small response to UDP was observed if hexokinase was not added to the cells (Fig. 1, *lower tracing*). Moreover, combined addition of 1 μM UDP and 10 μM ATP resulted in a slowly occurring but sustained increase in intracellular Ca^{2+} (Fig. 1, *lower tracing*). Taken together these results suggested that the stimulatory effects at the $P2Y_4$ receptor of the combined presence of UDP and ATP occurred as a consequence of formation of UTP by an endogenous transphosphorylating activity. Because 1321N1 cells readily release ATP upon mechanical stimulation (21), the small effect of UDP in the absence of

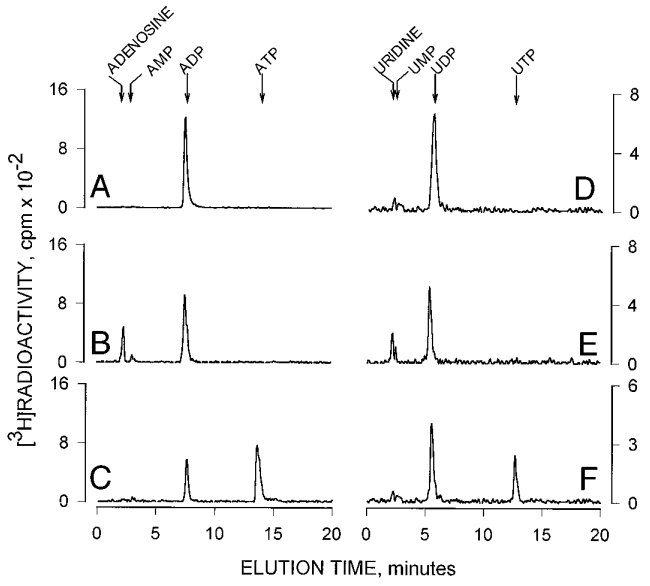

FIG. 2. **HPLC analysis of metabolic products of [^{3}H]ADP and [^{3}H]UDP.** P2Y$_4$ receptor-expressing 1321N1 cells were washed and preincubated for 1 h in 0.5 ml of serum-free medium and subsequently incubated in the presence of 1 μM [^{3}H]ADP (0.5 μCi) (*A*, *B*, and *C*) or in the presence of 1 μM [^{3}H]UDP (0.2 μCi) (*D*, *E*, and *F*). *Panels A* and *D* represent results from samples containing nonmetabolized ^{3}H-nucleotides (t = 0); *B* and *E* are results from samples incubated for 20 min, and *C* and *F* are from incubations carried out for 5 min in the presence of 10 μM ATP. ^{3}H-Labeled species were separated by HPLC as indicated under "Materials and Methods." The retention times for various standards of adenine and uridine nucleosides and nucleotides are indicated with *arrows*. The results are representative of at least four independent experiments performed in duplicate under similar conditions.

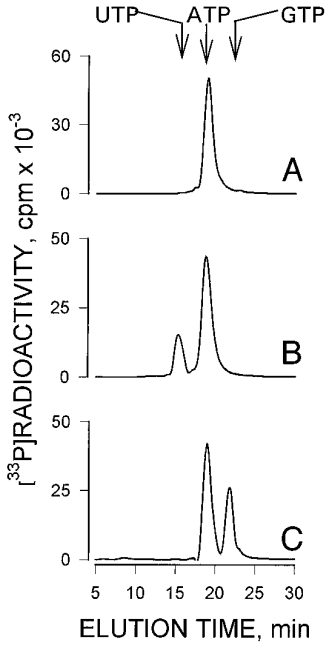

FIG. 3. **Transference of the γ-phosphate of ATP to UDP and GDP.** 1321N1 cells were incubated for 10 min with 100 μM [γ-^{33}P]ATP (0.5 μCi) alone (*A*) or in the presence of 100 μM UDP (*B*) or GDP (*C*). γ-^{33}P-Labeled species were separated by HPLC as detailed under "Materials and Methods." The results are representative of two experiments performed in duplicate.

