The Effects of Extracellular Purines and Pyrimidines on Human Airway Smooth Muscle Cells

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Received May 17, 2005; accepted August 10, 2005

ABSTRACT

Extracellular ATP and UTP modulate the function of many cell types through the stimulation of specific P2 receptors, and the inhalation of UTP has been proposed as a therapeutic means of increasing mucociliary clearance in cystic fibrosis patients. The aim of this study was to determine whether P2 receptors are present and functional in human airway smooth muscle (HASM) cells. Experiments were conducted on primary cultures of HASM cells. Reverse transcription-polymerase chain reaction and Western blot analysis showed that P2Y1, P2Y2, P2Y4, and P2Y6 receptor subtypes are expressed. Exposure to extracellular ATP, UTP, ADP, and UDP at concentrations ranging from 10^{-6} to 10^{-4} M, produced significant increases in intracellular Ca^{2+} that peaked to 491 ± 51 nM (p < 0.001) with ATP 10^{-5} M and to 321 ± 30 nM with UTP 10^{-4} M. ATP and UTP also induced HASM cell contraction, decreasing cell length by 9.9 ± 4.3 and 5.6 ± 2.0%, respectively. Pretreatment of the cells with UTP for short periods of time (10 and 30 min) enhanced the peak Ca^{2+} release to UTP, whereas repeated and prolonged pretreatment with UTP decreased it. These results indicate that several subtypes of P2Y receptors are present and functional in HASM cells. They also show that the response of the receptors is increased after short periods of exposure to UTP and decreased after prolonged and repeated exposure. Considering that ATP and UTP are endogenous mediators and that analogs of UTP could be used as a therapeutic modality, the role of extracellular triphosphate nucleotides in physiological and pathophysiological processes in the airways warrants further investigation.

Extracellular triphosphate nucleotides such as ATP, UTP, and related compounds have significant biological effects, including the modulation of synaptic transmission in the central and peripheral nervous systems, mast cell degranulation, and the modulation of visceral and vascular smooth muscle contractility (for review, see Ralevic and Burnstock, 1998). In the cardiovascular system, extracellular ATP has positive inotropic and chronotropic effects on the myocardium and induces contraction and relaxation of vascular smooth muscle cells (Ralevic and Burnstock, 1998). It also regulates vascular smooth muscle cell proliferation and apoptosis, and it is thought to be a significant mediator of vessel wall remodeling (Boarder and Hourani, 1998; Erlinge, 2004). Although the effects of extracellular purine nucleotides have been extensively investigated in the cardiovascular, urogenital, gastrointestinal, and nervous systems, research on the effects of these compounds in the respiratory system is more recent. In the respiratory system, ATP and UTP act on various cell types: ATP is one of the most powerful known agonists for surfactant secretion by alveolar type II cells, and it also increases Cl^- secretion and arachidonic acid metabolism when applied to the apical surface of cultured airway epithelial cells (Knowles et al., 1991; Hwang et al., 1996). Mucin secretion by submucosal glands and goblet cells is also increased in the presence of ATP (Kim and Lee, 1991). These effects are mediated by specific receptors, the P2 receptors, present on the cell plasma membrane. It has also been reported that endogenous sources of ATP and UTP are present in the airways, indicating that these compounds are physiological mediators. Indeed, airway epithelial cells are a significant source of ATP and UTP, which can be released after simple mechanical stimuli (Lazarowski et al., 1997a) and cellular swelling (Hazama et al., 1999). In addition, Lazarowski et al. (2000) have reported that the concentration of ATP in the extracellular fluid overlying cultured human bronchial epithelial cells remains stable, despite continuous ATP hydrolysis, indicating the presence of constant sources.

ABBREVIATIONS: HASM, human airway smooth muscle; AM, acetoxymethyl ester; PCR, polymerase chain reaction; 2-MeSATP, 2-methylthio-ATP; ANOVA, analysis of variance; RT, reverse transcription.
of ATP. The powerful effects of extracellular triphosphate nucleotides on mucociliary clearance have led some investigators to investigate the therapeutic potential of P2Y receptor stimulation for the improvement of mucociliary clearance in subjects with cystic fibrosis (Knowles et al., 1995; Bennett et al., 1996). Kreda et al. (2000) have also described another therapeutically oriented use of the P2Y2/4 receptor and its ligand UTP as a gene transfer vector.

