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Enzymatic synthesis of UTPγS, a potent hydrolysis resistant agonist of P_{2U}-purinoceptors

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1 The defective Cl^- secretion characteristic of cystic fibrosis airway epithelial cells can be bypassed by an alternative Ca^{2+} dependent Cl^- secretory pathway that is activated by extracellular nucleotides, e.g. uridine-5'triphosphate (UTP), acting on P_{2U} purinoceptors. Since UTP is susceptible to hydrolysis by nucleotidases and phosphatases present in the airways, the identification of stable P_{2U}-purinoceptor agonists would be of therapeutic relevance.

2 Uridine-5'-*O*-(3-thiotriphosphate) (UTPγS) was synthesized by nucleoside diphosphate kinase-catalyzed transfer of the γ-phosphorothioate from guanosine-5'-*O*-(3-thiotriphosphate) (GTPγS) or adenosine-5'=*O*-(3-thiotriphosphate) (ATPγS) to UDP. Formation of UTPγS was illustrated by observation of transfer of ^{35}S from [^{35}S]-GTPγS and transfer of ^{3}H from [^{3}H]-UDP. The chemical identity of high performance liquid chromatography (h.p.l.c.)-purified UTPγS was confirmed by nuclear magnetic resonance analysis.

3 Human 1321N1 astrocytoma cells stably expressing the phospholipase C-coupled human P_{2U}-purinoceptor were utilized to test the activity of UTPγS. UTPγS (EC_{50}=240 nM) was essentially equipotent to UTP and ATP for stimulation of inositol phosphate formation.

4 Unlike [^{3}H]-UTP, [^{3}H]-UTPγS was not hydrolyzed by alkaline phosphatase, acid phosphatase, or apyrase. Moreover, no hydrolysis was detected during a 1 h incubation with human nasal epithelial cells.

5 UTPγS was equally potent and efficacious with UTP for stimulation of Cl^- secretion by human nasal epithelium from both normal donors and cystic fibrosis patients. Based on its high potency and resistance to hydrolysis, UTPγS represents a promising compound for treatment of cystic fibrosis.

Keywords: UTPγS; P_{2U}-purinoceptors; cystic fibrosis; nucleoside diphosphate kinase

Introduction

Chloride transport by airway epithelia plays an important role in modulation of the volume and composition of pulmonary secretions, and defects in this mechanism may be involved in lung disease (Boucher, 1993). The defective adenosine 3':5'-cyclic monophosphate (cyclic AMP)-regulated Cl^- secretion, which is characteristic of cystic fibrosis (CF), can be bypassed by activation of an alternative Cl^- conductance that is CF transmembrane-regulator (CFTR)-independent and is regulated by intracellular Ca^{2+} levels (Clarke & Boucher, 1992; Clarke *et al.*, 1994). Extracellular nucleotides such as adenosine 5'-triphosphate (ATP) and uridine 5'-triphosphate (UTP) stimulate Cl^- secretion in CF airway epithelial cells, and this effect is associated with an elevation of intracellular Ca^{2+} secondary to P_{2U}-purinoceptor-promoted activation of phospholipase C (Brown *et al.*, 1991; Mason *et al.*, 1991).

P_{2U}-purinoceptors are a subclass of receptors for extracellular nucleotides found in a broad range of tissues (O'Connor *et al.*, 1991; Dubyak & El-Moatassim, 1993). The fact that both ATP and UTP are agonists at P_{2U}-purinoceptors distinguishes these receptors from other phospholipase C-linked nucleotide receptors such as the P_{2Y}-purinoceptor that is activated only by adenine nucleotides (Dubyak, 1991; Fredholm *et al.*, 1994; Harden *et al.*, 1995), and a recently described uridine nucleotide receptor that is activated only by uridine nucleotides (Lazarowski & Harden, 1994). The presence of P_{2U}-purinoceptors on the apical surface of airways (Mason *et al.*, 1991) raises the possibility that aerosolized nucleotides selective for these receptors might be used therapeutically to induce Cl^- secretion in individuals with CF or other airway diseases (Knowles *et al.*, 1991; 1992). UTP, the most potent and selective agonist for P_{2U}-purinoceptors, is currently undergoing clinical assessment as an agent to modify airway function in CF patients (Knowles *et al.*, 1994).

The duration of any therapeutic effect of UTP on the airways will be limited by the hydrolytic action of nucleotides and phosphatases normally present on the airway epithelial cell surface (Regnis *et al.*, 1994) and also abundant in secretions from inflamed airways of CF patients (Regnis, Knowles & Boucher, unpublished). Based on the resistance to hydrolysis observed with phosphate-modified derivatives of adenine and guanine nucleotides, analogues of UTP substituted in the phosphate side-chain might possess more long-lived effects on airway Cl^- secretion. However, such analogues of UTP have not been available.

Nucleotide 5'-diphosphate kinase (NDPK) is an enzyme that catalyzes the transfer of the γ-phosphate from nucleoside triphosphates to nucleoside diphosphates (Ratliff *et al.*, 1964). Goody and associates were the first to demonstrate that NDPK also is useful for the synthesis of phosphorothioates such as adenosine-5'-*O*-(3-thiotriphosphate) (ATPγS) and GTPγS (Goody *et al.*, 1972). Although UDP has been reported to be a poor substrate (Buczynski & Potter, 1990) or inhibitor (Seifert *et al.*, 1988) of NDPK, Seifert *et al.* have shown that NDPK can be utilized to synthesize UTPγS (Seifert *et al.*, 1989). Here, we describe in detail the enzymatic synthesis and purification of UTPγS, and present studies on both its suceptibility to hydrolysis and its nucleotide receptor selectivity. Our results indicate that UTPγS is a stable high potency P_{2U}-purinoceptor-selective agonist.

