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Quantitation of extracellular UTP using a sensitive enzymatic assay

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1 The wide distribution of the uridine nucleotide-activated $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors suggests a role for UTP as an important extracellular signalling molecule. However, direct evidence for UTP release and extracellular accumulation has been addressed only recently due to the lack of a sensitive assay for UTP mass. In the present study, we describe a method that is based on the uridinylation of [^{14}C]-glucose-1P by the enzyme UDP-glucose pyrophosphorylase which allows quantification of UTP in the sub-nanomolar concentration range.

2 The UTP-dependent conversion of [^{14}C]-glucose-1P to [^{14}C]-UDP-glucose was made irreversible by including the pyrophosphate scavenger inorganic pyrophosphatase in the reaction medium and [^{14}C]-glucose-1P and [^{14}C]-UDP-glucose were separated and quantified by HPLC. Formation of [^{14}C]-UDP-glucose was linearly observed between 1 and 300 nM UTP. The reaction was highly specific for UTP and was unaffected by a 1000 fold molar excess of ATP over UTP.

3 Release of UTP was measured with a variety of cells including platelets and leukocytes, primary airway epithelial cells, rat astrocytes and several cell lines. In most resting attached cultures, extracellular UTP concentrations were found in the low nanomolar range (1–10 nM in 0.5 ml medium bathing 2.5 cm^2 dish). Up to a 20 fold increase in extracellular UTP levels was observed in cells subjected to a medium change. Extracellular UTP levels were 10–30% of the ATP levels in both resting and mechanically-stimulated cultured cells. In unstirred platelets, a 1 : 100 ratio UTP/ATP was observed. Extracellular UTP and ATP increased 10 fold in thrombin-stimulated platelets.

4 Detection of UTP in nanomolar concentrations in the medium bathing resting cultures suggests that constitutive release of UTP may provide a mechanism of regulation of the basal activity of uridine nucleotide sensitive receptors.

Keywords: Extracellular nucleotides; UTP release; P2Y receptors; UTP measurement

Abbreviations: UDPG, uridine diphosphoglucose; HPLC, high performance liquid chromatography; DMEM, Dulbecco's modified Eagle's medium; HNE, human nasal epithelial cells; PMNs, polymorphonuclear leukocytes

Introduction

The physiological significance of ATP and ADP as extracellular signalling molecules is well established (Burnstock, 1972; Gordon, 1986; Dubyak & El-Moatassim, 1993). Both molecules are released in a regulated manner from excitatory and non-excitatory cells, and these released nucleotides interact with both the P2X receptor subfamily of ligand-gated ion channel receptors and the P2Y receptor subfamily of G protein-coupled receptors (Burnstock & Kennedy, 1985; Abbracchio *et al*., 1996; Fredholm *et al*., 1997).

The physiological role of extracellular uridine nucleotides is less well understood. However, extracellular UTP and UDP promote widespread and pronounced cellular responses, and three of the five cloned human P2Y receptors are potently activated by uridine nucleotides. The $P2Y_2$ receptor is activated equipotently by UTP and ATP (Lustig *et al*., 1993; Parr *et al*., 1994), the $P2Y_4$ receptor is specifically activated by UTP (Cohen *et al*., 1990; Nguyen *et al*., 1995; Nicholas *et al*., 1996), and the $P2Y_6$ receptor is specifically activated by UDP (Chang *et al*., 1995; Nicholas *et al*., 1996). Support for the physiological importance of extracellular uridine nucleotides would be provided by direct demonstration of the regulated release of UTP from a range of tissues. However, measurement of intracellular as well as extracellular UTP levels has been difficult due to the lack of a sensitive assay for the quantification of UTP mass at physiological or pharmacological levels (Anderson & Parkinson, 1997). Recently, we have developed an enzymatic approach for quantification of UTP based on the high selectivity of UDP-glucose pyrophosphorylase for UTP as a co-substrate for the conversion of glucose-1P to UDP-glucose (Lazarowski *et al*., 1997a). By utilizing [^{14}C]-glucose-1P as a tracer substrate, the UTP-dependent formation of [^{14}C]-UDP-glucose was quantified by HPLC and utilized to establish the occurrence of UTP release from 1321N1 human astrocytoma cells following mechanical stimulation (Lazarowski *et al*., 1997a). In the present study, we describe in detail the methodology that allows accurate quantification of UTP in the subnanomolar concentration range, and we establish conditions that illustrate the potential of measuring UTP at sub-picomolar levels. In addition, the intracellular and extracellular concentrations of UTP have been determined with a variety of cells, and our results suggest that both constitutive and regulated release of UTP may occur from many different cell types.

