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Direct Demonstration of Mechanically Induced Release of Cellular UTP and Its Implication for Uridine Nucleotide Receptor Activation*

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ATP is released from most cell types and functions as an extracellular signaling molecule through activation of members of the two large families of P2X and P2Y receptors. Although three mammalian P2Y receptors have been cloned that are selectively activated by uridine nucleotides, direct demonstration of the release of cellular UTP has not been reported. Pharmacological studies of the $P2Y_4$ receptor expressed in 1321N1 human astrocytoma cells indicated that this receptor is activated by UTP but not by ATP. Mechanical stimulation of 1321N1 cells also resulted in release of a molecule that markedly activated the expressed $P2Y_4$ receptor. This nucleotide was shown to be UTP by two means. First, high performance liquid chromatography analysis of the medium from $[^{33}P]H_3PO_4$-loaded 1321N1 cells illustrated that mechanical stimulation resulted in a large increase in a radioactive species that co-eluted with authentic UTP. This species was degraded by incubation with the nonspecific pyrophosphohydrolase apyrase or with hexokinase and was specifically lost by incubation with the UTP-specific enzyme UDP-glucose pyrophosphorylase. Second, a sensitive assay that quantitates UTP mass at low nanomolar concentrations was devised based on the nucleotide specificity of UDP-glucose pyrophosphorylase. Using this assay, mechanical stimulation of 1321N1 cells was shown to result in an increase of medium UTP levels from 2.6 to 36.4 pmol/10^6 cells within 2 min. This increase was paralleled by a similar augmentation of extracellular ATP levels. A calcein-based fluorescence quenching method was utilized to confirm that none of the increases in medium nucleotide levels could be accounted for by cell lysis. Taken together, these results directly demonstrate the mechanically induced release of UTP and illustrate the efficient coupling of this release to activation of $P2Y_4$ receptors.

Extracellular nucleotides regulate a myriad of physiological responses by interacting with two types of cell surface P2 receptors (1). The P2X receptors are ATP-activated cation channels, and seven members of this class of signaling proteins have been identified. In addition, four members of a G protein-coupled P2Y receptor family have been unambiguously identified. These include the $P2Y_1$ receptor, which is activated by adenine nucleotides (2, 3); the $P2Y_2$ receptor, which is activated equipotently by ATP and UTP (4); the $P2Y_4$ receptor, which is potently activated by UTP (5, 6); and the $P2Y_6$ receptor, which is selectively activated by UDP (7, 8).

The broad tissue distribution of these adenine and uridine nucleotide-activated P2 receptors supports the idea that endogenously released nucleotides act as important extracellular signaling molecules. Indeed, a large body of evidence exists demonstrating that ATP is released in a regulated fashion from most cell types and that its availability for target receptor activation is tightly controlled by cell surface nucleotidases (9, 10). In contrast, little is known about the regulated release of UTP from cells or tissues, although three out of the four cloned P2Y receptors recognize UTP or UDP as the most potent agonist. The lack of a sensitive assay for quantitation of UTP has constituted a major obstacle in examining its potential role as an extracellular signaling molecule.

In light of the wide distribution of uridine nucleotide-activated P2Y receptors, we have sought to determine whether cellular UTP is released in a regulated fashion. Mechanical stress, which is a known stimulator of the release of extracellular signaling molecules, has been applied to 1321N1 human astrocytoma cells, and the potential release of UTP has been measured by two independent methods. First, we have stably expressed the human $P2Y_4$ receptor in 1321N1 cells and shown it to be activated by UTP in a highly specific manner. Second, an enzyme-based assay has been developed that allows quantitation of the mass of UTP. We consequently have shown that 1321N1 cells release a soluble mediator that activates the expressed P2Y4 receptor and have identified this extracellular mediator as UTP. These results constitute the first direct demonstration of cellular release of UTP and provide biochemical approaches by which the cellular content and the release and extracellular presence of this molecule can be established in other tissues.

MATERIALS AND METHODS

Cell Culture—1321N1 Human astrocytoma cells were infected with retrovirus harboring DNA encoding the $P2Y_4$ receptor and selected with G-418 as described previously (8). A clonal cell line (P2Y4-1321N1 cells) exhibiting a high inositol phosphate response to UTP was isolated from $P2Y_4$ receptor-infected cell populations and was provided for this study by Drs. Rob Nicholas and Joel Schachter. Except where indicated otherwise, wild type and P2Y4-1321N1 cells were grown to near confluence on 24-well plastic plates (for inositol phosphate and nucleotide release measurements) or on 25-mm vitrogen-coated glass coverslips (for Ca^{2+} measurements) in DMEM-H[1] (high glucose) medium supplemented

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[1] The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; HPLC, high performance liquid chromatography; KBR, Krebs bicarbonate Ringer.

with 5% fetal bovine serum and antibiotics as described (8, 11).

Inositol Phosphate Accumulation—Cells were incubated overnight in 0.5 ml of inositol-free HEPES-buffered DMEM-H (pH 7.4) containing 0.5 μCi of *myo*-[^{3}H]inositol. At the end of the labeling period, 10 mM LiCl was added for 15 min followed by addition of the indicated drugs. Incubations were terminated by addition of 5% trichloroacetic acid. The [^{3}H]inositol phosphates were isolated on Dowex anion exchange columns and quantified as described previously (8).

Measurement of Intracellular Ca^{2+}—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.* (12). 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.

Calcein-based Assay of Cell Viability—Cells were grown on glass coverslips and then incubated with 1 μM calcein AM at 37 °C for 30 min. Following two gentle washes, the coverslips were mounted in a microscopic chamber, and the cells were maintained in 0.4 ml of Ringer solution under resting conditions for 30 min. Cellular calcein fluorescence from approximately 250 cells was monitored at >510 nm (excitation, 490 nm; bandwidth, 4 nm) using the equipment described for Ca^{2+} measurements. The external fluorescence was quenched by 1 mM $MnCl_2$. To confirm the sensitivity of this assay, single cells were disrupted by using a micropipette, and the entire cell population was lysed with 100 μM digitonin.