Methods

Synthesis and purification of UTPγS

UTPγS was synthesized by use of NDPK and modifications of the procedures originally described for the synthesis of ATPγS

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and GTPγS (Goody *et al.*, 1972; Buczynski & Potter, 1990). Except where indicated otherwise, incubations were carried out at 30°C for 1 h in a final volume of 1 ml containing (in mM) Tris (pH 7.4) 25, $MgCl_2$ 2, GTPγS 2, UDP 10, and NDPK 50 u ml^{-1}. The NDPK catalyzed reaction proceeded as follows:

$$\text{GTP}\gamma\text{S} + \text{UDP} \xleftrightarrow{\text{NDPK}} \text{GDP} + \text{UTP}\gamma\text{S}$$

To synthesize radioactively labelled UTPγS, 0.05–0.2 μCi of either [^{35}S]-GTPγS or [^{3}H]–UDP was included in the reaction mixture. Separation of the reaction products by high performance liquid chromatography (h.p.l.c.) was performed with a LC-10AD liquid chromatograph and a SCL-10A system controller (Shimadzu Scientific Instruments, Columbia, MD) utilizing an ion-exchange system (VYDAC, Hesperia, CA) with a linear ammonium phosphate gradient as the mobile phase as we have previously described (Lazarowski *et al.*, 1995). Absorbance at 260 nm was monitored with an on-line Model 490 multiwavelength detector (Waters Division, Millipore, Milford, MA). Species resolved after the NDPK-catalyzed reaction were identified by coelution with known standards and quantified by comparing peak areas with concentration curves established with standard nucleotides. Radioactivity was monitored on-line with a Radiomatic Flo-one detector (Radiomatic Instruments, Tampa, FL).

The species eluting from 30.5 to 32.5 min was purified, loaded onto an Accell Plus QMA Sep-Pak cartridge waters, and sequentially washed and eluted with triethylammonium bicarbonate as described previously (Goody *et al.*, 1972). The sample which contained purified UTPγS was lyophilized and stored at −80°C until use. Each preparation of UTPγS was re-chromatographed prior to use to insure purity. The concentration of purified UTPγS was determined by preparation of [^{3}H]-UTPγS from [^{3}H]-UDP of known specific radioactivity. ^{31}P nuclear magnetic resonance (n.m.r.) spectral analyses of nucleotides were performed at 202.46 Mhz with a Bruken AMX 500 spectrometer as described previously (Rao, 1989). Absorbance spectra (200–600 nm) were performed and recorded with a Shimadzu UV-265 spectrophotometer.

Synthesis of [^{3}H]-UDP

[^{3}H]-UDP was prepared by enzymatic dephosphorylation of [^{3}H]-UTP utilizing hexokinase. The incubation consisted of [^{3}H]-UTP (1–100 μCi, 0.1 μM), 4 mM glucose, and 1–5 u hexokinase in 1 ml HEPES, pH 7.4. Incubations were for 5–30 min at 37°C, and reactions were terminated by addition of 5% trichloroacetic acid (TCA) followed by ethyl ether extraction. Complete conversion of [^{3}H]-UTP into [^{3}H]-UDP was confirmed by h.p.l.c.

Measurement of inositol phosphates

Inositol phosphates were measured with HP2U-1321N1 human astrocytoma cells, a cell line infected with the LHP2USN retroviral vector to express stably the human airway epithelial cell P_{2U}-purinoceptor (Parr *et al.*, 1994). Cells were labelled for 18 h with [^{3}H]-myo-inositol, pre-incubated with 10 mM LiCl, and challenged with agonists for an additional 20 min (Lazarowski *et al.*, 1995). Inositol phosphates were separated on Dowex AG1-X8 columns as described by Brown *et al.* (1991). Experiments were performed with triplicate determinations and individual values differed by less than 10% from the mean.

Bioelectric studies

Confluent monolayers of primary nasal epithelium from normal donors or from CF patients were mounted in modified Ussing chambers. The submucosal bath solution was Krebs bicarbonate Ringer solution, and the mucosal bath solution was Krebs bicarbonate Ringer solution with Cl^- replaced by gluconate and 100 mM Na^+ replaced by 100 mM K^+. The mucosal bath solution contained 100 μM amiloride. Under these conditions, the short circuit current (I_{sc}) under zero voltage clamp conditions is equivalent to Cl^- secretion (Mason *et al.*, 1991).

Metabolism of [^{3}H]-UTP and [^{3}H]-UTPγS

Primary cultures of human nasal epithelial cells were grown to confluence on 6.5 mm collagen coated filters mounted on 24-transwell Costar plates. [^{3}H]-UTP and [^{3}H]-UTPγS (~300,000 c.p.m., 30 μM × 0.25 ml) was added to the mucosal surface and, after incubation for various times, the ^{3}H species present in the medium were separated and quantified by h.p.l.c. (Lazarowski *et al.*, 1995). To assess the activity of phosphatases on nucleotides *in vitro*, 10 μM [^{3}H]-UTP or [^{3}H]-UTPγS was incubated in the presence of 1 mM $MgCl_2$ at 25°C with various concentrations of acid phosphatase (pH 4.7), alkaline phosphatase (pH 9.5), or apyrase (pH 7.5). Incubations were terminated at the times indicated below by addition of 4 volumes of ice-cold 1 mM UTP and 2 mM EDTA, and the ^{3}H species were separated by h.p.l.c.

Reagents

Bovine liver NDPK (80 u mg^{-1}), yeast hexokinase (450 u mg^{-1}), acid phosphatase from potato (60 u mg^{-1}), calf alkaline phosphatase (400 u mg^{-1}), UTP, GTPγS, and other nucleotide standards were purchased from Boehringer (Indianapolis, IN). Apyrase from potato (80 u mg^{-1}) was from Sigma (St. Louis, MO). [^{3}H]-UTP (20 Ci $mmol^{-1}$) was obtained from Amersham (Arlington Heights, IL), [^{35}S]-GTPγS (1000 Ci $mmol^{-1}$) was purchased from DuPont NEN (Boston, MA), and myo-[2-^{3}H]-inositol (20 Ci $mmol^{-1}$) was from American Radiolabeled Chemicals (St. Louis, MO).

Results

NDPK exhibits a broad substrate specificity. We took advantage of this enzymatic activity to synthesize UTPγS by a reaction that utilized either GTPγS or ATPγS as the γ-phosphorothioate donor molecule and UDP as the acceptor substrate. Incubation of 2 μmol UDP, 1 μmol GTPγS, and 50 u of NDPK in the presence of 2 mM $MgCl_2$ resulted in formation of 0.65 μmol of GDP and in an equivalent molar decrease of UDP and GTPγS (Figure 1a,b). In addition, a novel species was generated that, as predicted for UTPγS, had an elution time (31.5 min) intermediate between UTP and GTPγS on an h.p.l.c. ion-exchange system (Figure 1). This species was not observed with incubations carried out in the absence of NDPK (Figure 1a and Figure 2b), UDP (Figure 2c), or GTPγS (not shown), or when Mg^{2+} ions were depleted by including 4 mM EDTA in the reaction mixture (data not shown). A species (UTPγS) that eluted at 31.5 min was also generated in addition to ADP when ATPγS was used as the donor substrate instead of GTPγS (data not shown).