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Methods

Cell culture

1321N1 Human astrocytoma cells and C6 rat glioma cells were grown on 24-well plastic plates (Costar) in the presence of high

glucose Dulbecco's Modified Eagles Medium (DMEM-H) supplemented with 5% foetal bovine serum (FBS) and antibiotics as described previously (Lazarowski & Harden, 1994; Lazarowski *et al*., 1997a). Human bronchial epithelial (16HBE14o$^-$) cells were grown on vitrogen coated plates as non-polarized epithelium in the presence of DMEM-H plus 10% FBS. Cystic fibrosis (CF) tracheal epithelial (CF/T43) cells were grown on 24-well plastic plates in keratinocyte growth medium (Brown *et al*., 1991). Primary human nasal epithelial cells (HNE) and T-84 human colonic epithelial cells were grown as a polarized epithelium on 12-mm transwells (costar) as described previously (Watt *et al*., 1998). PC12 rat pheochromocytoma cells were grown on collagen coated 24-well plastic plates in 85% DMEM-H, 10% FBS and 5% heat inactivated horse serum. During the last 5–7 days of culture, PC12 cells were differentiated with 100 ng ml^{-1} nerve growth factor (Upstate Biotechnology, NY, U.S.A.) in 94% DMEM-H, 1% inactivated horse serum and 5% FBS. Confluent cultures of primary rat astrocytes and nerve cells grown on 12-well plastic plates were generously provided by Dr Ken McCarthy. For the experimental designs, the cells were washed (i.e., the culture medium was aspirated and the dishes immediately rinsed gently twice with 0.5 ml of pre-warmed phenol red-free DMEM-H medium) and incubated in 0.5 ml of fresh DMEM-H medium. Medium samples were collected either immediately after the cell wash (medium change) or after a 4 h incubation period under undisturbed conditions (steady state). The collected medium was rapidly centrifuged ($400 \times g$, 2 min) to remove potentially detached cells and the resulting supernatant was boiled for 1 min. Aliquots of 400 and 50 μl were used for UTP and ATP measurements, respectively. For experiments with polarized HNE and T-84 cells, only the mucosal medium was processed.

Isolation of human platelets and neutrophils

Platelets were isolated from blood of healthy donors who had not taken medication for at least 2 weeks. Blood (60 ml) containing 3.8% trisodium citrate was centrifuged at $200 \times g$ for 20 min. The platelet-rich plasma was then centrifuged for 15 min at $800 \times g$. Contaminant red blood cells were depleted by rapid centrifugation in a microcentrifuge (2×10 s at 15,000 r.p.m.). Platelets were washed twice with 10 ml DMEM-H and resuspended in phenol red-free DMEM-H at a concentration of 1.25×10^8 cells ml^{-1}. Polymorphonuclear neutrophils (PMNs) were isolated from blood of healthy volunteers and kindly provided for this study by Dr Margrith Verghese (Verghese *et al*., 1996). PMNs were suspended in phenol red-free DMEM-H at a final concentration of 10^7 cells ml^{-1}.

Quantification of UTP mass

UTP concentrations were determined using a UDP-glucose pyrophosphorylase-catalyzed reaction. Except where indicated otherwise, incubations were for 60 min in 0.5 ml phenol red-free DMEM/HEPES (pH 8.0) containing 0.5 units ml^{-1} UDP-glucose pyrophosphorylase, 0.5 units ml^{-1} inorganic pyrophosphatase, and 1 μM 0.15 μCi [^{14}C]-glucose-1P. Reactions were terminated by boiling the samples for 1 min. The per cent conversion of [^{14}C]-glucose-1P to [^{14}C]-UDP-glucose was determined by HPLC.

HPLC analysis

Glucose-1P, UDP-glucose, and UTP were separated by HPLC (Shimadzu) *via* a 60-Å Dynamax C18 column (Varian, CA, U.S.A.) with the mobile phases consisting of KH_2PO_4 17 mM, tetrabutylethylhydrogen sulphate (TBEHS), pH 5.3 8 mM (Buffer A), and 10% methanol in KH_2PO_4 100 mM, TBEHS, pH 5.3 8 mM (Buffer B). The system developed isocratically in 100% buffer A for 10 min and was subsequently shifted to 100% buffer B for an additional 15 min. Absorbance at 264 nm was monitored with a SPD-10A UV detector (Shimadzu), and radioactivity was determined on-line with a Flo-One Radiomatic beta detector (Packard, Canberra, Australia) as described previously (Lazarowski *et al*., 1997a).

Measurement of ATP by the luciferin-luciferase assay

Samples (50 μl aliquots) were diluted 1 : 6 with water. A $4 \times$ luciferin-luciferase cocktail (300 μM luciferin, 5 μg ml^{-1} luciferase, HEPES (pH 7.8) 25 mM, $MgCl_2$ 6.25 mM, EDTA 0.63 mM, 75 μM dithiotritiol, 1 mg ml^{-1} bovine serum albumin) was added to the diluted samples *via* a LB953 AutoLumat luminometer (Berthold GmbH, Germany). Sample luminescence was compared to an ATP standard curve performed for each individual experiment as previously described (Watt *et al*., 1998).

Reagents

All nucleoside triphosphates were purchased from Pharmacia (Uppsala, Sweden). UDP-glucose pyrophosphorylase was from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Luciferin, luciferase, inorganic pyrophosphatase, UDP-glucose, glucose-1P, and TBEHS were from Sigma (St. Louis, MO, U.S.A.). [^{14}C]-glucose-1P (330 mCi mmol^{-1}), [^{3}H]-UTP (50 Ci mmol^{-1}), and [α^{33}P]-UTP (3000 Ci mmol^{-1}) were from Amersham (Arlington Heights, IL, U.S.A.).