The effects of ATP on the airway smooth muscle of various animal species have produced conflicting results. In vivo, Flezar et al. (1992) have reported that intratracheal instillation of ATP produces an increase in airway resistance in rats. In vitro, the published data on airway tone are conflicting; both relaxant and contractile effects of ATP on airway smooth muscle have been observed (Fedan et al., 1993a,b, 1994). These discrepancies are likely due to the type of preparation studied and the presence or absence of epithelium. In rat airway smooth muscle cells, extracellular ATP and UTP induce cell proliferation and trigger a substantial increase in intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]), one of the main determinants of smooth muscle contraction (Michoud et al., 1997, 1999). Also, a recent publication by Bergner and Sanderson (2002) shows that ATP and UTP induce airway smooth muscle cell contraction in mouse lung slices.

The effects of ATP and UTP on human airway smooth muscle (HASM) cells have not, to our knowledge, been determined. Thus, the aim of this work was to determine whether P2 receptors are present in HASM cells and to characterize the direct and indirect effects of ATP, UTP, and their metabolites on these cells. In an attempt to mimic the in vivo conditions that would prevail if cystic fibrosis patients were treated with inhaled P2Y receptor ligands, the modifying effects of short and prolonged periods of exposure to UTP on smooth muscle cell responses were also measured. Indeed, because cystic fibrosis patients frequently have inflamed and ulcerated airways, therapies based on the stimulation of P2Y receptor ligands to stimulate airway clearance could also affect the underlying airway smooth muscle cells.

### Materials and Methods

#### Cell Cultures

Cultures of HASM cells were prepared from surgical and lung transplant specimens as described by Panettieri et al. (1989). Segments of lobar or main bronchi measuring 5 × 2 mm were incubated for 90 min at 37°C in 10 ml of Hanks' balanced salt solution buffer (5 mM KCl, 0.3 mM KH\(_2\)PO\(_4\), 138 mM NaCl, 4 mM NaHCO\(_3\), and 5.6 mM Na\(_2\)HPO\(_4\)) to which 640 U of collagenase type IV, 10 mg/ml of both sense and antisense primers for P2Y\(_2\), MgCl\(_2\) (3 mM), and 20 pmol of the 5' and 3' primers, mRNA from P2Y\(_2\), P2Y\(_4\), P2Y\(_6\), and P2Y\(_{12}\) receptor and from cyclophilin (a housekeeping gene) was amplified from the resulting cDNA with the appropriate oligonucleotide primers (Table 1) by PCR. For the amplification of P2Y\(_2\) and P2Y\(_6\), the PCR conditions were as follows: initial denaturation for 5 min at 94°C followed by 35 cycles with each cycle consisting of denaturation at 94°C for 1 min, annealing at specific temperature for 1 min and extension at 72°C for 3 min. For P2Y\(_2\), 25 cycles for the first reaction followed by 20 cycles for the second reaction were performed. Real-time PCR was performed using a fluorescence temperature cycler (Light Cycler; Roche Diagnostics, Montreuil, QC, Canada). Twenty microliters of reaction mix contained 2 μl of cDNA and 4 μl of both sense and antisense primers for P2Y\(_{12}\), MgCl\(_2\) (3 mM), and 2 μl of fast start DNA SYBR Green with Taq polymerase and H\(_2\)O. PCR cycling parameters: initial denaturation step at 95°C for 10 min was followed by PCR (95°C for 15 s and extension at 72°C for 24 s) was performed for 40 cycles. After completion of the cycling process, samples were subjected to melting curve analysis for 1 cycle (denaturation at 95°C followed by 66°C and ended by 95°C and cooling). The amplified products were visualized by ethidium bromide staining after 2% agarose gel electrophoresis, and the size of the bands was determined by comparison with the DNA molecular markers (Roche Diagnostics). To confirm the presence of P2Y\(_2\) and P2Y\(_{12}\) mRNA, PCR products were purified with GFX PCR and gel purification kit (GE Healthcare, Piscataway, NJ) and sequenced at the Institute de Recherche en Sciences de la Vie et de la Santé (University of Laval, Quebec, Canada). Samples for RNA extraction were obtained from four different subjects. In all experiments, human placenta (BD Biosciences Clontech, Palo Alto, CA) was used.