The NDPK-catalyzed reaction also was carried out in the presence of radiolabelled precursors to confirm transference of the thiol moiety from GTPγS to UDP. This was demonstrated in two ways. First incubation of [^{35}S]-GTPγS and unlabelled UDP with NDPK resulted in ^{35}S labelling, i.e. [^{35}S]-UTPγS of the species eluting at 31.5 min (Figure 1c). Second, incubation of [^{3}H]-UDP and unlabelled GTPγS with NDPK resulted in ^{3}H labelling, i.e. [^{3}H]-UTPγS, of the species eluting in 31.5 min (Figure 1d). Thus, both uridine and the γ-thiophosphate could be transferred to the new species, i.e. UTPγS, by use of radioactive substrates. To support the conclusion that UTPγS was the molecular entity that was synthesized, the reverse NDPK reaction was carried out. That is, GDP and purified radioactive UTPγS were used as substrates and the production of UDP or GTPγS was measured. Incubations that contained

[^{3}H]-UTPγS resulted in production of [^{3}H]-UDP (data not shown) and incubations that contained [^{35}S]-UTPγS resulted in production of [^{35}S]-GTPγS (data not shown). Thus, the transfer of the γ-thiophosphate to GDP to produce GTPγS and the formation of UDP could be shown to occur by use of radioactive UTPγS.

NDPK-catalyzed transfer of γ-phosphate between nucleotides occurred rapidly under the conditions used, and steady state was reached within 5 min (data not shown; Buczynski & Potter, 1990). In contrast, synthesis of the γ-thio-substituted nucleotide triphosphates, i.e. GTPγS and ATPγS, has been shown to take place at a 1000 fold slower rate (Buczynski & Potter, 1990). As such, the time course and concentration-dependence for NDPK-catalyzed formation of [^{35}S]-UTPγS were determined (Figure 2). Formation of [^{35}S]-UTPγS in the presence of 50 u ml^{-1} NDPK, 2 mM UDP, and 1 mM [^{35}S]-GTPγS was time-dependent, and steady state was attained within 30 min (Figure 2a). Under these conditions the maximal rate of formation of [^{35}S]-UTPγS was obtained in the presence of approximately 30 u ml^{-1} of NDPK (Figure 2b). The effect of substrate concentration on the formation of UTPγS was examined under pseudo first order reaction conditions, i.e. the second substrate was present at near saturating concentrations. Rectangular hyperbolic curves were generated for both GTPγS (not shown) and UDP (Figure 2c), and the apparent K_m values (GTPγS = 0.54 mM, UDP = 1.44 mM) and the V_{max} (210 nmol min^{-1} mg^{-1}) were deduced from Eadie-Hofstee transformations (Figure 2c inset, and data not shown).

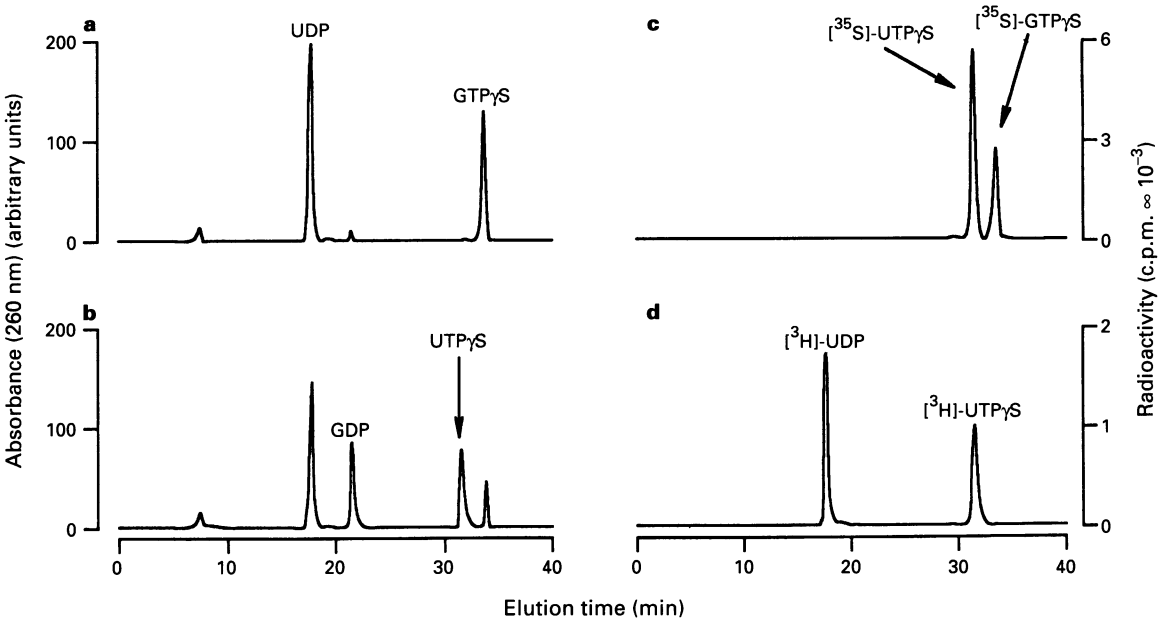

Figure 1 H.p.l.c. separation of the (NDPK)-reaction products. The incubation mixture consisted of 1 mM GTPγS, 2 mM UDP and 2 mM $MgCl_2$ in 1 ml 10 mM Tris, pH 7.5. Reactions were for 1 h in the absence (a) or in the presence (b, c, d) of 50 u ml^{-1} NDPK, and in the presence of 1 μCi of either [^{35}S]-GTPγS (c) or [^{3}H]-UDP (d). The data are representative of at least three experiments performed with duplicate samples.