Results

Lack of availability of reliable methodology for measuring UTP concentrations at low levels in biological tissues prompted us to develop a coupled enzymatic method for quantitation of UTP mass. This assay is based on the selectivity for UTP of the enzyme UDP-glucose pyrophosphorylase which converts UTP and glucose-1P into UDP-glucose in the obligatory step that precedes the incorporation of glucose into glycogen (Leloir, 1971): UTP + glucose-1P $\leftrightarrows$ UDP-glucose + PP_i.

Although the equilibrium constant of the reaction is 0.29–0.34 in the forward direction (Hansen *et al*., 1996), it can be forced quantitatively in the direction of UDP-glucose synthesis by including inorganic pyrophosphatase as an enzymatic scavenger of pyrophosphate: UTP + glucose-1P $\leftrightarrows$ UDP-glucose + $PP_i \rightarrow$ UDP-glucose + $2P_i$.

The fidelity of the two enzymatic steps was first established using [^{3}H]-UTP as a substrate. [^{3}H]-UTP (100 nM) was recovered unchanged following a 1 h incubation with UDP-glucose pyrophosphorylase and inorganic pyrophosphatase in the absence of glucose-1P (Figure 1a). In contrast, approximately 50% of [^{3}H]-UTP was converted to [^{3}H]-UDP-glucose by UDP-glucose pyrophosphorylase in the presence of 1 μM glucose-1P during the same period (Figure 1b). Inclusion of inorganic pyrophosphatase resulted in near 100% conversion of [^{3}H]-UTP to [^{3}H]-UDP-glucose (Figure 1c). Formation of [^{3}H]-UDP-glucose from [^{3}H]-UTP was equally efficient in the presence of a 1000 fold excess of ATP, GTP or CTP (not shown), indicating that these nucleotides do not compete with

UTP for the UDP-glucose pyrophosphorylase-catalyzed reaction. The substrate specificity of UDP-glucose pyrophosphorylase was further investigated in experiments in which [^{14}C]-glucose-1P (1 μM) was incubated with UTP or ATP (Figure 2). [^{14}C]-glucose-1P remained unchanged in the absence of UTP (Figure 2a). In contrast, formation of [^{14}C]-UDP-glucose was readily observed in the presence of 100 nM UTP (Figure 2b). Figure 2c shows that no formation of [^{14}C]-ADP-glucose occurred in the presence of 100 μM ATP (the elution time of ADP-glucose (ADPG) is indicated by an arrow on top of Figure 2a). These results are consistent with the reported substrate specificity of UDP-glucose pyrophosphorylase which does not utilize ATP as a substrate (Hansen *et al.*, 1996).

The time course of the UDP-glucose pyrophosphorylase-catalyzed reaction was determined in the presence of 1 μM glucose-1P and 100 nM UTP. Approximately 5% of the [^{14}C]-glucose-1P was converted to [^{14}C]-UDP-glucose after 1 h under the conditions detailed in Figure 3a, indicating that approximately 50% of the initial UTP was utilized in the reaction. Moreover, inclusion of 0.5 units ml^{-1} inorganic pyrophosphatase resulted in near full conversion of substrate to product within 40 min of incubation (Figure 3a). Conversion of [^{14}C]-glucose-1P to [^{14}C]-UDP-glucose was linearly dependent on the concentration of UDP-glucose pyrophosphorylase (Figure 3b) over a range from 5–50 μg enzyme ml^{-1} (0.1–1 units ml^{-1}). The reaction exhibited a pH optimum of pH 8.0 (Figure 3c).

Since our goal was to optimize conditions to quantify UTP at physiological levels, we further examined the efficiency of the conversion of UTP to UDP-glucose utilizing nanomolar and subnanomolar concentrations of UTP. In the presence of 1 μM glucose-1P, over 80% conversion of [α^{33}P]-UTP to [^{33}P]-UDP-glucose occurred at all concentrations (0.1–100 nM) of UTP (Figure 4a). Thus, UTP could be quantitated at concentrations that are several orders of magnitude below its Km for the UDP-glucose pyrophosphorylase-catalyzed reaction (Km UTP = 563 μM, Km glucose-1P = 172 μM, Duggleby *et al.*, 1996). The rate of the reaction was dramatically accelerated with 1 mM glucose-1P (100% conversion in 1 min; Figure 4b). However, the sensitivity of the assay ultimately is limited by the poor efficiency of conversion observed at concentrations less than 1 μM glucose-1P (Figure 4b). The relatively low specific activity of commercially available radioactive glucose-1P ([^{14}C]-UDP-glucose, SA ~300 mCi mmol^{-1}) also limits the total amount of radioactive substrate to <0.3 μCi ml^{-1}.