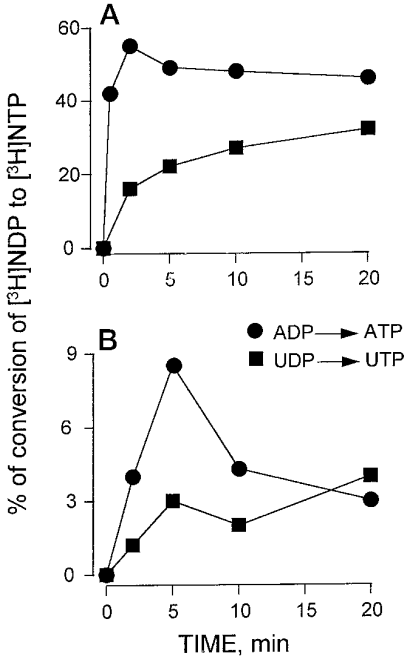

FIG. 4. **Time course for the conversion of [^{3}H]ADP to [^{3}H]ATP and [^{3}H]UDP to [^{3}H]UTP.** 1321N1 cells were washed, preincubated for 1 h in 0.5 ml of DMEM-H/HEPES, and further incubated for the indicated times in the presence of 10 μM (0.5 μCi) [^{3}H]ADP (●) or [^{3}H]UDP (■). The NDPK activity was assayed in the presence of exogenous 10 μM ATP (*A*) or after the release of endogenous ATP was promoted by a medium change (*B*). ^{3}H-Labeled species were quantified by HPLC as indicated under "Materials and Methods." The data represent the mean of two experiments performed with duplicate samples differing by less than 20%.

hexokinase might reflect formation of UTP from endogenous ATP.

To determine whether NDPK activity could be detected more directly, P2Y$_4$ receptor-expressing cells were incubated with either 1 μM [^{3}H]ADP or 1 μM [^{3}H]UDP in the absence or presence of 10 μM ATP, and the formation of [^{3}H]ATP or [^{3}H]UTP was assessed by HPLC. In the absence of added ATP, the radiolabeled nucleoside diphosphates were partially converted to the corresponding labeled nucleoside monophosphates, and no formation of ^{3}H-labeled nucleoside triphosphate was detected (Fig. 2). In contrast, in the presence of 10 μM ATP, [^{3}H]ADP and [^{3}H]UDP were converted to their respective nucleoside triphosphates with little evidence of formation of the monophosphate species.

Conversion of nucleoside diphosphates to triphosphates by transfer of γ-phosphate was confirmed in experiments carried out with [γ-^{33}P]ATP. Incubation of 1321N1 cells with [γ-^{33}P]ATP and UDP or GDP resulted in the rapid formation of [γ-^{33}P]UTP or [γ-^{33}P]GTP, respectively (Fig. 3). Thus, a highly active NDPK activity is associated with the extracellular environment of 1321N1 cells. This activity is not a result of overexpression of the P2Y$_4$ receptor in 1321N1 cells, since in the presence of 100 μM [^{3}H]ADP and 300 μM ATP similar formation of [^{3}H]ATP was observed with wild type cells (5.1 ± 0.7 nmol/10^6 cells/min), P2Y$_2$ receptor-expressing cells (4.4 ± 0.2 nmol/10^6 cells/min), and P2Y$_4$ receptor-expressing cells (2.7 ± 0.6 nmol/10^6 cells/min).

Time course experiments indicated that in the presence of unlabeled ATP, 10 μM [^{3}H]ADP was rapidly phosphorylated to [^{3}H]ATP, and a maximal conversion (approximately 60%) occurred within 2 min after drug addition to 1321N1 cells (Fig. 4*A*). Under the same conditions the rate of formation of [^{3}H]UTP was slower than was the formation of [^{3}H]ATP (Fig. 4*A*), and a maximal conversion of approximately 30% occurred within 20 min. We have reported previously that relatively large amounts of endogenous ATP are released from 1321N1 cells upon mechanical stimulation (21). Addition of [^{3}H]ADP or [^{3}H]UDP to mechanically stimulated 1321N1 cells also re-