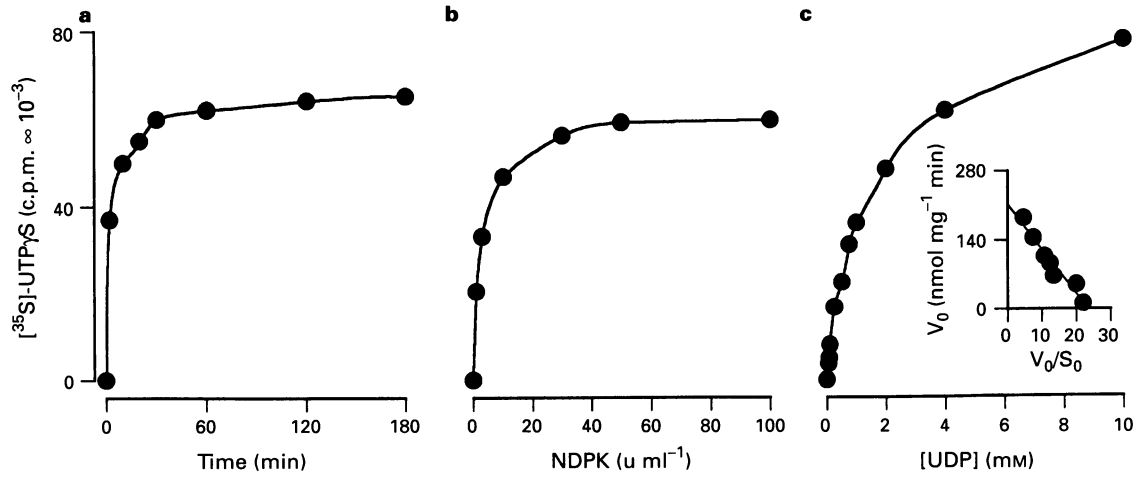

Figure 2 Nucleoside 5′-diphosphate kinase (NDPK)-catalyzed formation of uridine-5′-*O*-(3-thiotriphosphate) (UTPγS). Effect of time, enzyme and substrate concentration. (a) Incubations were for the times indicated in the presence of 50 u ml^{-1} NDPK, 2 mM UDP and (0.1 μCi) 1 mM [^{35}S]-GTPγS. (b) Incubations were for 30 min in the presence of 2 mM UDP and (0.1 μCi) 1 mM [^{35}S]-GTPγS. (c) The initial rate of UTPγS formation was determined for various UDP concentrations in the presence of (0.1 μCi) 2 mM [^{35}S]-GTPγS and 30 u ml^{-1} NDPK. An Eadie-Hofstee transformation of the data is shown in the inset. The data represent the mean value from two experiments performed in duplicate.

Scanning analysis of purified UTPγS revealed an absorbance peak at 262 nm that superimposed with that obtained with an equivalent concentration of UTP (Figure 3a). The observed molar extinction coefficient (ε_{262}, pH 7.4=

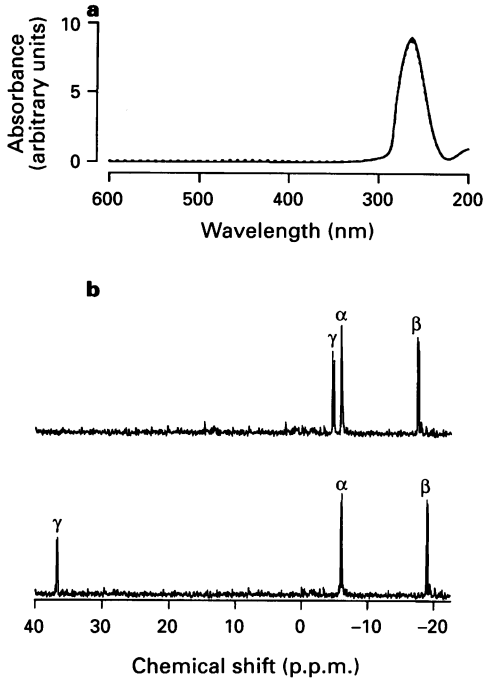

Figure 3 Spectral analysis of uridine-5′-*O*-(3-thiotriphosphate) (UTPγS). (a) 30 μM of either UTPγS (solid line) or UTP (dotted line) were scanned from 600 to 200 nm wavelength and the absorbance was simultaneously recorded as described in Methods. (b) ^{31}P-NMR spectra at 202.62 MHz of 1 mM UTP (upper panel) and 1 mM UTPγS (lower panel). The resonance frequency is quoted as part per million (p.p.m.) of the applied magentic field strength. The value p.p.m. = 1 was determined with standard ^{31}P-phosphoric acid.

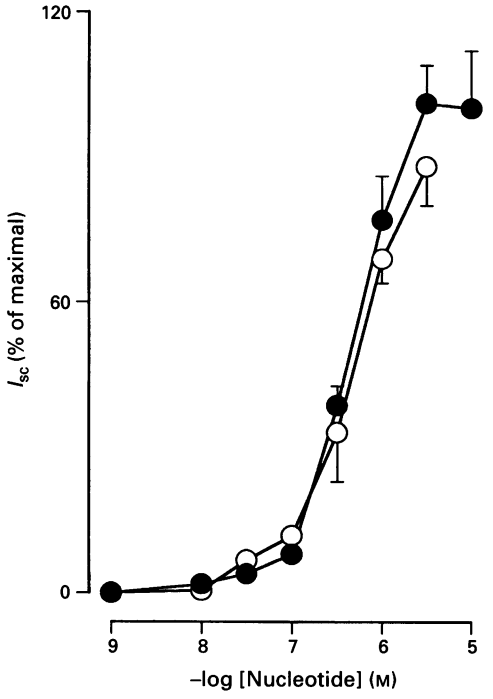

Figure 5 Uridine-5′-*O*-(3-thiotriphosphate) (UTPγS) and uridine 5′-triphosphate (UTP) stimulate short circuit currents (I_{sc}) in primary cultures of nasal epithelial cells from cystic fibrosis (CF) patients. Confluent monolayers of primary nasal epithelium obtained from CF patients were mounted in modified Ussing chambers and perfused with Krebs Ringer bicarbonate solution in the presence of 100 μM amiloride in the apical bath. Agonists were added to the apical bath (6 ml volume) and the short circuit current was measured under voltage clamp conditions. The data are expressed as the % of response obtained with 10 μM UTP ($\Delta I_{sc} = 24 \pm 4\ \mu A\ cm^{-2}$) and represent the mean value from cells cultured from two patients and assayed in duplicate. Error bars indicate the range of values obtained for each concentration of nucleotide. Symbols: (●) UTP; (○) UTPγS.