Incorporation of [^{33}P]P_i and Release of ^{33}P-Labeled Nucleotides—Confluent cells were incubated for 3 h in 0.5 ml of phosphate-free Eagle's medium containing 20 μCi of [^{33}P]orthophosphoric acid. These conditions resulted in maximal [^{33}P]nucleotide labeling. To assess intracellular [^{33}P]nucleotide content, cells were washed free of unincorporated radiolabel and lysed with 5% trichloroacetic acid. The trichloroacetic acid was extracted by ethyl ether, the aqueous solution was buffered to pH 7.5, and ^{33}P-labeled nucleoside triphosphates (NTPs) were identified by their susceptibility to treatment with 2 units/ml hexokinase and 25 mM glucose as described previously (13). In a typical experiment, 2–5 × 10^6 cpm were incorporated in the intracellular nucleotide pool. Extracellular ^{33}P-labeled nucleotides were assessed after washing the surface cultures and incubation in 0.5 ml of HEPES-buffered DMEM. Alternatively, the labeling medium was not changed to avoid mechanically stimulated ^{33}P-nucleotide release during a medium change (11). The extracellular medium (0.4 ml) was transferred to tubes at 0.5–5-min intervals, and samples were centrifuged to remove any detached cells. Trichloroacetic acid (5%) was added to the clarified medium, and the trichloroacetic acid then was extracted with ethyl ether. ^{33}P-Labeled species were resolved by HPLC as detailed below.

Identification and Quantification of UTP by a UDP-Glucose Pyrophosphorylase-catalyzed Reaction—UDP-glucose pyrophosphorylase catalyzes the uridinylation of glucose, *i.e.* glucose-1-P + UTP → UDP-glucose + PP_i, which precedes the incorporation of glucose into glycogen. The high selectivity of UDP-glucose pyrophosphorylase for UTP (ATP and other NTPs are not substrates; Ref. 14, Fig. 5, and data not shown) was utilized as a basis to identify [^{33}P]UTP in [^{33}P]H_3PO_4-loaded 1321N1 cells. ^{33}P-Species-containing samples (obtained as detailed above) were incubated for 30 min at 37 °C with 1 unit/ml of UDP-glucose pyrophosphorylase and 1 mM glucose-1-P. The resulting ^{33}P-species were separated by HPLC.

The UDP-glucose pyrophosphorylase catalyzed reaction also was utilized to develop a radiometric assay to quantitate the actual mass of UTP. In the presence of [^{14}C]glucose-1-P, the UDP-glucose pyrophosphorylase-catalyzed formation of [^{14}C]UDP-glucose was assayed as a function of UTP concentration. The reaction was made essentially irreversible by removing the co-product pyrophosphate with inorganic pyrophosphatase. Assays were for 45 min at 37 °C in 0.5 ml final volume of a standard Krebs-Ringer-bicarbonate HEPES buffer (pH 8.0) containing 0.5 units of UDP-glucose pyrophosphorylase, 0.5 units of inorganic pyrophosphatase, and 1 μM [^{14}C]glucose-1-P (0.15 μCi). Under these conditions, UTP concentration as low as 1 nM was accurately detected, and linearity was observed with up to 300 nM UTP. The formation of [^{14}C]UDP-glucose was determined by HPLC and quantified against a standard curve of UTP (as shown in Fig. 6*C*).

Nucleoside Diphosphokinase-catalyzed Phosphorylation of UDP—A 0.4-ml aliquot of medium bathing 1321N1 cells or cell extracts was incubated with 2 units/ml hexokinase and 25 mM glucose for 30 min at 37 °C to convert UTP to UDP as previously reported (13). The samples were subsequently boiled for 1 min to eliminate hexokinase activity and incubated for an additional 10 min at 37 °C with 0.5 units/ml nucleoside diphosphokinase in the presence of 10 nM (0.5 μCi) [γ-^{33}P]ATP. The conversion of [γ-^{33}P]ATP to [γ-^{33}P]UTP was determined by HPLC.

HPLC Separation of Nucleotides—Nucleotides were separated and quantified by HPLC (Shimadzu) via a Hypersil-SAX column (Bodman, Aston, PA) with a mobile phase developed (except where indicated otherwise) from 5 mM $NH_4H_2PO_4$, pH 2.8 (Buffer A), to 0.75 M $NH_4H_2PO_4$, pH 3.7 (Buffer B), in a 30-min linear gradient. Alternatively, samples were separated using an ion pairing system via a Dynamax-C18 column (Rainin) as described previously (11). Absorbance (λ = 264 nm) was monitored with a SPD-10A UV detector (Shimadzu), and radioactivity was determined on-line with a Flo-One Radiomatic β detector (Packard, Canberra, Australia).

Measurement of ATP by the Luciferin-Luciferase Method—ATP concentrations were determined by luciferin-luciferase bioluminescence (11). The luciferin-luciferase assay medium (300 μM luciferin, 5 μg/ml luciferase, 25 mM HEPES, pH 7.8, 6.25 mM $MgCl_2$, 0.63 mM EDTA, 75 μM dithiothreitol, 1 mg/ml bovine serum albumin) was added to samples via a LB953 AutoLumat luminometer (Berthold GmbH, Germany). To assess intracellular ATP content, cells were lysed with 5% trichloroacetic acid followed by ethyl ether extraction.

Mechanical Stimulation of Cells—A medium displacement method was used as a mechanical stimuli of 1321N1 cells. For the inositol phosphate assays and for nucleotide release measurements, half of the volume of the bathing medium was gently pipetted up and down twice with a micropipette. In addition to a similar procedure used in Ca^{2+} measurements, coverslips also were mounted in a perfusion chamber connected to a 1-ml syringe containing assay buffer. A gravitational flow was applied to the cells by increasing the height of the syringe. A flow rate of 70 μl/s resulted in a linear flow of 6 ± 1 mm/s at the entry of the cell chamber.