#### Measurement of Intracellular Ca\(^{2+}\)

Cells grown on 25-mm-diameter coverslips in 35-mm-diameter wells were used 10 to 14 days postplating. As described previously, cells were incubated for 20 to 30 min at 37°C with Hanks' buffer (137 mM NaCl, 4.2 mM NaHCO\(_3\), 10 mM glucose, 3 mM Na\(_2\)HPO\(_4\), 5.4 mM KCl, 0.4 mM KH\(_2\)PO\(_4\), 1.3 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 0.8 mM MgSO\(_4\), and 5 mM HEPEs) containing 5 μM Fura 2-acetoxymethyl ester (Fura-2AM) and 0.02% polyoxyethylene/polyoxypropylene copolymer (Pluronic F-127) (Michoud et al., 1997). The loaded cells were then washed and the coverslips placed in a Leiden chamber (Medical Systems Corp., Greenville, NY) containing 450 μl of Hanks' buffer on the stage of an inverted microscope equipped for cell imaging with 40× oil objective (Nikon, Montreal, QC, Canada). The cells were imaged using an intensified camera (VideoScope IC 200) and PTI software (Photon Technology International, Lawrenceville, NJ) at a single emission wavelength (510 nm) with double excitation wavelengths (340 and 380 nm). The fluorescence ratio (340/380) was measured in individual cells (n = 8 per slide), and [Ca\(^{2+}\)] was calculated using a dissociation constant of Ca\(^{2+}\) to Fura-2 of 224 nM (Tsien et al., 1985). Maximum ratio was determined in cells exposed to 10^{-5} M ionomycin in the presence of 1.3 mM CaCl\(_2\) and minimum ratio in Ca\(^{2+}\)-free Hanks' buffer to which EGTA (10^{-3} M) and ionomycin (10^{-5} M) had been added. Background fluorescence and autofluorescence were automatically subtracted.

#### Detection of P2-Receptor Subtype mRNA Expression

Total RNA was extracted from confluent cells (10th day after passage) by TRIzol reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions. To analyze mRNA, single-strand cDNA was synthesized in a 20-μl reaction using 2 μg of total RNA as a template, oligo(dT)\(_{12-18}\) primer, and Superscript II enzyme in the presence of acetylated bovine serum albumin (Invitrogen) and RNAGuard ribonuclease inhibitor (GE Healthcare, Montreal, QC, Canada). Conventional PCR was done for the detection of P2Y\(_1\), P2Y\(_4\), and P2Y\(_6\) subtypes and real-time PCR for P2Y\(_2\). The PCR mixture consisted of 1.5 mM MgCl\(_2\), 1× PCR buffer, 0.2 μM dNTP mix, 2.5 units of Platinum Taq polymerase (Invitrogen), and 20 pmol of the 5' and 3' primers, mRNA from P2Y\(_1\), P2Y\(_2\), P2Y\(_4\), and P2Y\(_6\) receptor and from cyclophilin (a housekeeping gene) was amplified from the resulting cDNA with the appropriate oligonucleotide primers (Table 1) by PCR. For the amplification of P2Y\(_2\) and P2Y\(_6\), the PCR conditions were as follows: initial denaturation for 5 min at 94°C followed by 35 cycles with each cycle consisting of denaturation at 94°C for 1 min, annealing at specific temperature for 1 min and extension at 72°C for 3 min. For P2Y\(_2\), 25 cycles for the first reaction followed by 20 cycles for the second reaction were performed. Real-time PCR was performed using a fluorescence temperature cycler (Light Cycler; Roche Diagnostics, Montreuil, QC, Canada). Twenty microliters of reaction mix contained 2 μl of cDNA and 4 μl of both sense and antisense primers for P2Y\(_{12}\), MgCl\(_2\) (3 mM), and 2 μl of fast start DNA SYBR Green with Taq polymerase and H\(_2\)O. PCR cycling parameters: initial denaturation step at 95°C for 10 min was followed by PCR (95°C for 15 s and extension at 72°C for 24 s) was performed for 40 cycles. After completion of the cycling process, samples were subjected to melting curve analysis for 1 cycle (denaturation at 95°C followed by 66°C and ended by 95°C and cooling). The amplified products were visualized by ethidium bromide staining after 2% agarose gel electrophoresis, and the size of the bands was determined by comparison with the DNA molecular markers (Roche Diagnostics). To confirm the presence of P2Y\(_2\) and P2Y\(_{12}\) mRNA, PCR products were purified with GFX PCR and gel purification kit (GE Healthcare, Piscataway, NJ) and sequenced at the Institute de Recherche en Sciences de la Vie et de la Santé (University of Laval, Quebec, Canada). Samples for RNA extraction were obtained from four different subjects. In all experiments, human placenta (BD Biosciences Clontech, Palo Alto, CA) was used.
Measurements of Contraction