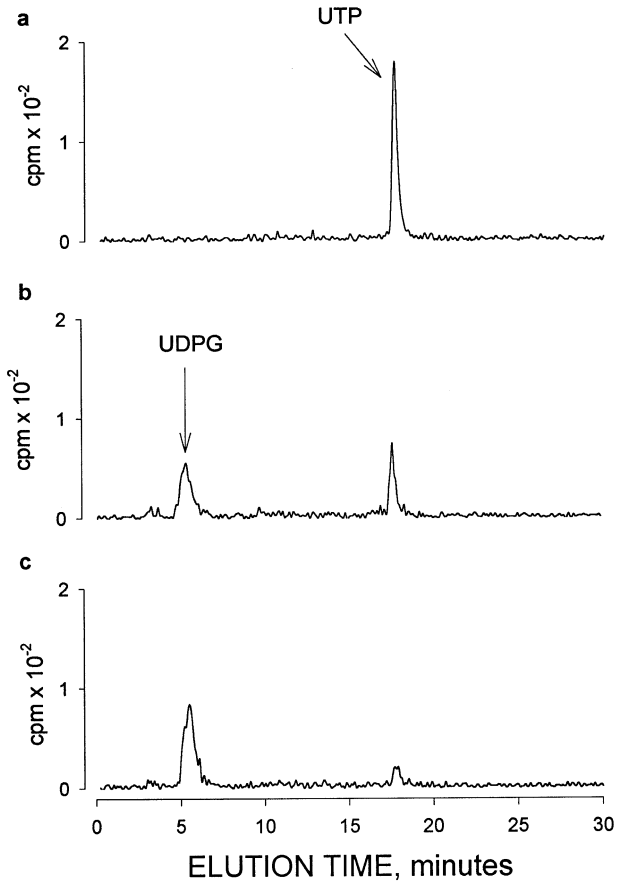

Figure 1 Glucose-1P-dependent conversion of [^{3}H]-UTP to [^{3}H]-UDP-glucose. Effect of inorganic pyrophosphatase. HPLC tracings showing the conversion of 100 nM 0.5 μCi [^{3}H]-UTP to [^{3}H]-UDP-glucose. (a) [^{3}H]-UTP remained intact after a 1 h incubation with 0.5 units ml^{-1} UDP-glucose pyrophosphorylase and inorganic pyrophosphatase in the absence of glucose-1P. (b) In the presence of 1 μM glucose-1P, approximately 50% of the initial [^{3}H]-UTP was converted to [^{3}H]-UDP-glucose (UDPG) by 0.5 units ml^{-1} UDP-glucose pyrophosphorylase after 1 h. (c) Inclusion of inorganic pyrophosphatase resulted in near full conversion of [^{3}H]-UTP to [^{3}H]-UDP-glucose during the same incubation period. The data are representative of six experiments performed in duplicate.

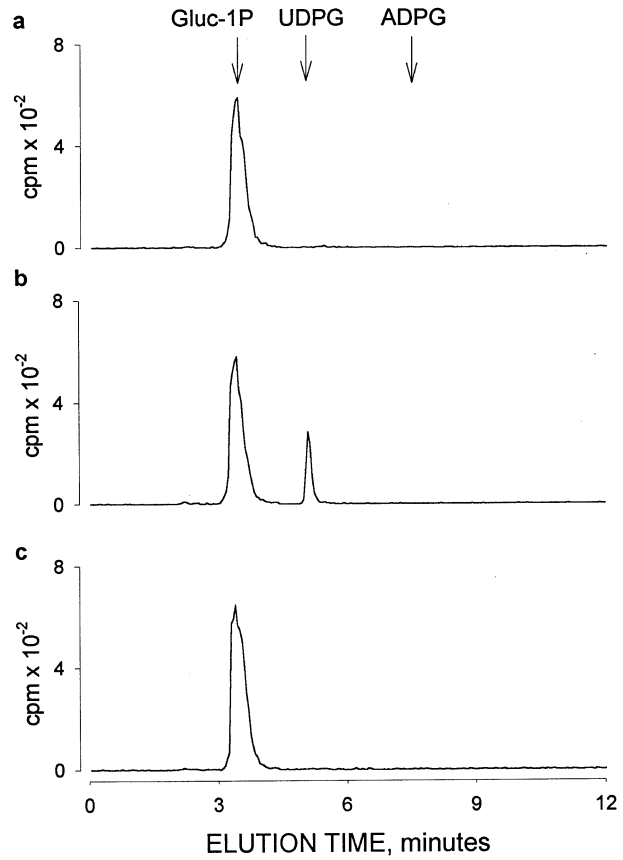

Figure 2 UTP-dependent UDP-glucose pyrophosphorylase-catalyzed conversion of [^{14}C]-glucose-1P to [^{14}C]-UDP-glucose. HPLC tracings corresponding to 1 h incubations in the presence of 0.5 units ml^{-1} of both UDP-glucose pyrophosphorylase and inorganic pyrophosphatase, 1 μM [^{14}C]-glucose-1P and either vehicle (a), 100 nM UTP (b) or 100 μM ATP (c). The data are representative of at least three experiments performed with duplicate samples. The elution time of glucose-1P (Gluc-1P), UDP-glucose (UDPG), and ADP-glucose (ADPG) are indicated with arrows on top of a.

Thus, the assay developed here utilizing 1 μM [^{14}C]-glucose-1P (~200,000 c.p.m. per assay) exhibits a threshold sensitivity of ~1 nM UTP (Figure 5a, left). That is, in the presence of 1 nM UTP in a 0.5 ml reaction volume, the predicted accumulation of approximately 200 c.p.m. [^{14}C]-UDP-glucose (0.1% of the total counts) can be reliably detected over baseline (Figure 5a, left).