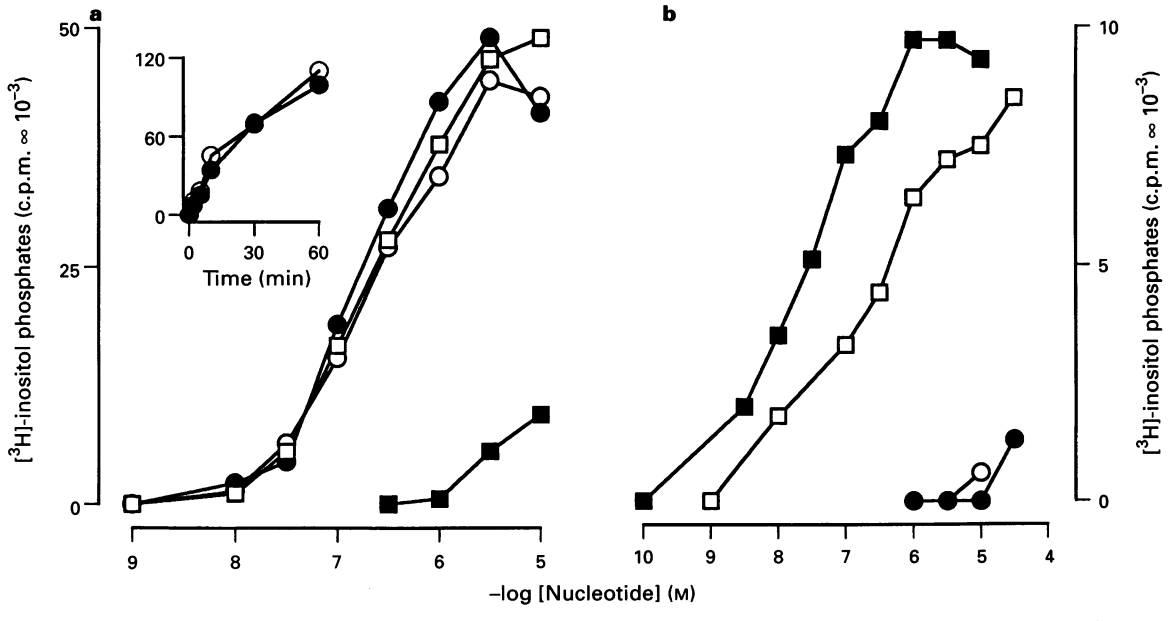

Figure 4 Uridine-5′-*O*-(3-thiotriphosphate) (UTPγS) and uridine 5′-triphosphate (UTP) stimulate the formation of [^{3}H]-inositol phosphates in HP2U-1321N1 human astrocytoma cells. Cells were labelled with [^{3}H]-myo-inositol, preincubated with 10 mM LiCl, and challenged with either 3 μM UTPγS or 3 μM UTP for the times indicated (a inset), or for 20 min in the presence of the indicated nucleotide concentrations (a). (b) Concentration-effect relationship of nucleotide-stimulated inositol phosphate formation in P2Y-1321N1 cells. Incubations were terminated by addition of 5% TCA and the individual [^{3}H]-inositol phosphates were separated on Dowex columns and quantified as described in Methods. The data are the mean values from at least 2 different experiments performed with triplicate samples. Symbols: (●) UTP; (□) ATP; (○) UTPγS; (■) 2MeSATP.

10.2 nmol l^{-1} cm^{-1}) of UTPγS indicated that the γ-phosphorothioate substitution has little or no effect on ε (ε_{262} = 10.0 nmol l^{-1} cm^{-1}) of UTP. ^{31}P n.m.r. spectra for UTP and UTPγS are shown in Figure 3b. The resonances of the γ-phosphates exhibited a large chemical shift difference consistent with the presence of different substitutions on the γ-residues. In contrast, no chemical shift difference was observed between the two α-phosphate resonances of UTP and UTPγS, and only a small difference was observed between the two β-phosphate resonances. The same spectral differences were observed between ATP and ATPγS standards (data not shown).

The activity of UTPγS at P_{2U}-purinoceptors was tested by measuring agonist-stimulated inositol phosphate formation in HP2U-1321N1 cells, an astrocytoma cell subclone stably expressing the phospholipase C-coupled human P_{2U}-purinoceptor (Parr *et al.*, 1994). UTPγS (3 μM) induced a sustained formation of inositol phosphates over a 60 min incubation period (Figure 4a inset). The time course for inositol phosphate formation was similar to that observed with an equivalent concentration of the potent P_{2U}-purinoceptor agonist, UTP. (In contrast to results obtained with airway epithelial cells (see below), only a minor (12%) hydrolysis of [^{3}H]-UTP was observed during a 20 min incubation of this nucleotide (0.1 μM) in the presence of HP2U-1321N1 cells). Concentration-effect curves for stimulation of inositol phosphate formation in HP2U-1321N1 cells illustrated that [^{3}H]-UTPγS was a very potent agonist for the P_{2U}-purinoceptor, with an EC_{50} (240 nM) very similar to the EC_{50} values of UTP (120 nM) and ATP (230 nM) (Figure 4a; (Lazarowski *et al.*, 1995)). In contrast to the effects observed with HP2U-1321N1 cells, UTPγS at concentrations as high as 10 μM produced little stimulation (i.e. approximately 20% of maximal stimulation obtained with 100 nM 2MeSATP) of inositol phosphate formation in P2Y-1321N1 cells (Figure 4b), a cell line expressing the recombinant P_{2Y}-purinoceptor (Filtz *et al.*, 1994).

An ultimate goal of this work was to test whether UTPγS is a stable and effective stimulus for Cl^- secretion in airway epithelial cells. Thus, the capacity of UTPγS to stimulate short circuit current (I_{sc}) in primary cultures of human nasal epithelial cells was examined. UTPγS was equally potent and efficacious to UTP for stimulation of chloride secretion in airway cells obtained from normal donors (data not shown) and cystic fibrosis patients (Figure 5).

The resistance of UTPγS to hydrolysis was examined with purified enzymes and on airway tissues. [^{3}H]-UTP (10 μM) was extensively hydrolyzed during a 10 s incubation with 0.01 u ml^{-1} of alkaline phosphatase or apyrase. In contrast, [^{3}H]-UTPγS remained intact after a 20 min incubation with a 10 fold higher concentration of either enzyme (not shown). Although minor (3%) hydrolysis of [^{3}H]-UTPγS was observed after a 20 min incubation with acid phosphatase, this breakdown occurred at an approximately 2000 times slower rate (0.0019 μmol min^{-1} mg^{-1}) than did hydrolysis of UTP (4.6 μmol min^{-1} mg^{-1}) (Figure 6). The susceptibility of UTPγS and UTP to the hydrolytic action of extracellular nucleotidases present on the human airway epithelial cell surface was also determined. Primary cultures of human nasal epithelial cells were incubated with 30 μM [^{3}H]-UTP or [^{3}H]-UTPγS added on the mucosal surface. [^{3}H]-UTP was substantially hydrolyzed over a 1 h incubation confirming our previous observations on the presence of phosphatases and nucleotidases on mucosal airway cell surfaces (Regnis *et al.*, 1994). In contrast, [^{3}H]-UTPγS remained essentially intact after a 1 h incubation (Figure 7). These results indicate that UTPγS is a poor substrate for extracellular airway epithelial cell hydrolases.