HASM cells were plated in six-well plates onto 25-mm-diameter coverslips coated with laminin (1 μg/cm²) at an approximate density of 1500 cells/cm² (Hirst et al., 2000a). They were grown for 4 days in a culture medium containing 5% fetal bovine serum. The coverslips were then transferred into a Leiden chamber (Medical Systems Corp.) containing 450 μl of Hanks’ buffer. Images were taken using an inverted microscope (Nikon) at 15× magnification using Nomarski optics. A charge-coupled device camera (Hamamatsu C 2400) and commercial software (Photon Technology International) were used to acquire and record the images. Images were taken once before and up to 10 min after the addition of the agonists or vehicle. Images were analyzed with the Scion software (National Institutes of Health, Bethesda, MD). The length of the cells was measured before and 10 min after the addition of the agonists and contraction expressed as the percentage decrease in cell length from the initial value.

Experimental Protocols

Pharmacological P2 Receptor Identification. Peaks in [Ca²⁺], were determined after stimulation with a range of concentrations (10⁻⁶–10⁻⁴ M) of ATP, ADP, adenosine UTP, UDP, and 2-methylthio-ATP (2-MeSATP). All test drugs were diluted in Hanks’ buffer from frozen stock solutions. They were prewarmed to 37°C and 2-methylthio-ATP (2-MeSATP). All test drugs were diluted in Hanks’ buffer. Images were taken using an inverted microscope (Nikon) at 15× magnification using Nomarski optics. A charge-coupled device camera (Hamamatsu C 2400) and commercial software (Photon Technology International) were used to acquire and record the images. Images were taken once before and up to 10 min after the addition of the agonists or vehicle. Images were analyzed with the Scion software (National Institutes of Health, Bethesda, MD). The length of the cells was measured before and 10 min after the addition of the agonists and contraction expressed as the percentage decrease in cell length from the initial value.

Short-Term Exposure of HASM Cells to UTP. Cells were cultured on glass coverslips in six-well plates. On the 10th day postplating, UTP (10⁻⁴ M) or vehicle (Hanks’ buffer) was added for 5, 10, 30, or 60 min to the culture medium. After exposure to UTP, the cells were washed and loaded with Fura-2-AM for measurement of [Ca²⁺].

Prolonged and Repeated Exposures of HASM Cells to UTP. Cells were cultured on glass coverslips in six-well plates. On the 10th day postplating, either vehicle or UTP (10⁻⁴ M) was added once a day for 3 days and [Ca²⁺], was measured in response to various agonists on the 10th day. The experiments on short- and long-term exposure to UTP were carried out on cells originating from four different patients.