One approach that would increase the sensitivity of this assay for UTP would include the use of glucose-1P with higher specific radioactivity, e.g., [^{32}P]-glucose-1P or [^{33}P]-glucose-1P. Since these compounds are not yet available, we examined the effectiveness of the conversion of UTP to UDP-glucose utilizing [α^{33}P]-UTP at picomolar concentrations in the presence of 1 μM glucose-1P. Quantitative conversion of [^{33}P]-UTP to [^{33}P]-UDP-glucose occurred in a 1 h incubation with UTP at concentrations as low as 20 pM (Figure 5b, left). Thus, the UDP-glucose pyrophosphorylase catalyzed-reaction as described here exhibits a linear dependence on UTP concentration of near five orders of magnitude (20 pM UTP to 0.3 μM UTP).

In recent studies with a human astrocytoma cell line, we have observed release of UTP in pharmacologically important concentrations from mechanically-stimulated cells (Lazarowski *et al*., 1997a). As a first step in determining whether UTP release reflects a more general phenomenon, we have measured the UTP content and extracellular levels of UTP in other tissues. The concentration of UTP in each sample was compared with ATP concentration measured by the luciferin-luciferase assay (Table 1).

The intracellular amount of UTP ranged between 500 and 8000 pmol per million cells in the different cells studied. The cellular concentration of UTP was 10–30% of that determined for ATP. Extracellular UTP was present in detectable amounts for many cells under resting conditions, but extracellular UTP was not detected (i.e., <1 nM) with non-stimulated C6 glioma cells, T84 cells, primary nerve cells and astrocytes. However, enhanced accumulation of extracellular UTP was observed after mechanical stimulation of the cells by a change of medium. As we previously observed with 1321N1 human astrocytoma cells, the ratio of extracellular UTP to ATP typically was in the range of 1:3 to 1:10 for most cells studied. The extracellular concentration of UTP was the highest with neutrophils and platelets in suspension (Table 1) probably reflecting cell stress and activation or damage during isolation procedures. Notably, stimulation of platelets with thrombin (2 units ml^{-1}) caused a 10 fold increase in accumulation of extracellular UTP and ATP. Unlike most cells, where extracellular nucleotide ratios reflected the cellular content, a ratio ~1:100 of

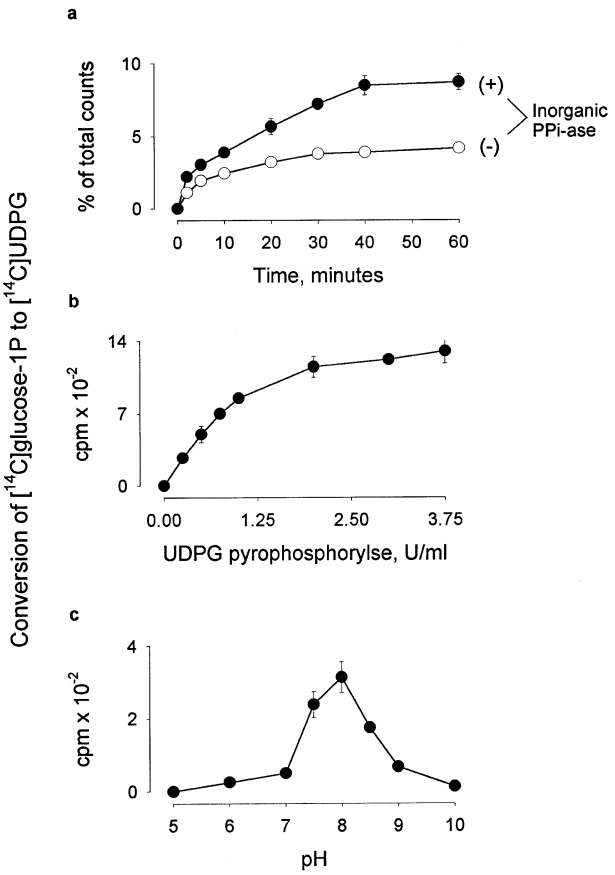

Figure 3 UDP-glucose pyrophosphorylase-catalyzed conversion of [^{14}C]-glucose-1P to [^{14}C]-UDP-glucose. (a) Incubations were for the indicated times in the presence of 1 μM [^{14}C]-glucose-1P, 100 nM UTP and 0.5 units ml^{-1} UDP-glucose pyrophosphorylase with or without the addition of 0.5 units ml^{-1} inorganic pyrophosphatase (Inorganic PP_i-ase). (b) The effect of increased concentrations of UDP-glucose pyrophosphorylase was determined after a 15 min incubation period in the presence of 0.5 units ml^{-1} inorganic pyrophosphatase, 1 μM [^{14}C]-glucose-1P and 100 nM UTP. (c) The pH dependence was examined in the presence of 0.5 units ml^{-1} of UDP-glucose pyrophosphorylase and inorganic pyrophosphatase. The data represent the mean value (±s.e.mean) from three experiments performed with duplicate samples.