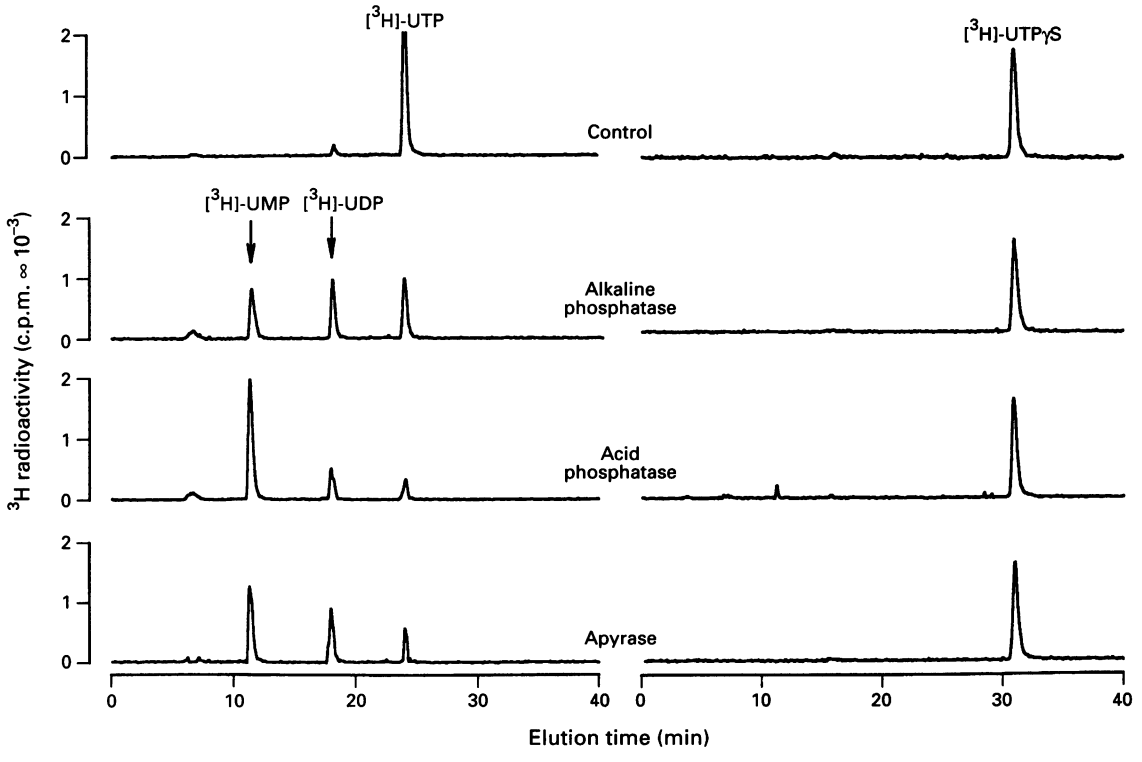

Figure 6 Effect of various phosphatases on [^{3}H]-uridine 5′-triphosphate ([^{3}H]-UTP) and [^{3}H]-uridine-5′-*O*-(3-thiotriphosphate) ([^{3}H]-UTPγS) *in vitro*. Approximately 300,000 c.p.m. ml^{-1} of either 10 μM [^{3}H]-UTPγS or 10 μM [^{3}H]-UTP were incubated with alkaline phosphatase, acid phosphatase, or apyrase for various periods of time and the resulting nucleotides were separated by h.p.l.c. as detailed in Methods. The tracings shown on the left panels are representative of a 10 s incubation of [^{3}H]-UTP with vehicle (top panel) or with 0.01 u ml^{-1} of the indicated enzyme. The tracings shown on the right panels correspond to [^{3}H]-UTPγS samples after a 20 min incubation period with vehicle (top panel) or with 0.1 u ml^{-1} of the indicated enzyme.

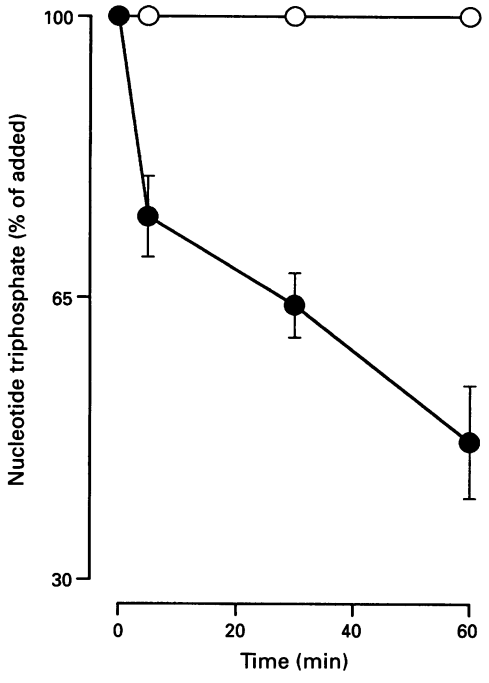

Figure 7 Susceptibility of uridine 5'triphosphate (UTP) and uridine-5'-*O*-(3-thiotriphosphate) (UTPγS) to the hydrolytic action of extracellular airway epithelial cell nucleotidases. Primary cultures of human nasal epithelial cells were incubated with approximately 300,000 c.p.m. ml^{-1} of either 30 μM [^{3}H]-UTPγS (○) or 30 μM [^{3}H]-UTP (●) added in the mucosal medium (0.25 ml volume). At the times indicated the medium was collected and ^{3}H nucleotides were quantified by h.p.l.c. as described in Methods. The data represent the mean value from two experiments performed with duplicate samples. Error bars indicate the range of values obtained for each time of incubation.

Discussion

The localization and regulatory activity of the P_{2U}-purinoceptor on human airways suggests that this protein is an important therapeutic target for diseases of the lung. Our results with UTPγS now place this uridine nucleotide analogue at the forefront of molecules that could prove clinically useful due to their activity at P_{2U}-purinoceptors. In tests of P_{2U}-purinoceptor activity, UTPγS was equipotent to ATP and UTP and was more potent than ATPγS. Its resistance to hydrolysis relative to ATP and UTP and its receptor selectivity over ATPγS, which also activates P_{2Y}-purinoceptors, are remarkable. Thus, UTPγS is a unique P_{2U}-purinoceptor agonist based on its potency, P_{2U}-over P_{2Y}-receptor selectivity, and stability in biological tissues.