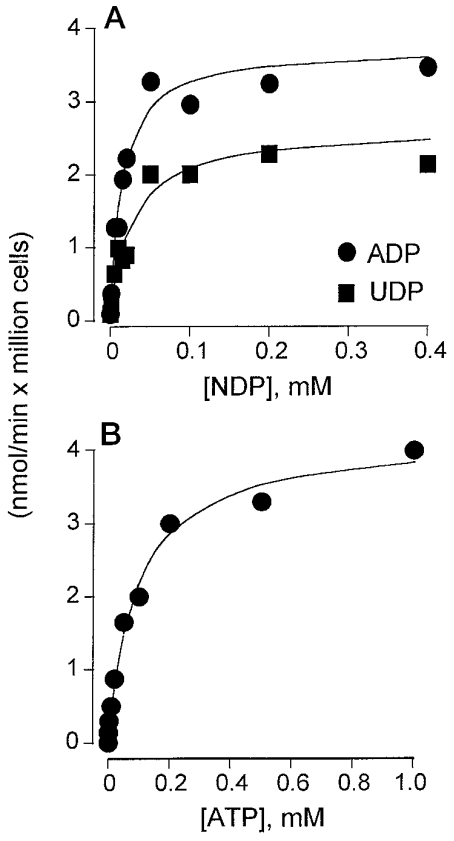

FIG. 5. **Substrate dependence for the conversion of ^{3}H-nucleoside diphosphates to ^{3}H-nucleoside triphosphates.** 1321N1 cells were incubated in the presence of 300 μM ATP and the indicated concentrations of [^{3}H]ADP (0.5 μCi) or [^{3}H]UDP (0.5 μCi) (*A*) or in the presence of the indicated concentrations of ATP and 100 μM [^{3}H]ADP (0.5 μCi) (*B*). The rates of formation of ^{3}H-nucleoside triphosphates determined by HPLC are expressed as nmol/min/million cells and were obtained from incubations in which less than 10% of the [^{3}H]diphosphate was converted to [^{3}H]triphosphate. The data are the mean value from a single experiment performed with duplicate samples differing by less than 20%. Similar results were obtained with at least three independent experiments performed under the same conditions.

sulted in rapid formation of [^{3}H]ATP or [^{3}H]UTP (Fig. 4*B*), and the extent of formation of ATP was greater than that of UTP. Observation of rapid conversion of extracellular ADP to ATP and of UDP to UTP by intact 1321N1 cells under conditions in which nucleotide released from cells serves as a γ-phosphate donor suggests that NDPK activity is important at pharmacologically relevant concentrations of nucleotides. For example, in the presence of endogenously released ATP, addition of 10 μM UDP (to 0.5 ml of medium bathing 0.3×10^6 cells) resulted in accumulation of approximately 0.3 μM UTP (Fig. 4*B*), a concentration that markedly activates the $P2Y_2$ (21) and $P2Y_4$ (18) receptors.

The concentration dependence for ADP and UDP for promotion of NDPK activity was determined in the presence of a near-saturating concentration (300 μM) of ATP. Assays were carried out under apparently initial rate conditions where <10% of the diphosphate was converted to the corresponding triphosphate. Conversion of [^{3}H]UDP to [^{3}H]UTP by 1321N1 cells occurred with $V_{max(app)}$ and $K_{m(app)}$ values that were approximately one-half and two-fold, respectively, the values obtained in analogous experiments examining the conversion of [^{3}H]ADP to [^{3}H]ATP (Fig. 5*A* and Table I). UDP inhibited the ATP-promoted formation of [^{3}H]ATP from [^{3}H]ADP and ADP inhibited the ATP-promoted formation of [^{3}H]UTP from [^{3}H]UDP (Table II). Furthermore, 50 μM UDP caused an approximately 3-fold shift to the right of the concentration effect curve for ADP but had no effect on the $V_{max(app)}$ for ADP (data not shown). The concentration dependence for ATP also was determined in the presence of a fixed concentration (100 μM) of [^{3}H]ADP (Fig. 5*B*). The observed $K_{m(app)}$ for ATP was 4–7-fold higher than the values determined for the nucleoside diphosphates (Table I).