Reagents—ATP, UTP, and other nucleoside triphosphates were purchased from Pharmacia (Uppsala, Sweden). Hexokinase, nucleoside diphosphokinase, UDP-glucose pyrophosphorylase, and UDP were from Boehringer Mannheim. Glucose-1-P, UDP-glucose, inorganic pyrophosphatase, luciferin, and luciferase were purchased from Sigma. Fura-2/AM and calcein AM were from Molecular Probes (Eugene, OR). *myo*-[^{3}H]Inositol (17 Ci/mmol) was from ARC (St. Louis, MO). [^{14}C]Glucose-1-P (287 mCi/mmol), [γ-^{33}P]ATP (3000 Ci/mmol), and [^{33}P]H_3PO_4 (3000 Ci/mmol) were from Amersham Corp.

RESULTS

Pharmacological Characterization of the Human $P2Y_4$ Receptor—Our initial evidence for the release of UTP emanated from studies designed to examine the pharmacological action of ATP on the $P2Y_4$ receptor. The $P2Y_4$ receptor recently was cloned by Communi *et al.* (5) and by Nguyen *et al.* (6) and characterized as a uridine nucleotide-selective receptor. Results from these and other laboratories on the effects of ATP at this receptor have differed. Although ATP was reported to be a partial $P2Y_4$ receptor agonist in the stimulation of inositol phosphate formation by Communi *et al.* (5), no ATP-promoted Ca^{2+} responses were observed by Nguyen and co-workers (6). Other investigators have reported that ATP stimulates inositol lipid hydrolysis in $P2Y_4$ receptor-expressing 1321N1 cells (8, 15). To further examine this apparent discrepancy, both inositol phosphate and intracellular Ca^{2+} responses to ATP and other nucleosides were examined in P2Y4-1321N1 cells, a cell line stably expressing the human $P2Y_4$ receptor after retrovirus infection (8). Manipulation of the cells during the assay was minimized to reduce the likelihood of release of endogenous ATP (11). As previously reported, UTP was a potent agonist at $P2Y_4$ receptors with EC_{50} values for promotion of inositol phosphate formation and Ca^{2+} mobilization of 0.4 and 0.9 μM, respectively (Fig. 1). As in our previous study (8) as well as in

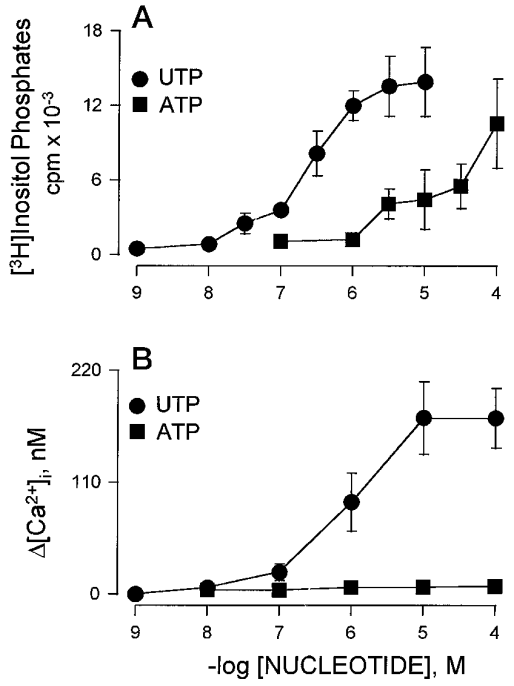

FIG. 1. **Concentration-effect curves for UTP- and ATP-stimulated [^{3}H]inositol phosphate formation and Ca^{2+} mobilization in P2Y4-1321N1 cells.** *A*, [^{3}H]inositol-labeled cells were preincubated with LiCl as detailed under "Materials and Methods." The cells were subsequently challenged with the indicated nucleotide for 20 min. The data represent the mean value ± S.E. from two experiments performed with triplicate samples. *B*, changes in $[Ca^{2+}]_i$ were measured immediately after the addition of agonist to Fura-2-loaded cells. The data are the mean value ± S.E. from at least three different experiments.

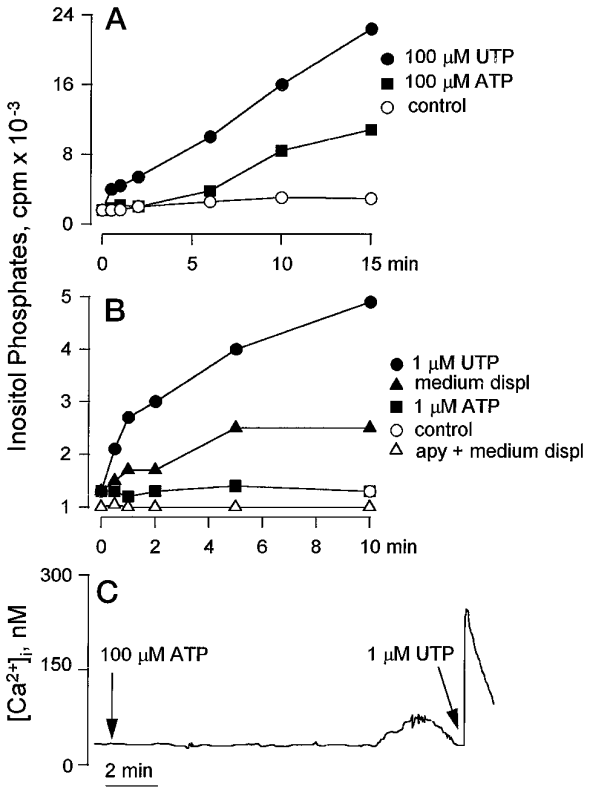

FIG. 2. **Time course for UTP- and ATP-stimulated [^{3}H]inositol phosphate formation and Ca^{2+} mobilization in P2Y4-1321N1 cells.** *A*, [^{3}H]inositol-labeled cells were preincubated with LiCl for 15 min and subsequently challenged with 100 μM of either ATP or UTP for the times indicated. *B*, the cells were challenged with 1 μM of the indicated nucleotide, or they were subjected to a medium displacement (*medium displ.*) in the absence or in the presence of 2 units/ml apyrase (*apy*) added simultaneously with LiCl. The inositol phosphate data are the mean from at least three experiments performed in triplicate. *Error bars* were omitted for clarity. *C*, $[Ca^{2+}]_i$ was continuously recorded during a ~15-min period following the addition of 100 μM ATP. The cells were further stimulated with 1 μM UTP. The Ca^{2+} tracing is representative of three experiments. Other details are as indicated under "Materials and Methods."