NDPK-catalyzed transfer of thiophosphoryl groups between nucleosides has displaced lower yield chemical procedures for preparation of radioactively labelled nucleoside γ-thiotriphophates, e.g. GTPγS (Goody *et al.*, 1972). However, UDP has been shown to be a poor substrate (Buczynski & Potter, 1990) or an inhibitor (Seifert *et al.*, 1988) of NDPK-catalyzed reactions. Although chemical characterization and confirmation of structure were not presented, Seifert *et al.* have demonstrated that NDPK can be utilized to synthesize UTPγS (Seifert *et al.*, 1989). These investigators also found that this uridine nucleotide analogue produced a concentration-dependent potentiation of the effect of formyl methionyl leuncyl phenylaltanine (fMLP) on superoxide formation in human neutrophils. The selectivity of nucleotides for activation of this response did not fit that subsequently delineated for P_{2U}-purincoeptors in neutrophils and other tissues (Dubyak & El-Moatassim, 1993). However, the responses measured by Seifert *et al.* (1989) were relatively complex and other work has clearly established the presence of P_{2U}-purinoceptors on neutrophils (Cowen *et al.*, 1989). Thus, the effects studied by Seifert *et al.* most likely occurred through a P_{2U}-purinoceptor.

Our results illustrate that UTPγS can be synthesized by a simple procedure. In the presence of 30 u ml^{-1} of NDPK and near saturating initial concentrations of UDP (10 mM) and GTPγS (2 mM), a 60–70% transfer of the γ-thiophosphate to UTPγS was obtained in a 20–30 min incubation. Amounts of UTPγS sufficient for detailed pharmacological studies can be produced as can low quantities of radioactively-labelled compound.

ATPγS heretofore has been the only hydrolysis resistant agonist available for activation of P_{2U}-purinoceptors (Lazarowski *et al.*, 1995). The lower potency of ATPγS relative to ATP usually observed at P_{2U}-purinoceptors suggested that thiol-substitution of the γ-phosphate of UTP would result in a molecule that was less potent than UTP. However, UTPγS stimulated inositol phosphate formation in HP2U-1321N1 cells with a potency (EC_{50} = 240 nM) that was approximately 10 fold higher than the potency of ATPγS (EC_{50} = 1.72 μM) and essentially the same as the potencies of ATP and UTP, which previously were shown to be the most potent P_{2U}-purinoceptor agonists (Lazarowski *et al.*, 1995). UTPγS is the first nucleotide derivative to be shown to stimulate P_{2U}-purinoceptors with a potency similar to its natural congener. Our data showing little or very weak effects of UTPγS on P2Y-1321N1 cells, a cell line transfected with the avian P_{2Y}-purinoceptor (Filtz *et al.*, 1994), indicate that UTPγS is selective for the P_{2U}-purinoceptor over the P_{2Y}-purinoceptor. Although not tested, UTPγS also is unlikely to be an agonist at P_{2X}-, P_{2T}-, or P_{2Z}-purinoceptors. It will be important to synthesize and establish the potencies of other uridine nucleotide analogues. For example, diuridine tetraphosphate may be a very potent agonist at P_{2U}-purinoceptors based on the potency of UTPγS and on the higher potency of diadenosine tetraphosphate relative to ATPγS on HP2U-1321N1 cells (Lazarowski *et al.*, 1995).

We reasoned that UTP and UTPγS labelled in the uridine base would be useful molecules for examining the relative resistance of these nucleotides to hydrolysis. By using hexokinase to remove the γ-phosphate of [^{3}H]-UTP and h.p.l.c.-based resolution of products, highly purified [^{3}H]-UDP was obtained and subsequently used to synthesize [^{3}H]-UTPγS. Phosphatases are a group of widely distributed enzymes that act on a broad range of monoesters of orthophosphoric acid. Both alkaline phosphatase and acid phosphatase hydrolyze sulphur-containing esters, although with different selectivity. Alkaline phosphatase cannot hydrolyze *O*-substituted monoesters of phosphorothioic acids, e.g. nucleoside 5'-*O*-(2-thiodiphosphates) and nucleoside 5'-*O*-(3-thiotriphosphates). In contrast, acid phosphatase appears to require oxygen in the linkage that is split (Dixon *et al.*, 1979). Consistent with the known activities of phosphatases, UTPγS was resistant to hydrolysis by alkaline phosphatase, and although acid phosphatase hydrolyzed UTPγS, the rate was several thousand times slower than that observed with UTP. In addition to results showing little or no breakdown of UTPγS by non-specific phosphatases, the ATPase apyrase (0.1 u ml^{-1}), which completeley hydrolyzed UTP in a 10 s incubation, had no effect on UTPγS during a 20 min incubation.

Although it was important to show that UTPγS was resistant to hydrolysis by various phosphatases or nucleotidases, the most important analyses came in comparative studies of its stability in the presence of tissue. Although UTP was metabolized, UTPγS was unchanged after a prolonged incubation in the presence of airway tissue. Taken together, these results suggest that UTPγS is a poor substrate for phosphatases and nucleotidases, and represents a P_{2U}-purinoceptor agonist with actions that should be long-lasting.

In summary, our data indicate that UTPγS is a non-hydrolyzable nucleotide analogue that potently and selectively activates the P_{2U}-purinoceptor. The high potency of UTPγS for promotion of Cl^- secretion in primary cultures of CF

nasal epithelial cells along with the high stability of UTPγS relative to UTP on an airway cell surface strongly suggest that UTPγS has considerable potential as a therapeutic agent.

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References

BOUCHER, R.C. (1993). Human airway ion transport. *Am. J. Respir. Crit. Care Med.*, **150,** 581–593.

BROWN, H.A., LAZAROWSKI, E.R., BOUCHER, R.C. & HARDEN, T.K. (1991). Evidence that UTP and ATP regulate phospholipase C through a common extracellular 5'-nucleotide receptor in human airway epithelial cells. *Mol. Pharmacol.*, **40,** 648–655.

BUCZYNSKI, G. & POTTER, R.L. (1990). Nucleoside diphosphate kinase from *Xenopus* oocytes; partial purification and characterization. *Biochim. Biophys. Acta*, **1041,** 296–304.

CLARKE, L.L. & BOUCHER, R.C. (1992). Chloride secretory response to extracellular ATP in human normal and cystic fibrosis nasal epithelia. *Am. J. Physiol.*, **263,** C348–C356.

