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 P2Y4 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 P2Y4 receptor. This nucleotide was shown to be UTP by two means. First, high performance liquid chromatography analysis of the medium from [32P]HPO42−-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/10⁶ 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 P2Y4 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 P2Y1 receptor, which is activated by adenosine nucleotides (2, 3); the P2Y2 receptor, which is activated equipotently by ATP and UTP (4); the P2Y4 receptor, which is potently activated by UTP (5, 6); and the P2Y6 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 extracellular UTP has been measured by two independent methods. First, we have stably expressed the human P2Y4 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 P2Y4 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 P2Y4 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 nitroen-coated glass coverslips (for Ca²⁺ measurements) in DMEM-HCO₃ (high glucose) medium supplemented with 10% FCS.

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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-[3H]inositol. At the end of the labeling period, 10 μl LiCl was added for 15 min followed by addition of the indicated drugs. Incubations were terminated by addition of 150 μl of trichloroacetic acid followed by ethyl ether extraction. Partial P2Y4 receptor agonist in the stimulation of inositol phosphate formation 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 P2Y4 receptor agonist in the stimulation of inositol phosphate formation by Communi et al. (5), no ATP-promoted Ca2+ responses were observed by Nguyen and co-workers (6). Other investigators have reported that ATP stimulates inositol lipid hydrolysis in P2Y4 receptor-expressing 1321N1 cells (8, 15). To further examine this apparent discrepancy, both inositol phosphate and intracellular Ca2+ responses to ATP and other nucleosides were examined in P2Y4-1321N1 cells, a cell line stably expressing the human P2Y4 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, ATP was a potent agonist at P2Y4 receptors with EC50 values for promotion of inositol phosphate formation and Ca2+ mobilization of 0.4 and 9 μM, respectively (Fig. 1). As in our previous study (8) as well as in

**UTP Release**

Nucleoside Diphosphokinase-catalyzed Phosphorylation of UTP—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-32P]ATP. The conversion of [γ-32P]ATP to [γ-32P]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 NH4H2PO4, pH 2.8 (Buffer A), to 0.75 M NH4H2PO4, pH 3.7 (Buffer B), in a 30-min linear gradient. Alternatively, samples were separated using an ion pairing system via a Dymax 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 Radiometric 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 7.5, 6.25 mM MgCl2, 0.63 mM EDTA, 75 mM NaCl, 5 mM glucose-1-P, 1 mM glucose-6-P, 10 mM HEPES, pH 7.4) 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 Ca2+ measurements, coverslips were also 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 calcine AM were from Molecular Probes (Eugene, OR). myo-[3H]inositol (17 Ci/mmol) was from ARC (St. Louis, MO). [14C]Glucose-1-P (287 mCi/mmol), [γ-32P]ATP (3000 Ci/mmol), and [32P]orthophosphoric acid were from Amersham Corp.

**RESULTS**

**Pharmacological Characterization of the Human P2Y4 Receptor**—Our initial evidence for the release of UTP emanated from studies designed to examine the pharmacological action of ATP on the P2Y4 receptor. The P2Y4 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 P2Y4 receptor agonist in the stimulation of inositol phosphate formation by Communi et al. (5), no ATP-promoted Ca2+ responses were observed by Nguyen and co-workers (6). Other investigators have reported that ATP stimulates inositol lipid hydrolysis in P2Y4 receptor-expressing 1321N1 cells (8, 15). To further examine this apparent discrepancy, both inositol phosphate and intracellular Ca2+ responses to ATP and other nucleosides were examined in P2Y4-1321N1 cells, a cell line stably expressing the human P2Y4 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, ATP was a potent agonist at P2Y4 receptors with EC50 values for promotion of inositol phosphate formation and Ca2+ mobilization of 0.4 and 9 μM, respectively (Fig. 1). As in our previous study (8) as well as in
the report by Charlton et al. (15), ATP was a relatively weak (EC\textsubscript{50} \approx 30 \mu M) agonist for promotion of inositol phosphate accumulation in P2Y4-1321N1 cells (Fig. 1A). In contrast to the stimulatory effect observed on inositol phosphate accumulation, the addition of ATP (1–100\mu M) to P2Y4-1321N1 cells did not elicit rapid \( \text{Ca}^{2+} \) mobilization (Fig. 1B), 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 \mu M, promoted a rapid and sustained accumulation of inositol phosphates (Fig. 2, A and B). In marked contrast, the inositol phosphate response to ATP (100 \mu M) was preceded by a 5–10-min delay (Fig. 2A). As with the inositol phosphate response, a \( \text{Ca}^{2+} \) response to 100 \mu M ATP could be observed, but this also occurred only after a ~10-min delay (Fig. 2C). No effect on inositol phosphate formation or \( \text{Ca}^{2+} \) mobilization (Fig. 2B and data not shown) was observed with 1 \mu M 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 \mu M) 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. 2B). 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. 2B).

Although no effect was observed in wild type 1321N1 cells (Fig. 3A), mechanical stimulation by manual medium displacement resulted in an immediate \( \text{Ca}^{2+} \) mobilization in P2Y4-1321N1 cells (Fig. 3B). Mechanical stimulation-promoted \( \text{Ca}^{2+} \) changes also were promoted by applying a flow pulse (70 \mu l/s during a 5-s period) (Fig. 3B). The \( \text{Ca}^{2+} \) response to mechanical stimulation but not to carbachol, also was completely abolished by apyrase (Fig. 3B) or by the UTP-specific enzyme UDP-glucose pyrophosphorylase in the presence of glucose-1-P (Fig. 3C). UTP (1 \mu M) promoted a rapid \( \text{Ca}^{2+} \) response in P2Y4-1321N1 cells (Fig. 2C), but no rapid effect was observed with 100 \mu M ATP (Fig. 2C), 100 \mu M CTP, 100 \mu M UDP, or 100 \mu M 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 \( ^{32} \text{P} \)H\textsubscript{2}PO\textsubscript{4}\textsuperscript{-}-labeled cells (Fig. 4). Two \( ^{32} \text{P} \)-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 \( ^{32} \text{P} \)H\textsubscript{2}PO\textsubscript{4}\textsuperscript{-}). A 25-fold increase in the level of the 13.0-min \( ^{32} \text{P} \)-species was observed following medium displacement (Fig. 4B), 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 \( ^{32} \text{P} \)-species that co-eluted with UTP following mechan-
phokinase (data not shown), which transfers the 

\[ \text{UTP} \]

could be recovered after the hexokinase treatment by 

glu-1-P

cells were subjected to a mechanical stimulation by pipetting the medium (0.2 ml) up and down twice. The Ca\(^{2+}\) response 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.

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 \(^{33}\)P[UTP and \(^{33}\)P[ATP as a cocrustubstrate (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 \(\mu\)M had no effect (in the presence of 1 \(\mu\)M 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 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 P2Y4 receptor, since identical results were obtained with wild type 1321N1 cells (data not shown).

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observed for ATP release using a luciferin-luciferase assay (Fig. 7). ATP measurements by the luciferin-luciferase indicated that up to 104 ± 12 pmol/10⁶ 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 Mn²⁺-sensitive fluorescent dye, and 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 (P2Y₂, P2Y₄, and P2Y₆) have 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).

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., <10⁴ to 10⁵ 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 P2Y₄ 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).
P2Y₆ 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 and that was present in a concentrate of the extracellular medium of mammary tumor epithelial cells. However, UTP detected in the medium in the absence of this nucleotide. UTP detected in the medium in the absence of...
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 P2Y4 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 coordinate 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 P2Y4 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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