Chemicals

Elastase and collagenase were purchased from Sigma-Aldrich (St. Louis, MO), and Fura-2AM and Fura-AM F-127 were from Molecular Probes (Eugene, OR). ATP, ADP, and 2-MeSATP were purchased from Sigma-Aldrich. UDP was from Roche Diagnostics. EGTA was from Calbiochem (San Diego, CA).

TABLE 1

Summary of PCR primers sequences for P₂Y receptor

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences (5’-3’)</th>
<th>Target Size</th>
<th>Annealing</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₂Y₁</td>
<td>F: CCGCCCGCCCTAAGTCGAG</td>
<td>640 bp</td>
<td>52 °C</td>
<td>25 cycles followed by 20 cycles</td>
</tr>
<tr>
<td></td>
<td>R: GGTGTGTAACAGGTGATGG</td>
<td></td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>P₂Y₂</td>
<td>F: CAGGGCCCCCGTGCTACTTTTG</td>
<td>367 bp</td>
<td>56 °C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: CATGTGATGGGCTTGAAGGTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P₂Y₄</td>
<td>F: CCACCTGCGGATTGTCAGAC</td>
<td>425 bp</td>
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<td>35</td>
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<tr>
<td></td>
<td>R: GAGTACCCAGCAGGACGAC</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>P₂Y₆</td>
<td>F: CGCTTCCTCCTCTTATGCGAAC</td>
<td>65 bp</td>
<td>60 °C</td>
<td>35</td>
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<tr>
<td></td>
<td>R: CCATCTGGGGCGGCACAGGGGC</td>
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</tr>
<tr>
<td>Cyclophilin</td>
<td>F: GTGCAAACCCACCGTTGTTCTTG</td>
<td>566 bp</td>
<td>60 °C</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>R: GTGCUCCTCCTGAGCTACAG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

bp, base pair(s); F, forward; R, reverse.

as positive control. PCR primers were synthesized at the Sheldon Biotechnology Centre (Montreal, QC, Canada).

Statistical Analysis

The data are expressed as mean ± S.E.M. (n, number of cells). In pharmacological experiments, the cells were obtained from four subjects. Comparison of means was performed using paired or unpaired Student’s t tests as appropriate, and when multiple comparisons were made, the Bonferroni correction was applied. ANOVA followed by Student’s t test was used for acute and chronic UTP exposure studies. p < 0.05 was considered to be statistically significant.

Results

Pharmacological Identification of P₂ Receptors. After exposure to 10⁻⁶ M UTP, cells responded with a rapid rise in [Ca²⁺], reflected by the change in fluorescence ratio for Fura-2 and a subsequent decline to a more sustained plateau (n = 3; Fig. 1A). Concentration-response curves for ATP, ADP, adenosine, UTP, and UDP are shown in Fig. 1B. ATP and ADP induced significant increases in [Ca²⁺], at all concentrations tested (10⁻⁶–10⁻⁴ M). After ATP stimulation at a concentration of 10⁻⁵ M, the release of peak [Ca²⁺], was 491 ± 51 nM (p < 0.001; n = 27), whereas a concentration of 10⁻⁴ M elicited a smaller release of Ca²⁺ with peak [Ca²⁺], reaching 389 ± 34 nM, indicating that a plateau had been reached. ADP also induced a significant Ca²⁺ release that peaked at 10⁻⁵ M with a [Ca²⁺], of 349 ± 27 nM (p < 0.001; n = 32). Exposure to adenosine, the metabolite of ATP produced very small, albeit statistically significant increases in [Ca²⁺], with a peak of 150 ± 7 nM (n = 54) at 10⁻⁵ M, indicating the presence of functional adenosine receptors. The cells also responded to pyrimidine nucleotides: at a concentration of 10⁻⁴ M, peak [Ca²⁺], release in response to UTP with peak [Ca²⁺], was 480 ± 5 M (p < 0.005; n = 58) and 255 ± 41 nM (p < 0.005; n = 57) in response to UDP.