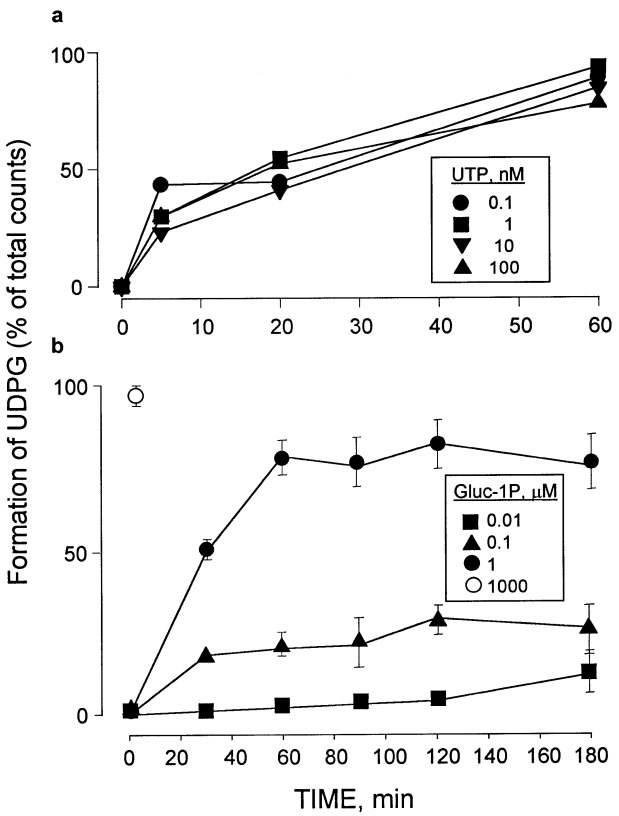

Figure 4 Effect of substrate concentration on the conversion of [α^{33}P]-UTP to [^{33}P]-UDP-glucose. Incubations were in the presence of 0.1 μCi [α^{33}P]-UTP, 0.5 units ml^{-1} UDP-glucose pyrophosphorylase and inorganic pyrophosphatase and (a) 1 μM glucose-1P (Gluc-1P) and the indicated concentration of UTP and (b) 100 nM UTP and the indicated concentration of glucose-1P. The data represent the mean value from at least three experiments performed with duplicate samples (in a, error bars were omitted for clarity).

extracellular UTP to ATP was observed with platelets while the total intracellular UTP:ATP ratio was 1:25 (Table 1).

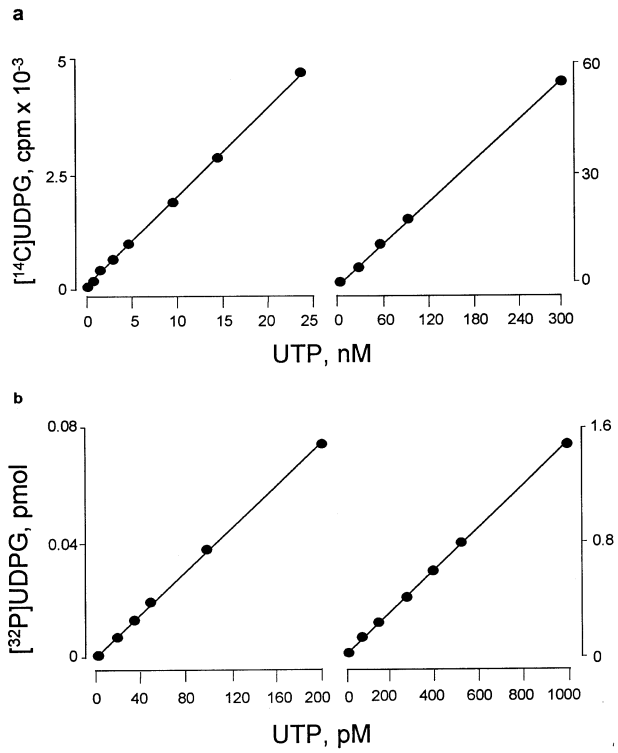

Figure 5 The formation of UDP-glucose is linear over a broad range of UTP concentrations. (a) Conversion of [^{14}C]-glucose-1P to [^{14}C]-UDP-glucose. (b) Conversion of [α^{33}P]-UTP to [^{33}P]-UDP-glucose. All incubations were for 1 h in the presence of 1 μM glucose 1-P and 0.5 units ml^{-1} of UDP-glucose pyrophosphorylase and inorganic pyrophosphatase. The data represent the mean value from one experiment performed with duplicate samples that differed by less than 10% from each other. The experiments were repeated at least three times and similar results were obtained.

Discussion

The assay for UTP described here provides reliable detection of UTP for the first time in biological samples such as cell supernatants and cell lysates. The sensitivity of this coupled enzymatic assay using [^{14}C]-glucose as a radiolabelled co-substrate is at least two orders of magnitude greater than other methods previously available (Keppler *et al*., 1970 ; Sasvari-Szekely *et al*., 1975). Moreover, we show here using radiolabelled UTP in proof of principle experiments that the assay is linear to at least the low picomolar range of concentrations of UTP. Therefore, use of [^{33}P]-glucose-1P or [^{32}P]-glucose-1P as a substrate will provide an assay of UTP mass to concentrations much lower than our results indicate exist under resting levels in the extracellular medium of a variety of cell preparations. As predicted from the previously established selectivity of UDP-glucose pyrophosphorylase for UTP, concentrations of ATP and other nucleoside triphosphates that exceeded the concentration of UTP by three orders of magnitude do not interfere with the quantitation of UTP using this assay. Further, no evidence of other interfering molecules in tissues could be ascertained from experiments in which UTP was added as an internal standard to extracts from various cell culture supernatants (data not shown). Thus, we describe a very sensitive and specific assay that should find usefulness in accurate quantitation of UTP mass under a variety of experimental conditions.