CLARKE, L.L., GRUBB, B.R., YANKASKAS, J.R., COTTON, C.U., MCKENZIE, A. & BOUCHER, R.C. (1994). Relationship of a non-cystic fibrosis transmembrane conductance regulator-mediated chloride conductance to organ-level disease in *Cftr* (-/-) mice. *Proc. Natl. Acad. Sci. U.S.A.*, **91,** 479–483.

COWEN, D.S., LAZARUS, H.M., SHURIN, S.B., STOLL, S.E. & DUBYAK, G.R. (1989). Extracellular adenosine triphosphate activates calcium mobilization in human phagocytic leukocytes and neutrophil/monocyte progenitor cells. *J. Clin. Invest.*, **83,** 1651–1660.

DIXON, M., WEBB, E.C., THORNE, C.J.R. & TIPTON, K.F. (1979). Enzyme specificity. In *Enzymes*. 3rd Ed. ed. Anonymous. pp. 231–270. New York: Academic Press Inc.

DUBYAK, G.R. (1991). Signal transduction by P_2-purinergic receptors for extracellular ATP. *Am. J. Respir. Cell Mol. Biol.*, **4,** 295–300.

DUBYAK, G.R. & EL-MOATASSIM, C. (1993). Signal transduction via P_2-purinergic receptor for extracellular ATP and other nucleotides. *Am. J. Physiol.*, **265,** C577–C606.

FILTZ, T.M., LI, Q., BOYER, J.L., NICHOLAS, R.A. & HARDEN, T.K. (1994). Expression of a cloned P_{2Y}-purinergic receptor that couples to phospholipase C. *Mol. Pharmacol.*, **46,** 8–14.

FREDHOLM, B.B., ABBRACCHIO, M.P., BURNSTOCK, G., DALY, J.W., HARDEN, T.K., JACOBSON, K.A., LEFF, P. & WILLIAMS, M. (1994). Nomenclature and classification of purinoceptors. *Pharmacol. Rev.*, **46,** 143–156.

GOODY, R.S., ECKSTEIN, F. & SCHIRMER, R.H. (1972). The enzymatic synthesis of thiophosphate analogs of nucleotides. *Biochim. Biophys. Acta*, **276,** 155–161.

HARDEN, T.K., BOYER, J.L. & NICHOLAS, R.A. (1995). P_2-purinergic receptors: subtype-associated signalling responses and structure. *Ann. Rev. Pharmacol. Toxicol.*, **35,** 541–579.

KNOWLES, M.R., CLARKE, L.L. & BOUCHER, R.C. (1991). Extracellular nucleotides activate chloride secretion in cytstic fibrosis airway epithelia in vivo. *N. Engl. J. Med.*, **325,** 533–538.

KNOWLES, M.R., CLARKE, L.L. & BOUCHER, R.C. (1992). Extracellular ATP and UTP induce chloride secretion in nasal epithelia of cystic fibrosis patients and normal subjects in vivo. *Chest*, **101,** 60S–63S.

KNOWLES, M.R., OLIVIER, K.N., BENNETT, W., HOHNEKER, K., GEARY, C., DAVIS, C.W. & BOUCHER, R.C. (1994). Aerosolized uridine triphosphate (UTP) +/− amiloride: safety and effect on mucociliary clearance in normal subjects and CF patients. *Pediatric Pulmonology*, **Suppl. 10,** 99(Abstract).

LAZAROWSKI, E.R. & HARDEN, T.K. (1994). Identification of a uridine nucleotide-selective G-protein-linked receptor that activates phospholipase C. *J. Biol. Chem.*, **269,** 11830–11836.

LAZAROWSKI, E.R., WATT, W.C., STUTTS, M.J., BOUCHER, R.C. & HARDEN, T.K. (1995). Pharmacological selectivity of the cloned human P_{2U}-purinergic receptor: potent activation by diadenosine tetraphosphate. *Br. J. Pharmacol.*, **115,** 1619–1627.

MASON, S.J., PARADISO, A.M. & BOUCHER, R.C. (1991). Regulation of transepithelial ion transport and intracellular calcium by extracellular adenosine triphosphate in normal and cystic fibrosis airway epithelium. *Br. J. Pharmacol.*, **103,** 1649–1656.

O'CONNOR, S.E., DAINTY, I.A. & LEFF, P. (1991). Further subclassification of ATP receptors based on agonist studies. *Trends Pharmacol. Sci.*, **12,** 137–141.

PARR, C.E., SULLIVAN, D.M., PARADISO, A.M., LAZAROWSKI, E.R., BURCH, L.H., OLSEN, J.C., ERB, L., WEISMAN, G.A., BOUCHER, R.C. & TURNER, J.T. (1994). Cloning and expression of a human P_{2U} nucleotide receptor, a target for cystic fibrosis pharmacology. *Proc. Natl. Acad. Sci. U.S.A.*, **91,** 3275–3279.

RAO, B.D.N. (1989). Nuclear magnetic resonance line-shape analysis and determination and exchange rates. *Methods Enzymol.*, **176,** 279–311.

RATLIFF, R.L., WEAVER, R.H., LARDY, H.A. & KUBY, S.A. (1964). Nucleoside triphosphate-nucleoside diphosphate transphosphorylase (nucleoside diphosphokinase): isolation of the crystalline enzyme from brewer's yeast. *J. Biol. Chem.*, **239,** 301–309.

REGNIS, J.A., LAZAROWSKI, E.R., HENKE, D.C., MASON, S.J., OLIVIER, K.N., FOY, C.E., WATT, W.C., BOUCHER, R.C. & KNOWLES, M.R. (1994). Metabolism of uridine 5'-triphosphate in vitro by human airway epithelial cells. bronchoalveolar lavage fluid, blood and T-84 cells. *Pediatric Pulmonology*, **Suppl. 10,** 240–241 (Abstract).

SEIFERT, R., ROSENTHAL, W., SCHULTZ, G., WIELAND, T., GIERSCHICK, P. & JAKOBS, K.H. (1988). The role of nucleoside-diphosphate kinase reactions in G protein activation of NADPH oxidase by guanine and adenine nucleotides. *Eur. J. Biochem.*, **175,** 51–55.

SEIFERT, R., WENZEL, K., ECKSTEIN, F. & SCHULTZ, G. (1989). Purine and pyrimidine nucleotides potentiate activation of NADPH oxidase and degranulation by chemotactic peptides and induce aggregation of human neutrophils via G-proteins. *Eur. J. Biochem.*, **181,** 277–285.

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