Figure 2A shows the increase in [Ca²⁺], produced by 2-MeSATP, an agonist for P₂Y₃ and P₂X₁ and P₂X₃ receptors, in the presence and in the absence of Ca²⁺ in the extracellular fluid. In the presence of Ca²⁺, the agonist caused significant increases in [Ca²⁺]: 26.0 ± 5 M (p < 0.05; n = 48) at 10⁻⁵ M and 143 ± 17 nM (p < 0.005; n = 51) at 10⁻⁴ M. In Ca²⁺-free medium, the increases in [Ca²⁺], were not statistically different from those observed in the Ca²⁺ containing Hanks’ buffer (38 ± 12 nM; n = 40 and 121 ± 20 nM; n = 31, respectively), indicating that the increase in [Ca²⁺], is due to release from intracellular stores rather than influx from extracellular sources. Thus, these data indicate that the response is mediated by the P₂Y₁ receptor subtype, a G protein-coupled receptor, rather than the P₂X₁ or P₂X₃ receptors that are ligand-gated ion channels. Figure 2B shows
the release of Ca\textsuperscript{2+} produced by ATP (10^{-5} M) in Ca\textsuperscript{2+}-containing and Ca\textsuperscript{2+}-free extracellular medium. The increase in [Ca\textsuperscript{2+}]\textsubscript{i} was comparable in both conditions: 112 ± 15 nM (n = 32) in presence of Ca\textsuperscript{2+} versus 106 ± 16 nM (n = 24) in the absence of Ca\textsuperscript{2+}, indicating that Ca\textsuperscript{2+} release was not dependent on extracellular Ca\textsuperscript{2+} influx and confirming that the agonist is acting on a G protein-coupled receptor, possibly P2Y, rather than on ligand-gated ion channels.

Expression of mRNA for P2Y Receptor Subtypes.

Conventional and real-time PCR analysis using different sets of primers for P2Y receptors (Fig. 3) showed the expression of mRNA coding for P2Y\textsubscript{1}, P2Y\textsubscript{2}, P2Y\textsubscript{4}, and P2Y\textsubscript{6} in HASM cells. mRNA expression for P2Y\textsubscript{1}, P2Y\textsubscript{4}, and P2Y\textsubscript{6} receptors was detected with conventional PCR. P2Y\textsubscript{2} receptor mRNA was detected with real-time PCR. Because we had difficulty in obtaining the signals from positive controls (human placenta) with P2Y\textsubscript{2} receptor primers, the PCR products from P2Y\textsubscript{2} receptor reactions were sent for sequencing. The sequences were matched with the reported gene sequences for the P2Y\textsubscript{2} gene (GenBank no. NM_002564), confirming the expression of the P2Y\textsubscript{2} receptor. In P2Y\textsubscript{4}, two bands were detected. Hence, the PCR products from the most prominent band were purified and sequenced. The sequenced products matched the published P2Y\textsubscript{4} gene sequence (GenBank no. NM_002565). We did not explore the other weakly expressed band, which might be a splice variant.

Contraction of HASM by UTP and ATP. Figure 4 shows that after a 10-min exposure to UTP (10^{-5} M), HASM cell length decreased by 9.9 ± 4.3% (p = 0.004; n = 14). ATP (10^{-5} M) also produced a decrease in cell length but of lesser magnitude; 5.6 ± 2.0% (p < 0.01; n = 9).

Effects of Short-Term Pretreatment of HASM Cells with UTP on Responses to P2Y Receptor Stimulation. Peak [Ca\textsuperscript{2+}]\textsubscript{i}, was measured in response to 10^{-4} M UTP in cells that were previously incubated for 5, 10, 30, and 60 min with 10^{-4} M UTP, and the results are shown in Fig. 5. A and B. Figure 5A shows representative tracings of the response to 10^{-4} M UTP of two cells exposed for 10 min to vehicle

Fig. 1. Effects of ATP, UTP, and metabolites on [Ca\textsuperscript{2+}]\textsubscript{i}. A, typical response of three individual cells to UTP 10^{-5} M. Noncumulative concentration-response curves to ATP, ADP, adenosine, UTP, and UDP are shown on B. The ordinate represents peak [Ca\textsuperscript{2+}]\textsubscript{i}. The data presented are means ± S.E.M. *, p < 0.01; **, p < 0.001.