the report by Charlton *et al.* (15), ATP was a relatively weak (EC_{50} ~= 30 μM) agonist for promotion of inositol phosphate accumulation in P2Y4-1321N1 cells (Fig. 1*A*). In contrast to the stimulatory effect observed on inositol phosphate accumulation, the addition of ATP (1–100 μM) to P2Y4-1321N1 cells did not elicit rapid Ca^{2+} mobilization (Fig. 1*B*), a result that was consistent with that reported previously by Nguyen *et al.* (6).

Time course experiments revealed that UTP, at both 1 and 100 μM, promoted a rapid and sustained accumulation of inositol phosphates (Fig. 2, *A* and *B*). In marked contrast, the inositol phosphate response to ATP (100 μM) was preceded by a 5–10-min delay (Fig. 2*A*). As with the inositol phosphate response, a Ca^{2+} response to 100 μM ATP could be observed, but this also occurred only after a~ 10-min delay (Fig. 2*C*). No effect on inositol phosphate formation or Ca^{2+} mobilization (Fig. 2*B* and data not shown) was observed with 1 μM ATP over a 20-min incubation period.

We previously have reported that mechanical stimulation of 1321N1 cells results in release of ATP (11). ATP, added at concentrations (1 μM) in excess of those that we have quantitated in the medium after mechanical stimulation (11), had no effect on inositol phosphate accumulation in P2Y4-1321N1 cells (Fig. 2). Nevertheless, accumulation of inositol phosphates occurred after mechanical stimulation of the cells (Fig. 2*B*). Inclusion of apyrase in the medium prevented this mechanical stimulation-promoted increase in inositol phosphate levels. Apyrase also caused a 30% reduction of basal inositol phosphates that accumulated during preincubation with LiCl (control, 1457 ± 109 cpm; apyrase-treated, 1078 ± 121 cpm; n = 4; also see Fig. 2*B*).

Although no effect was observed in wild type 1321N1 cells (Fig. 3*A*), mechanical stimulation by manual medium displacement resulted in an immediate Ca^{2+} mobilization in P2Y4-1321N1 cells (Fig. 3*B*). Mechanical stimulation-promoted Ca^{2+} changes also were promoted by applying a flow pulse (70 μl/s during a 5-s period) (Fig. 3*B*). The Ca^{2+} response to mechanical stimulation but not to carbachol, also was completely abolished by apyrase (Fig. 3*B*) or by the UTP-specific enzyme UDP-glucose pyrophosphorylase in the presence of glucose-1-P (Fig. 3*C*). UTP (1 μM) promoted a rapid Ca^{2+} response in P2Y4-1321N1 cells (Fig. 2*C*), but no rapid effect was observed with 100 μM ATP (Fig. 2*C*), 100 μM CTP, 100 μM UDP, or 100 μM GTP (data not shown). Taken together, these results suggested to us that mechanical stimulation of 1321N1 cells results in release of UTP and that higher levels of accumulation of UTP may occur in the presence of exogenous ATP.

Release of UTP from 1321N1 Cells—To directly assess whether UTP was released into the medium of mechanically stimulated 1321N1 cells, we performed HPLC analysis of the radioactive species released from [^{33}P]H_3PO_4-loaded cells (Fig. 4). Two ^{33}P-species with retention times of approximately 12.3 and 13.0 min co-eluted with authentic UTP and ATP, respectively (the large peak eluting at 8–10 min represents nonincorporated [^{33}P]H_3PO_4). A 25-fold increase in the level of the 13.0-min ^{33}P-species was observed following medium displacement (Fig. 4*B*), a result consistent with our previous studies showing release of ATP from mechanically stimulated 1321N1 cells (11). A 17-fold increase occurred in the extracellular level of the [^{33}P]species that co-eluted with UTP following mechan-