TABLE I
Kinetic constants for the NDPK activity of 1321N1 cells

Concentration-response curves were generated under conditions that approximated first order rates of reaction, *i.e.* one substrate was maintained at a near maximally effective concentration whereas the concentration of the second nucleotide was varied as detailed in Fig. 5. Data were obtained from incubations resulting in less than 10% conversion of ^{3}H-nucleoside diphosphate to ^{3}H-nucleoside triphosphate. The data were fitted to a hyperbolic function, and the parameters were calculated utilizing a Sigma Plot software (Jandel). The results represent the mean ± S.D. from at least three different experiments performed in duplicate.

	K_m	V_{max}
	μM	*nmol/min/10^6 cells*
ATP	92.7 ± 8.1	4.2 ± 0.6
ADP	17.4 ± 3.8	3.7 ± 0.4
UDP	37.0 ± 6.7	2.3 ± 0.3

TABLE II
Substrate competition for NDPK activity

The conversion of 100 μM [^{3}H]ADP to [^{3}H]ATP was assayed in the absence or in the presence of 100 μM UDP, and the conversion of 100 μM [^{3}H]UDP to [^{3}H]UTP was assayed in the absence or in the presence of 100 μM ADP. Incubations were for 20 min. The results are expressed as nanomoles of [^{3}H]NTP formed per well (mean ± S.D., $n = 4$).

Addition	None	ADP	UDP
[^{3}H]ADP	32.5 ± 1.0		24.2 ± 0.7
[^{3}H]UDP	11.5 ± 0.6	5.3 ± 0.9	

EDTA (5 mM) completely inhibited the conversion of [^{3}H]ADP to [^{3}H]ATP whereas 5 mM EGTA had no effect (data not shown). These results suggest that the ecto-NDPK activity is Mg^{2+}-dependent. Ap_5A, a well characterized inhibitor of adenylate kinase (25), had no effect on the conversion of [^{3}H]ADP to [^{3}H]ATP, suggesting that adenylate kinase activity does not contribute in the conversion of diphosphates to triphosphates by 1321N1 cells.

The substrate selectivity of the NDPK activity was determined for both nucleoside triphosphates and nucleoside diphosphates (Table III). GTP and ATP were equally effective in promoting conversion of [^{3}H]ADP to [^{3}H]ATP, whereas a significantly slower rate of phosphorylation of [^{3}H]ADP was obtained with CTP (30–40% relative to ATP). GDP and ADP also were preferred acceptor substrates relative to CDP (Table III).

The localization of the extracellular NDPK activity also was determined. Approximately 70% of the total extracellularly measured NDPK activity was associated with the cell surface of 1321N1 cells (Fig. 6). An approximate doubling of medium NDPK activity occurred over a 60-min period after an extensive wash (rapid change of the medium three times) of 1321N1 cells (data not shown). Under no conditions did medium NDPK activity approximate that of cell surface NDPK activity. The total cellular NDPK activity (determined as described under "Materials and Methods") was approximately 10-fold higher than the total activity associated with the cell surface (data not shown).

The role of ecto-nucleotidases in the metabolism of extracellular adenine nucleotides has been widely studied in various tissues. In light of the ecto-NDPK activity found associated with 1321N1 cells, comparative experiments were carried out measuring the rates of hydrolysis of extracellular nucleoside tri- and diphosphates. Since essentially all analyses of ecto-

TABLE III
Substrate specificity for NDPK activity

Upper, confluent 1321N1 cells grown on 12-well plastic plates were incubated for 10 min in 0.5 ml of DMEM-H/HEPES with 100 μM [^{3}H]ADP and 300 μM of the indicated nucleoside triphosphates. Bottom, cells were incubated for 10 min with 100 μM GDP, UDP, or CDP and with 300 μM [γ-^{33}P]ATP or with 100 μM [^{3}H]ADP and 300 μM unlabeled ATP. Conversions of [^{33}P]ATP to other ^{33}P-nucleoside triphosphates and [^{3}H]ADP to [^{3}H]ATP were quantified by HPLC as described under "Materials and Methods." The results are expressed as percent of NDPK activity relative to ATP (upper) or ADP (bottom), and the numbers in parentheses indicate the net activity in nmol/well. The results represent the mean (±S.D.) from three experiments performed in duplicate.