Fig. 2. Peak Ca\textsuperscript{2+} release in response to 2-MeSATP and ATP is independent of extracellular Ca\textsuperscript{2+} concentration. A, increases in [Ca\textsuperscript{2+}]\textsubscript{i}, after increasing doses of 2-MeSATP in Ca\textsuperscript{2+} rich (white column) and in Ca\textsuperscript{2+}-free medium (hatched columns). B, increase in [Ca\textsuperscript{2+}]\textsubscript{i}, produced by ATP. The data presented are means ± S.E.M.

Fig. 3. Expression of mRNA for P2Y receptor subtypes. mRNA expression of P2Y\textsubscript{1}, P2Y\textsubscript{2}, P2Y\textsubscript{4}, and P2Y\textsubscript{6} receptors was detected by PCR. Lanes 1–4 represent mRNA from four different individuals. M, DNA molecular marker; bp, base pair(s). The positive control is human placenta, and cyclophilin was used as the housekeeping gene.
(Hanks’ buffer) and of two cells exposed to UTP. Figure 5B shows the average data obtained from four different experiments in each group. The resting \([\text{Ca}^{2+}]_{i}\) values were comparable (ANOVA; N.S.) between the vehicle and the UTP-pretreated groups after 5, 10, 30, and 60 min of pretreatment with a mean of 131 ± 3 nM (n = 148) in the vehicle-treated group and 141 ± 8 nM (n = 152) in UTP-exposed cells. Exposure to 10^{-4} M UTP elicited significant increases in \([\text{Ca}^{2+}]_{i}\) in both vehicle and UTP-exposed cells, but peak \([\text{Ca}^{2+}]_{i}\) was significantly greater in the cells previously incubated with UTP for 10 and 30 min (617 ± 30 nM; n = 36 and 458 ± 42 nM; n = 38) compared with cells incubated with vehicle alone (357 ± 42 nM; n = 37 and 289 ± 35 nM; n = 40; p < 0.05). After 5- and 60-min incubations with UTP, peak \([\text{Ca}^{2+}]_{i}\) was comparable with that measured in vehicle-treated cells: 229 ± 24 nM (n = 37) and 221 ± 14 nM (n = 37) versus 295 ± 40 nM (n = 35) and 245 ± 25 nM (n = 37; p = N.S.).

Effects of Prolonged Periods of Pretreatment of HASM Cells with UTP on Responses to P2Y Receptor Stimulation. After prolonged and repeated exposures of HASM cells to 10^{-4} M UTP (72 h, three doses, each at the intervals of 24 h), \([\text{Ca}^{2+}]_{i}\) was measured in response to 10^{-4} M UTP, 10^{-4} M ATP, and 10^{-4} M histamine, and the results are shown in Fig. 6. Resting \([\text{Ca}^{2+}]_{i}\) was comparable in vehicle- and UTP-treated groups: 105 ± 2.3 nM (n = 105) in the vehicle-treated groups and 99 ± 2.5 nM (n = 107) in the UTP-treated groups (ANOVA; N.S.). Peak \([\text{Ca}^{2+}]_{i}\) levels were significantly decreased in response to 10^{-4} M UTP: 152 ± 12 nM (n = 35) in the cells pretreated with UTP versus 349 ± 24 nM (n = 36; p < 0.001) in the cells exposed to vehicle alone. Peak \(\text{Ca}^{2+}\) release in response to 10^{-4} M ATP was also decreased in cells exposed to UTP for prolonged periods compared with those exposed to vehicle alone: 148 ± 7 nM (n = 38) versus 383 ± 17 nM (n = 38; p < 0.001). In contrast, the peak \([\text{Ca}^{2+}]_{i}\) measured in response to 10^{-4} M histamine was comparable in both vehicle (413 ± 45 nM; n = 31) and UTP-pretreated cells (368 ± 36 nM; n = 34).