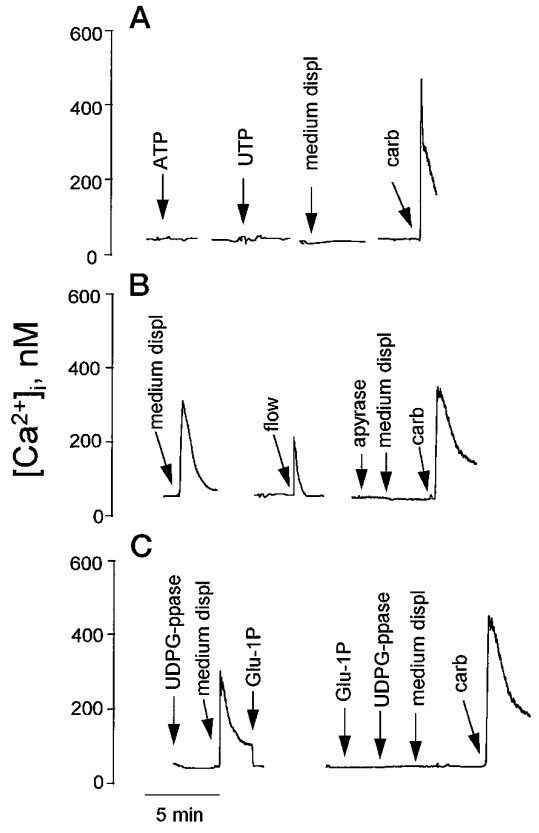

FIG. 3. **Evidence for a mechanically stimulated release of a P2Y4 receptor agonist that increases $[Ca^{2+}]_i$ in 1321N1 cells.** *A*, Fura-2-loaded wild type cells mounted on glass coverslips were bathed in 0.4 ml Ringer/HEPES buffer and challenged with 100 μM ATP or UTP, or they were subjected to a mechanical stimulation by pipetting the medium (0.2 ml) up and down twice. The Ca^{2+} responses to activation by carbachol (1 mM) of the natively expressed muscarinic cholinergic receptor of 1321N1 cells also were examined at the completion of the experiments shown in *panels A*, *B*, and *C*. *B*, P2Y4-1321N1 Fura-2-loaded cells were subjected to a medium displacement (*medium displ.*) as indicated above prior to (*left trace*) or after (*right trace*) the addition of 2 units/ml apyrase. The $[Ca^{2+}]_i$ response to a pulse of medium (flow rate 70 ml/s for 5 s) is also indicated (*flow*) in the middle tracing. *C*, the effect of 10 units/ml UDP-glucose pyrophosphorylase (*UDPG-ppase*) on mechanically stimulated $[Ca^{2+}]_i$ responses was investigated in P2Y4-1321N1 cells. *Glu-1-P* indicates the addition of 1 mM glucose-1-P to the cells.

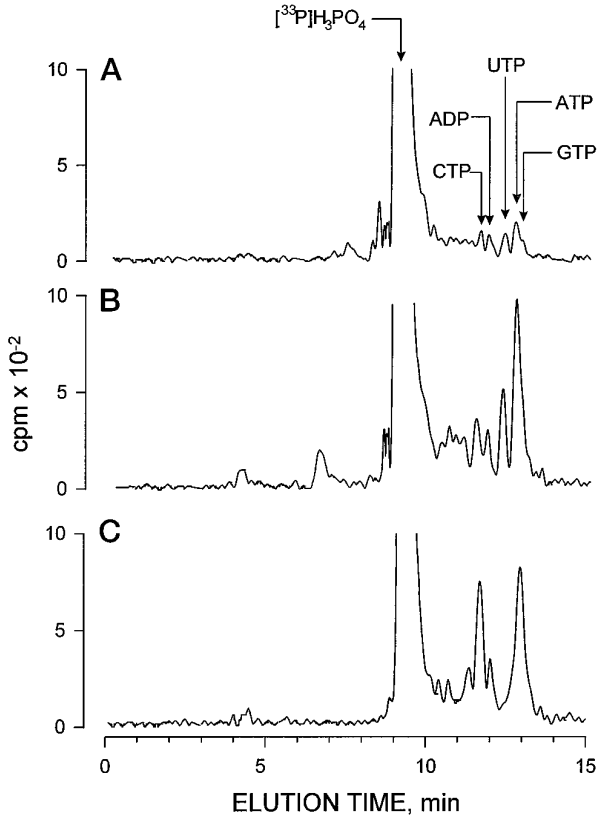

FIG. 4. **Release of ^{33}P-species from 1321N1 cells.** ^{33}P-Labeled P2Y4-1321N1 cells were incubated for 2 min undisturbed (*A*) or following a mechanical stimulation applied in the absence (*B*) or in the presence of 10 units/ml UDP-glucose pyrophosphorylase and 1 mM glucose-1-P (*C*). A 0.4-ml aliquot of the extracellular medium was analyzed by HPLC as indicated under "Materials and Methods." The results are representative of three experiments performed with duplicate samples. The retention times of various nucleotide standards are indicated.

ical stimulation (Fig. 4*B*). Incubation of samples derived from mechanically stimulated cells with hexokinase and glucose (to remove the terminal phosphate of nucleoside triphosphates (13)) or with apyrase resulted in loss of the species tentatively identified as [^{33}P]UTP and [^{33}P]ATP (data not shown). [^{33}P]UTP could be recovered after the hexokinase treatment by incubating the samples with [γ-^{33}P]ATP and nucleoside diphosphokinase (data not shown), which transfers the γ-phosphate from nucleoside triphosphates to nucleoside diphosphates. The identity of UTP was further confirmed using the UTP-selective (14) enzyme UDP-glucose pyrophosphorylase (Fig. 4*C*). Incubation with UDP-glucose pyrophosphorylase resulted in loss of the [^{33}P]species migrating as [^{33}P]UTP, but the [^{33}P]ATP species was not affected. Although all of the analyses described above were carried out on a SAX ion exchange column, similar results were obtained using a C18-column. The retention times of authentic UTP, but not of other nucleotide standards (ATP, GTP, CTP, dGTP, dCTP, TTP, dATP), matched the retention times in both columns of the UDP-glucose pyrophosphorylase-sensitive ^{33}P-species (Fig. 4 and data not shown). In addition to [^{33}P]ATP and [^{33}P]UTP, other minor apyrase-sensitive [^{33}P]species, which eluted as CTP, ADP, and GTP, were detected in the medium of mechanically stimulated cells (Fig. 4 and data not shown). Time course experiments indicated that the extracellular accumulation of [^{33}P]UTP and [^{33}P]ATP was maximal within 1–2 min after a mechanical stimulus and that an increase in extracellular [^{33}P]species was still observed after 10 min (Fig. 5). These results were not a consequence of the expression of the $P2Y_4$ receptor, since identical results were obtained with wild type 1321N1 cells (data not shown).