Nucleotide	NDPK activity	
	%	*nmol*
ATP	100	(32)
GTP	103 ± 1	(33)
dATP	90 ± 2	(29)
UTP	77 ± 9	(28)
dGTP	72 ± 2	(23)
CTP	34 ± 1	(11)
ADP	100	(32)
GDP	109 ± 1	(35)
UDP	82 ± 2	(26)
TDP	56 ± 3	(18)
CDP	39 ± 6	(12)

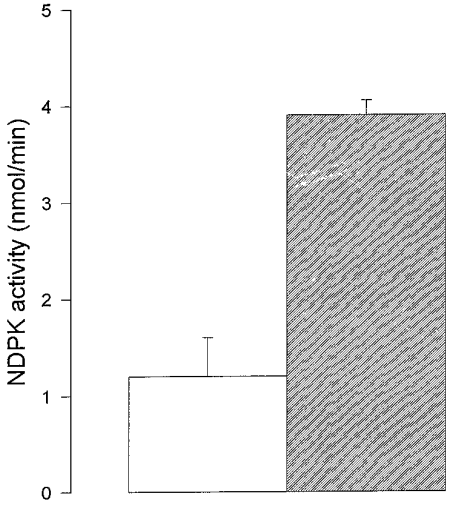

FIG. 6. **Distribution of extracellular NDPK activity.** The conversion of [^{3}H]ADP to [^{3}H]ATP was measured in medium bathing cells (*hatched bar*) or in cell-free medium (*open bar*) collected from surface cultures of 1321N1 cells as described under "Materials and Methods." The data (mean ± S.D., n = 3) are representative of results from two different experiments.

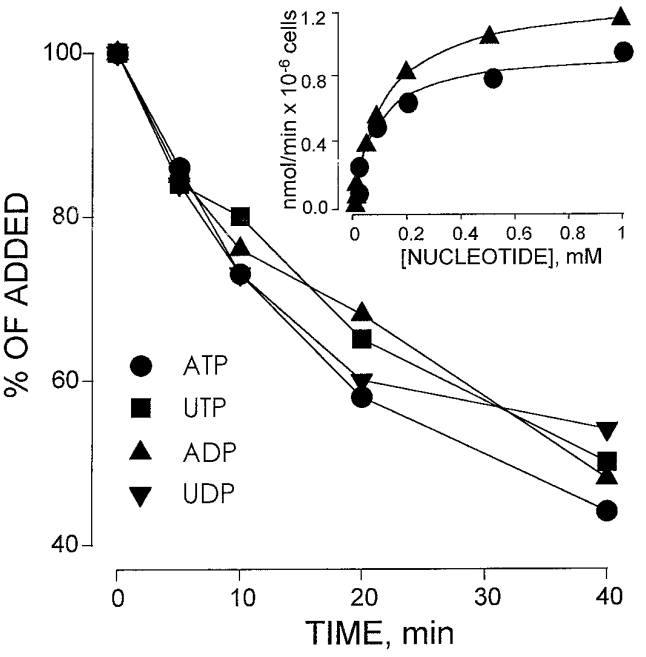

FIG. 7. **Hydrolysis of ^{3}H-nucleotides by 1321N1 cells.** Confluent 1321N1 cells grown on 24-well plastic plates were incubated in 0.5 ml of serum-free DMEM-H/HEPES in the presence of 1 μM (0.5 μCi) [^{3}H]ATP (●), [^{3}H]UTP (■), [^{3}H]ADP (▲), or [^{3}H]UDP (▼). Reactions were terminated at the times indicated, and the ^{3}H-labeled species were quantified by HPLC. *Inset*, the hydrolysis of [^{3}H]ATP (●) and [^{3}H]ADP (▲) was measured at different substrate concentrations, and values for the initial rates of hydrolysis were determined as detailed in legend of Fig. 5. The data represent the mean of at least two different experiments performed with duplicate samples which differed by less than 20%.