The luciferin/luciferase assay for ATP has provided widely used methodology for quantitation of ATP mass in biological tissues at sub-nanomolar concentrations. Thus, ATP has been known for decades to exist extracellularly and to function both as an extracellular signalling molecule as well as a source for extracellular produced adenosine, which in turn activates a group of G protein-coupled adenosine receptors (Burnstock & Kennedy, 1985; Gordon, 1986). In contrast, no information on extracellular UTP has been available until recently. Saiag *et al*. (1995) reported that bovine endothelial cells incorporated [^{3}H]-uridine into UTP, which then was shown to be released by mechanical stimulation of the cells. We recently reported the first direct quantitation of UTP mass in extracellular medium

Table 1 Measurement of ATP and UTP in various cell types

	ATP					*UTP*				
	Extracellular				*Intracellular*	*Extracellular*				*Intracellular*
	Steady state		*Medium change*		*pmol*	*Steady state*		*Medium change*		*pmol*
Cell type	nM	%	nM	%	10^6 cells	nM	%	nM	%	10^6 cells
HNE	6.1±2	0.13	18.9	0.4	8450	2.8±1	0.06	10.3±3	2.4	3250
16HBE14o-	4.1±2	0.15	88±14	2.4	6250	1.2±0.2	0.2	9.8±3	3.4	1160
CF/T43	6.2±1.4	0.08	40.9±12	0.5	7790	1.9±0.6	0.07	10.9±0.9	0.4	3376
T-84	5.9	0.02	25.6±7	0.1	26600	<1	–	3.5±1	0.1	7970
Nerve cells	<0.1	–	3.7±0.02	0.1	17200	<1	–	<1	–	7100
Astrocytes	1±0.03	0.01	34±6	0.4	26410	<1	–	13.5±6	1.0	5170
1321N1	12±1.2	0.43	139.5±65	4.5	7510	4.6	0.33	49.2±12	3.5	2140
PC12	3.4±0.6	0.01	nd*	–	10300	1.2±0.4	0.02	nd	–	2710
C6	3.2±1	0.34	21±5	2.0	1860	<1	–	2.1±1	0.8	575
PMNs	145±39	0.36			4020	13.3±0.5	0.45			190
Platelets (Control)	561±62	6.3			80	5.3±1	1.8			3
Platelets (Thrombin)	3889±976	74.1			–	47±4.1	16			–

Top: ATP and UTP concentrations were determined in the medium (0.5 ml) bathing confluent cultures of undisturbed cells (steady state) or cells that were subjected to a medium change. Bottom: PMNs were suspended at a concentration of 10^7 ml^{-1} and incubated for 5 min at 37°C without stirring. Platelets (1.25×10^8 ml^{-1}) were incubated unstirred for 2 min in the absence or presence of 2 U ml^{-1} thrombin. Extracellular ATP and UTP concentrations (nM) are expressed as the mean value±s.e.mean and as the per cent of the total ATP and UTP cell content, respectively. The intracellular nucleotide concentrations are expressed as picomoles per million cells. *Not determined.

and further illustrated that mechanical stimulation of 1321N1 human astrocytoma cells resulted in co-release of UTP with ATP (Lazarowski *et al*., 1997a).

The assay of mass has been extended here to show that extracellular UTP can be detected in the medium from other neural as well as non-neural cells including normal and cystic fibrosis airway epithelial cell preparations, and from circulating leukocytes and platelets. These observations are important for a number of reasons. For example, the principle Ca^{2+}-mobilizing receptor on the apical surface of human airway epithelial cells is a UTP-activated $P2Y_2$ receptor (Mason *et al*., 1991; Lazarowski *et al*., 1997c). Indeed, this receptor has become an important therapeutic target for treatment of cystic fibrosis and potentially other obstructive airway disease (Knowles *et al*., 1991). The availability of an assay for UTP and the observation of UTP accumulation in the extracellular medium of airway epithelial cells will provide impetus for experiments addressing the potential role that extracellular UTP may play in the physiological regulation of the airway receptor. Likewise, receptors for ATP and UTP, e.g. the $P2Y_1$ and $P2Y_2$ receptors, are differentially expressed developmentally in various blood cell lineages (Clifford *et al*., 1997). The role of UTP as an extracellular signalling molecule in inflammatory and immunological responses is an important topic for future investigation. Release of UTP from human platelets has not been reported previously, although UTP is thought to be co-stored with ATP in the platelet secretory granules (Goetz *et al*., 1971). Our data indicates that thrombin promotes a 10 fold increase in extracellular UTP levels. However, the physiological significance of this release remains to be determined since extracellular concentrations of UTP are much lower than that of ATP.