An assay was devised to assess directly for the first time the release of cellular UTP. This assay takes advantage of the selectivity of UDP-glucose pyrophosphorylase for UTP and utilizes [^{14}C]glucose-1-P as a cosubstrate (see "Materials and Methods"). A standard curve for UTP was established, and, consistent with previous reports on the selectivity of the enzyme (14), ATP, GTP, and CTP were not substrates (data not shown). Moreover, ATP, GTP, and CTP at concentrations as high as 100 μM had no effect (in the presence of 1 μM UTP) on the uridinylation reaction (data not shown). The results illustrated in Fig. 6 indicate that approximately 5.1 nM UTP (2.6 pmol/10^6 cells) could be readily detected in the medium (0.5 ml in an individual well of a 12-well plate; surface area = 3.15 cm^2) of resting wild type 1321N1 cells. A 14-fold increase in UTP concentration (72 nM; 36.4 pmol/10^6 cells; Fig. 6*B*) was observed in the extracellular medium 2 min after mechanical stimulation. This represented 1.7 ± 0.5% of the total amount of intracellular UTP. Time course experiments indicated that maximal UTP release occurred within 1–4 min after mechanical stimulation of the cells and that extracellular UTP decreased gradually thereafter (Fig. 7). A similar pattern was

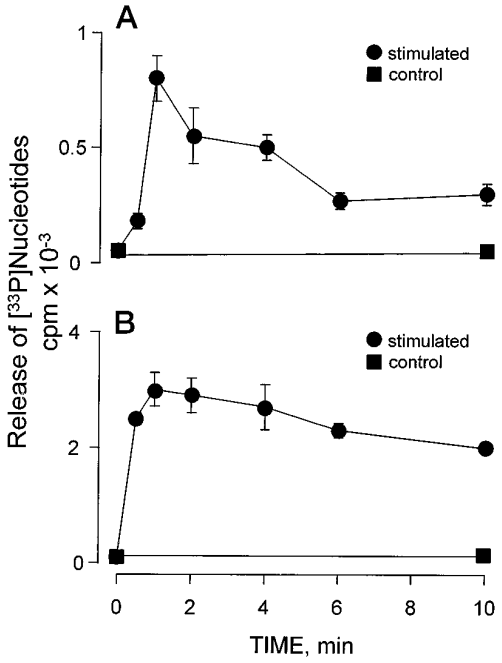

FIG. 5. **Time course of [^{33}P]ATP and [^{33}P]UTP release from 1321N1 cells.** The release of ^{33}P-labeled nucleotides from nonstimulated (*filled squares*) or mechanically stimulated (*filled circles*) P2Y4-1321N1 cells was quantified by HPLC as indicated under "Materials and Methods." The data are the mean value ± S.E. obtained from two experiments performed in duplicate.

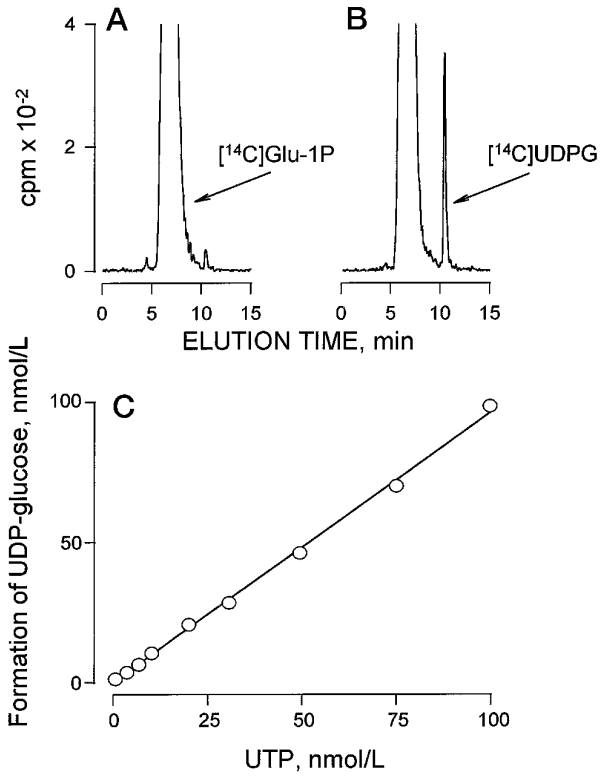

FIG. 6. **UDP-glucose pyrophosphorylase-based assay for measurement of extracellular UTP.** The incubation medium (0.5 ml) bathing resting (*A*) or mechanically stimulated (*B*) 1321N1 wild type cells (grown on 12-well plastic plates) was collected after a 2-min incubation period. Samples were assayed for UTP content by a 1-h incubation in the presence of 0.5 units/ml UDP-glucose pyrophosphorylase, 0.5 units/ml inorganic pyrophosphorylase, and 1 μM [^{14}C]glucose-1-P (0.15 μCi). The retention times of [^{14}C]glucose-1-P ([^{14}C]GLU-1-P) and [^{14}C]UDP glucose ([^{14}*C*]*UDPG*) are indicated with *arrows*. A calibration curve showing the UTP concentration-dependent formation of UDP-glucose is also illustrated (*C*). The data are representative of six experiments performed in duplicate. The resulting ^{14}C-species were resolved by HPLC via a SAX column as detailed under "Materials and Methods" except that a 15-min gradient from 0 to 50% buffer B was employed.

observed for ATP release using a luciferin-luciferase assay (Fig. 7). ATP measurements by the luciferin-luciferase indicated that up to 104 ± 12 pmol/10^6 cells was released by mechanical stimulation (Fig. 7), which represents 1.3 ± 0.2% of the total intracellular ATP content. No difference in the extent of UTP or ATP content or release was observed between wild type cells and P2Y4-1321N1 cells (data not shown). Also, the addition of 100 μM ATP to the medium of resting wild type or P2Y4-1321N1 cells for 10 min resulted in a 3–5-fold increase in extracellular UTP concentration (data not shown).

We previously have shown that mechanically stimulated release of ATP from 1321N1 cells was not accompanied by detectable release of lactate dehydrogenase (11). To further examine the possibility that cell lysis led to misleading results during mechanical stimulation, wild type 1321N1 cells were loaded with calcein, a Mn^{2+}-sensitive fluorescent dye, and changes in fluorescence were examined in the presence of 1 mM extracellular $MnCl_2$. Mechanical stimulation caused no change in the calcein-fluorescence signal (Fig. 8). In contrast, the signal obtained from impaling a single cell in a population of 250 cells was approximately 0.4% of the total signal and was approximately 4 times background. Identical results were obtained with P2Y4-1321N1 cells. Thus, cell lysis could be observed with a sensitivity of at least 0.4%, and these results indicate that the measurable increases in the release of UTP triggered by mechanical stimulation of the cells are not a consequence of cell damage.