TABLE IV
Kinetic constants for the ecto-nucleotidase activity of 1321N1 cells

Concentration-response curves were generated from incubations in which less than 10% of ^{3}H-labeled substrate was converted to its hydrolysis products (as in Fig. 7, *inset*). The data were fit to a hyperbolic function, and values for constants were calculated utilizing a Sigma Plot software. K_m and V_{max} values are expressed in μM and nmol min^{-1} cells $\times 10^{-6}$, respectively. The results are the mean ± S.D. from three experiments performed in duplicate.

	K_m	V_{max}
	μM	*nmol/min/10^6 cells*
ATP	66 ± 13	0.8 ± 0.3
UTP	87 ± 11	0.6 ± 0.1
ADP	90 ± 17	0.9 ± 0.2
UDP	122 ± 34	1.1 ± 0.2

nucleotidases to date have focused on the hydrolysis of adenine nucleotides, we also determined the relative effectiveness of 1321N1 cells for hydrolysis of extracellular uridine nucleotides. Extracellular [^{3}H]ATP (1 μM) was hydrolyzed (Fig. 7) by 1321N1 cells with a half-time of approximately 25 min (in a volume of 500 μl on a 1-cm^2 well of a 12-well dish). The time courses of disappearance of UTP, ADP, and UDP were essentially superimposable with that observed for ATP (Fig. 7). The similarity of adenine and uridine nucleotide tri- and diphosphates as substrates for ecto-nucleotidase activity was confirmed in kinetic experiments. Thus, the observed K_m and V_{max} values for all four molecules were very similar (Table IV). This nucleotidase activity was unchanged in the presence of 2 mM ouabain (a P-type ATPase inhibitor), 5 mM tetramisol (an alkaline phosphate inhibitor), 10 mM NaN_3 (a mitochondrial ATPase inhibitor), or 10 mM NaF (a nonspecific inhibitor of phosphatase), but it was abolished by chelators of Ca^{2+}/Mg^{2+} (data not shown). The similarity of the kinetic constants calculated for nucleotidase activity against the four adenine and uridine nucleotides and the lack of inhibitory effects of several ATPase and phosphatase inhibitors suggest that the nucleotidase activity is an apyrase type, *i.e.* it is an ATP-diphosphohydrolase (8, 9, 26). The results obtained in kinetic analysis of the nucleotidase activity of 1321N1 cells also were consistent with the data illustrated in Fig. 2, *B* and *C*. That is, the NDPK activity associated with 1321N1 cells would be expected to exceed the ecto-nucleotidase activity by up to 20-fold in the presence of low micromolar concentrations of nucleoside diphosphate and an excess concentration of a γ-phosphate-donating nucleoside triphosphate.

DISCUSSION

This study identifies an ecto-NDPK activity associated with the extracellular surface of 1321N1 cells. Transphosphorylating activity apparently emanates from a single enzymatic species that utilizes either ADP or UDP similarly well as substrates for formation of the corresponding triphosphate.

Although formation of ATP from ADP can be effected by adenylate kinase (2ADP → ATP + AMP), no evidence for the occurrence of this reaction was detected. Moreover, the rate of transfer of [γ-^{33}P]phosphate from extracellular ATP to nucleoside diphosphates approximated the rates of conversion of ^{3}H-labeled nucleoside diphosphates to nucleoside triphosphates. Therefore, the majority of this conversion must occur due to an extracellular NDPK activity.

The observed NDPK activity is not trivial. Indeed, kinetic analyses of the NDPK and nucleotidase activities indicated that at relatively low diphosphate concentrations the NDPK activity exceeds that of the extracellular nucleotidase activity by up to 20-fold. This calculated surfeit of NDPK activity relative to nucleotidase activity was confirmed directly. That is, addition of [^{3}H]ADP or [^{3}H]UDP to the medium of 1321N1 cells in the presence of ATP resulted in relatively large formation of the corresponding radiolabeled triphosphates with little evidence of conversion of the diphosphates to monophosphates.