The enzyme, UDP-glucose pyrophosphorylase, which was used for quantitation of UTP levels in this study, also potentially can be utilized in studies assessing the nucleotide selectivity of the P2Y receptors. For example, we and others originally reported that the human $P2Y_4$ receptor is activated by high concentrations of ATP (Communi *et al*., 1995; Nicholas *et al*., 1996), although no effect of ATP on this receptor was observed by Nguyen *et al*. (1995). Subsequent studies in which the release and accumulation of UTP and UDP were minimized indicated that ATP is not an agonist at the human $P2Y_4$ receptor (Lazarowski *et al*., 1997a), suggesting that the reported effect of ATP was likely due to its competitive action on ecto-nucleotidases acting on endogenous UTP or due to transfer of the terminal phosphate from ATP to endogenous UDP *via* nucleoside diphosphokinase (Lazarowski *et al*, 1997b). UDP-glucose pyrophosphorylase should prove useful in addressing contributions of UTP to biological responses since ATP is not a substrate for UDP-glucose pyrophosphorylase and this enzyme efficiently and rapidly metabolizes UTP when glucose-IP is present in millimolar amounts (Figure 4). That is, UDP-glucose pyrophosphorylase provides a means for the assessment of the effect of ATP as well as other nucleotides without any confounding influence of endogenous or contaminant UTP. Similarly, UDP-glucose pyrophosphorylase will be useful to assess the true potencies of non-uridine nucleotides at a pair of recently cloned non-mammalian P2Y receptors that share relatively high homology with the human $P2Y_4$ receptor and appear to be activated by all nucleoside triphosphates (Bogdanov *et al*., 1997; Boyer *et al*., 1997).

The mechanism whereby non-excitatory cells release ATP (and now UTP) remains unclear. A proposed role for the cystic fibrosis transmembrane regulator CFTR in the release of nucleotides (Watt *et al*., 1998) was not substantiated by a number of studies including experiments with cells from the CFTR mouse as well as in experiments in which CFTR was expressed to high levels and ATP mass directly quantitated in the medium or indirectly measured at the level of the cell surface by assessing activation of co-expressed P2Y receptors (Watt *et al*., 1998). The results described here comparing the concentration of UTP in the extracellular medium of a variety of non-secretory cells address neither the mechanism whereby the extracellular nucleotide appeared nor its intracellular source. Nonetheless, the similar (but not always identical) ratio of UTP to ATP in the medium relative to the cell content suggests the occurrence of a common mechanism and further suggests that these nucleotides are released by a transport mechanism that simply reflects the relative intracellular concentrations. It will be important to establish whether other nucleotides, e.g. CTP, GTP, are released in amounts that reflect their relative intracellular levels. This question takes on added significance in face of the observation that in contrast to the UTP specificity of the human $P2Y_4$ receptor, the rat $P2Y_4$ receptor is activated by both UTP and ATP as well as by ITP (Bogdanov *et al*., 1998). As discussed above, an avian and a xenopus P2Y receptor have been cloned that are potently activated by all nucleoside triphosphates that have been tested.

Difficulties in sampling the liquid layer immediately surrounding the cell surface limit our understanding of the potential autocrine roles of UTP and ATP. Basal levels of UTP measured in the bulk medium of 'non-stimulated' cultured cells are below threshold values to promote $P2Y_2$, $P2Y_4$ or $P2Y_6$ receptor stimulation (Nicholas *et al*., 1996). However, as suggested from recent studies, measurements of nucleotide levels in bulk medium samples may underestimate local accumulation, e.g., at cell surface receptors. Utilizing a fusion system to anchor the ATP-specific enzyme luciferase to the outer cell surface of human platelets, Dubyak and colleagues observed that accumulation of ATP in the bulk medium from thrombin-stimulated platelets underestimates by at least one order of magnitude the ATP concentration in the environment near the cell surface (Beigi *et al*., 1999). Experiments with mechanically stimulated hP2Y4-1321N1 cells also suggested that local accumulation of UTP was several fold higher than levels measured in the bulk medium (Lazarowski *et al*., 1997a). Similarly, baseline accumulation of inositol phosphates in resting hP2Y4-1321N1 cells decreased significantly after the addition of the non-specific nucleotidase apyrase (Lazarowski *et al*., 1997a). Since ATP is not an agonist at the human $P2Y_4$ receptor, the most likely explanation for these results is that under resting conditions, UTP accumulates locally on the surface of 1321N1 cells in amounts sufficient to activate phospholipase C through the $P2Y_4$ receptor. Consistent with this notion, the baseline $[Ca^{2+}]$ signal of non-stimulated hP2Y4-1321N1 cells was markedly reduced upon inclusion of UDP-glucose pyrophosphorylase and glucose-1P in the bathing solution. (Homolya, L. & Lazarowski, E.R., unpublished). 'Constitutive' release of UTP may provide a mechanism whereby the $P2Y_4$ receptor, and by analogy the $P2Y_2$ and the $P2Y_6$ receptors, regulate 'baseline functions' of resting cells.

In summary, the biological significance of extracellular UTP has been supported in recent years by pharmacological studies of a series of G protein-coupled receptors that are potently activated by UTP or UDP. The availability of an assay capable of detecting UTP at low levels under a variety of conditions should provide important methodology for linking the release of cellular UTP to physiological responses through these receptors in a variety of tissues.

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