DISCUSSION

Two major conclusions follow from this work. First, by developing a sensitive assay that quantitates trace amounts of UTP (<1 nM UTP), we could accurately measure the cell content of UTP with a limited number of cells (*e.g.* $<10^4$ to 10^5 cells). Moreover, this methodology provides direct quantification of release of endogenous UTP into the extracellular medium bathing cells. Second, this work not only demonstrates for the first time that mechanical stimulation of cells results in nonlytic release of UTP, but it also illustrates that releasable UTP efficiently effects an autocrine stimulation of $P2Y_4$ receptors. Thus, UTP release is placed in a physiologically relevant context.

The original report by Burnstock (16) of release of ATP during nerve transmission has been followed by demonstration of nonlytic mechanically promoted ATP release from essentially all tissues. For example, changes in flow rates induce the release of ATP from freshly harvested aortic endothelial cells (17), and shear forces trigger ATP release by cultured mouse fibroblasts (18). ATP release by mechanical stimulation also has been described for rat basophilic cells (19), hepatocytes (20), T84 colonic cells (21), and Calu-3 lung adenocarcinoma cells (21), and we recently have reported ATP release following mechanical stimulation of 1321N1 human astrocytoma cells (11), NIH 3T3 cells (22), and primary cultures of human nasal epithelial cells (22).

Unlike the well documented autocrine/paracrine function of extracellular adenine nucleotides, the potential existence and role of uridine nucleotide release have not been established. A major obstacle in assessing the significance of extracellular UTP has been the lack of a reliable method for accurately detecting the mass of UTP within the low nanomolar range. Despite the absence of direct evidence for UTP release, three G protein-linked mammalian P2Y receptors ($P2Y_2$, $P2Y_4$, and

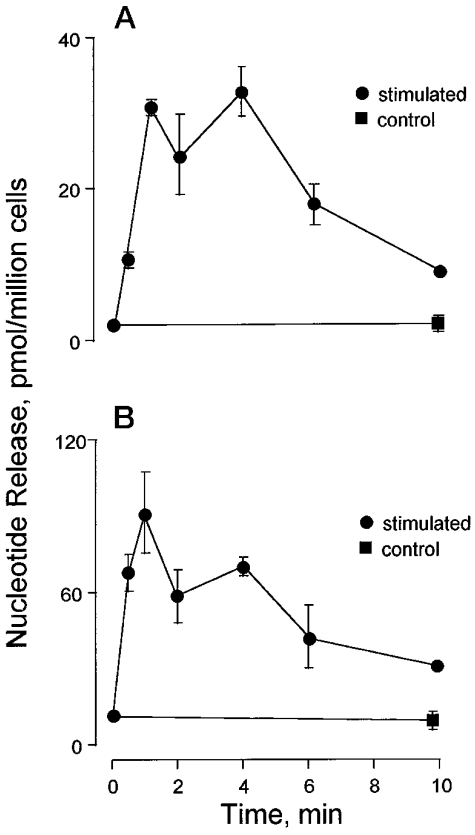

FIG. 7. **Quantification of UTP and ATP released from 1321N1 cells.** Wild type 1321N1 cells were grown on 12-well plastic plates and preincubated in KBR/HEPES buffer. The cells were mechanically stimulated and incubated for the indicated times, and the extracellular UTP (*A*) and ATP (*B*) were assayed as detailed in the legend to Fig. 6. The data represent the mean value ± S.E. from three independent experiments performed with triplicate samples.

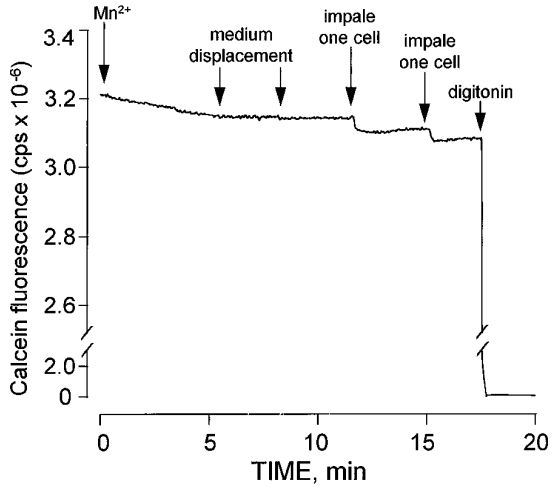

FIG. 8. **Assessment of cell viability by a calcein fluorescence-based assay.** Wild type 1321N1 cells were loaded with calcein, and changes in fluorescence were continuously recorded as detailed under "Materials and Methods." After the addition of 1 mM $MnCl_2$ (Mn^{2+}), the cells were mechanically stimulated twice (*medium displ.*), and then two individual cells were consecutively impaled. Digitonin (100 μM) was added at the end of the experiment. The *trace* is representative of a field with 250 cells, and similar results were obtained with four experiments with wild type 1321N1 cells and four experiments with P2Y4-1321N1 cells. The results are in an arbitrary fluorescence scale as counts/s (cps).

$P2Y_6$) have been identified that recognize UTP or UDP as the most potent and/or selective agonist. Thus, whereas the discovery of three different uridine nucleotide-activated receptors has suggested that UTP is an extracellular signaling molecule, the present results demonstrating nonlytic release of UTP in pharmacologically effective concentrations directly support this hypothesis.