The extracellular NDPK activity is largely, but not exclusively, found as a surface membrane-associated ectoenzyme rather than as an enzymatic activity in the extracellular medium. The identity of this extracellular NDPK is not yet clear. Two putative tumor suppressor genes, *nm23-H1* and *mm23-H2*, have been cloned and shown to encode for approximately 17-kDa proteins (also called NDPK A and NDPK B) that exhibit NDPK activity (14, 15). It is unclear whether these enzymes account for all of the intracellular NDPK activity, and we have not yet addressed whether the ecto-NDPK activity is the same species as the previously molecularly identified forms of NDPK activity.

Extracellular NDPK has functional significance in 1321N1 cells. Although kinetic analyses required addition of known amounts of nucleoside triphosphate to the medium, substantial conversion of radiolabeled diphosphate to triphosphate occurred under conditions, *e.g.* a change of medium, in which ATP is released from 1321N1 cells (21). Thus, the released triphosphate readily serves as a γ-phosphate donor for exogenously added nucleoside diphosphates. Such interconversion of nucleotides also has a major influence on pharmacological effects observed with exogenously applied nucleotide agonists. Thus, as was illustrated in Fig. 1, UDP in the absence but not in the presence of hexokinase raised Ca^{2+} in $P2Y_4$ receptor-expressing 1321N1 cells. Coaddition of UDP with ATP resulted in a marked Ca^{2+} response. Thus, observations (22, 24) indicating that UDP was an agonist at the $P2Y_4$ receptor expressed in 1321N1 cells likely were due to conversion of UDP to UTP in the presence of released endogenous ATP during the 15–20-min measurements of inositol phosphate accumulation. Similarly, we have shown that the full agonist effects that we and others originally reported for UDP and ADP at the $P2Y_2$ receptor could be at least in part due to NDPK-promoted conversion of these diphosphates to their corresponding triphosphates (18, 21), which are potent full agonists at the $P2Y_2$ receptor. We also have reported that since hexokinase in the presence of glucose converts ATP and UTP to their corresponding diphosphates, this enzyme can be included in the medium in experiments designed to determine the pharmacological effects of nucleoside diphosphates (18, 20), a strategy that blocked the UDP effect on Ca^{2+} in $P2Y_4$ expressing cells (Fig. 1). Since 1321N1 cells represent the principle null cell line in which P2Y receptors have been expressed, the presence of a heretofore unrecognized ecto-NDPK activity on these cells may explain in large part the discrepant results reported by various laboratories in studies of the pharmacological selectivities of these receptors.

Not only is the ecto-NDPK similarly active against adenine and uridine nucleotide substrates, but our results indicate that the ecto-nucleotidase activity of 1321N1 cells also hydrolyzes uridine nucleotides at rates similar to that observed with adenine nucleotides. Thus, the extracellular hydrolytic machinery that has been widely studied and established as an important component of the extracellular adenine nucleotide signaling apparatus likely has a similarly important role in terminating the action of extracellular uridine nucleotides. Identification of an ecto-NDPK activity that is equally active against adenine and uridine nucleotides now adds a second level of complexity in understanding the physiological roles of adenine and uridine nucleotides as extracellular signaling molecules.

Although the data presented here have arisen entirely from studies of 1321N1 cells, the extracellular conversion of diphosphates to triphosphates is not restricted to this tumor cell line. For example, we have observed similar extracellular interconversion of nucleotides in studies of polarized human airway epithelial cells that maintain many of the phenotypical characteristics associated with these cells *in vivo* (20). Whether extracellular NDPK activity is expressed in a cell- or tissue-specific manner will need to be established. The occurrence of extracellular levels of enzyme activity similar to those observed with 1321N1 cells would have major physiological significance in regulating the extracellular signaling properties of adenine and uridine nucleotides.

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