An enzymatic assay for UTP content in tissues has been reported (23). However, this assay, which is based on the NADH:NAD^+ ratio resulting from UDP-glucose dehydrogenase-catalyzed oxidation of UDP-glucose, is not sensitive for quantitation of UTP below the micromolar range (23). Similarly, Enomoto *et al.* (24), utilizing UV light detection, have described a species that co-eluted with UTP on an HPLC system and that was present in a concentrate of the extracellular medium of mammary tumor epithelial cells. However, UTP was not unambiguously identified in this study, and extracellular UTP could have resulted from cells that were damaged (up to 50%) as a result of dislodging during sample collection.

UTP potentially can be detected in biological samples by utilizing radioactive precursors to radiolabel the intracellular uridine nucleotide pool (25). The disadvantage of such an approach is that the specific radioactivity of UTP cannot be determined and, therefore, no quantitation of mass can be made. Aspartate is utilized during *de novo* synthesis of pyrimidines (25). However, incorporation of this nonessential amino acid is difficult to achieve with cultured human cell lines. Alternatively, [^{3}H]Uridine potentially can be incorporated into the pyrimidine salvage cycle (25), provided that active RNA synthesis is occurring and an active nucleoside transporter is available in the target cells. Indeed, qualitative evidence for UTP release from intact cells recently was reported by Saiag and co-workers (26), who demonstrated that an increase in the flow rate of perfused [^{3}H]uridine-labeled endothelial cells resulted in the release of a species tentatively identified as [^{3}H]UTP.

Since neither [^{3}H]uridine, [^{3}H]uracil, [^{3}H]orotate, nor [^{3}H]aspartic acid was significantly incorporated into 1321N1 cells under a variety of conditions (data not shown), [^{33}P]H_3PO_4 was used to label the entire intracellular nucleotide pool. Mechanically induced release of [^{33}P]UTP from ^{32}P-labeled 1321N1 cells was subsequently demonstrated. This observation led to the development of a methodology that provides for the first time a means for quantitation of the mass of UTP at low nanomolar levels and has allowed an accurate determination of the extracellular concentration of released UTP.

The concentration of UTP (up to 70 nM) detected in the extracellular medium after mechanical stimulation was somewhat lower than expected from receptor response studies; *i.e.* mechanically stimulated P2Y4-1321N1 cells exhibited elevated Ca^{2+} levels comparable with the levels observed with 0.3–1 μM UTP. This difference could not be explained by rapid degradation of UTP, since the half-life of [^{3}H]UTP added to 0.5 ml of medium bathing 1321N1 cells (at 0.1 μM) is 10–15 min (Ref. 27 and data not shown). The simplest explanation is that transient accumulation of released nucleotides at the cell surface can differ significantly from the accumulation measured in the bulk medium. A highly efficient coupling of mechanically released UTP with $P2Y_4$ receptors also could be promoted by undefined cell surface structures, although this has not been proven.

Our results suggest that the stimulatory effect of ATP observed in P2Y4-1321N1 cells occurs due to an indirect action of this nucleotide. UTP detected in the medium in the absence of mechanical stimulation probably represents a balance of tonic release and metabolism of nucleotide(s) and provides a potential mechanism for the effects of ATP observed on P2Y4-1321N1 cells. We previously have characterized a nonspecific ectonucleotidase activity in 1321N1 cells that hydrolyzes both ATP and UTP with similar K_m and V_{max} values (27). We also

have identified a very active nucleoside diphosphokinase on the surface of 1321N1 cells that, in the presence of excess ATP, phosphorylates UDP to UTP (27). Thus, the addition of ATP to the medium of 1321N1 cells can result in increased accumulation of endogenously released UTP by two means. First, ATP can compete with endogenously released UTP for hydrolysis by ectonucleotidases. Second, ATP can serve as a co-substrate with UDP for nucleoside diphosphokinase-promoted formation of UTP. The increased UTP concentration found in the presence of exogenous ATP supports this hypothesis. A corollary to the latter observation is that UDP, which we have shown not to be a $P2Y_4$ receptor agonist (8), can indirectly activate this receptor as a consequence of nucleoside diphosphokinase-promoted formation of UTP from ATP present in the medium (27).

The mechanism whereby UTP and ATP are released from mechanically stimulated cells is unclear. Schwiebert and coworkers (28) have proposed that the cystic fibrosis transmembrane conductance regulator (CFTR) acts as an ATP channel. However, CFTR is not expressed in wild type 1321N1 cells, and heterologous expression of CFTR in 1321N1 cells resulted in no differences in mechanically stimulated ATP release (22). It also will be important to establish whether release of ATP and UTP is coordinately or independently regulated. The amount of ATP released from 1321N1 cells exceeded that of UTP, but this may be a cell-specific phenomenon.

Whether mechanical stimulation-promoted release of UTP from human astrocytoma cells is reflective of a function of glial cells *in vivo* is not known. However, the recent description of a uridine nucleotide-selective receptor on C6–2B glioma cells (29), an astrocyte-like cell line, and of uridine nucleotide receptors on primary cultures of astrocytes (30, 31) open the possibility of a specific target for UTP in glial cells. Astrocytes are known to be active elements in normal brain function and development and respond to various forms of injury with increased cell proliferation and hypertrophy (32). Gliotic-like responses can be induced by extracellular nucleotides, which are known to have trophic effects on astrocytes via activation of various P2 receptors (32, 33). It will be important to determine whether mechanically stimulated release of UTP and ATP occurs from astrocytes *in vivo* and whether such a release from 1321N1 human astrocytoma cells is reflective of an autocrine signaling function of these molecules in astrocytes following trauma, stroke, or seizure.

In summary, we have developed an assay for detection of UTP in subnanomolar concentrations, and we have shown that mechanically stimulated 1321N1 human astrocytoma cells release UTP into the extracellular medium in the absence of detectable cell lysis. The release of UTP is efficiently coupled to stimulation of heterologously expressed $P2Y_4$ receptors. To our knowledge, this represents the first report of a physiologically relevant release of UTP from intact cells. Considering the existence of at least three receptors that are specifically activated by uridine nucleotides, this release likely presages a general occurrence of this phenomenon. The methodology developed here should be valuable in addressing this hypothesis.

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