**Fig. 5.** Short-term incubation with UTP enhances the response to UTP. A, representative tracings of the response to UTP 10^{-4} M of two cells exposed for 10 min to vehicle (dash lines) and of two cells exposed to UTP (solid lines). B, peak \(\text{Ca}^{2+}\) release in response to UTP (10^{-4} M) after cell incubation in UTP 10^{-4} M (hatched columns) for 10 and 30 min was significantly enhanced compared with incubation in Hanks’ buffer (white columns). R represents the resting \(\text{Ca}^{2+}\) levels from all experiments done at different time periods. The data presented are means ± S.E.M. *, p < 0.05.

**Discussion**

Although the effects of extracellular ATP and other triphosphate nucleotides have been studied in various lung cell types, including alveolar macrophages, mast cells, alve-
cloned from nonmammalian vertebrates or receptors that are currently under functional characterization. The P2Y1 receptor responds to ADP > ATP but not to UTP, whereas the P2Y2 receptor responds to both ATP and UTP, and the P2Y4 receptor responds to UTP > ATP in humans (Kennedy et al., 2000). The P2Y6 subtype responds preferentially to UDP and has little affinity for ATP, and the P2Y12 responds to ATP but not to UTP. The results we obtained showing that ATP, ADP, UTP, and UDP produce an increase in [Ca\(^{2+}\)]i, suggest that the P2Y1,2,4,6 receptor subtypes are present on HASM cells. The response to ADP being comparable in amplitude to that of ATP suggests that the P2Y1 subtype is present. To confirm this latter observation, a dose-response curve to 2-MeSATP, a selective agonist of the P2Y1, P2X1, and P2X4 receptors, was obtained. Exposure to this agonist produced an increase in [Ca\(^{2+}\)]i, that was independent of the presence of Ca\(^{2+}\) in the extracellular medium, indicating that the peak [Ca\(^{2+}\)]i was due to Ca\(^{2+}\) release from intracellular stores rather than from extracellular sources, which is consistent with the response being mediated by the P2Y1 (G-protein coupled) rather than by a P2X (ion gated channel) receptor subtype.

Although selective antagonists are available for P2Y1, and P2Y12, highly selective antagonists are not available for other subtypes of P2Y receptors. This makes further pharmacological characterization of the receptors difficult. Hence, we used RT-PCR analysis to confirm the presence of P2Y receptor mRNA transcripts. Because our pharmacological data indicated the presence of G protein-coupled P2Y subtypes of the receptors rather than P2X receptors, identification of mRNA transcripts of P2X receptors was not explored. These results obtained in cultured cells do not preclude the possibility of a more significant contribution of the P2X receptors in vivo. Indeed, the P2X receptor, which is present and functional in contractile vascular smooth muscle cells, is down-regulated in cultured vascular smooth muscle cells (Erlinge, 2004). Thus, it is possible that the same phenomenon occurs in airway smooth muscle cells. The RT-PCR analysis of mRNA expression confirmed that HASM cells express P2Y1, P2Y2, P2Y4, and P2Y6 receptors.

The P2 receptors can be stimulated by extracellular ATP and/or UTP through endogenous as well as exogenous sources. The airway epithelium is one endogenous source of extracellular ATP and UTP. Indeed, airway epithelial cells constitutively secrete ATP and UTP and can also release ATP and/or UTP after various biological and mechanical stimuli (Lazarowski et al., 1997a, 2000). Considering that airway epithelium and smooth muscle cells are in proximity, it is likely that the ATP and/or UTP released by epithelial cells can have a direct effect on HASM cells, particularly in pathological conditions such as asthma and cystic fibrosis, in which the airway epithelium is disrupted. Also, aerosolized UTP has been proposed as a therapeutic agent to enhance mucociliary clearance in cystic fibrosis patients as the stimulation of P2Y2 receptors and to a lesser extent of P2Y4 and of P2Y6 (via UDP, the metabolic product of UTP) (Lazarowski and...
Boucher, 2001) on human epithelial cells produces an increase in chloride ion secretion and ciliary beat frequency. The potential limitation of this therapy is that G protein-coupled receptors, including P2Y2 receptors, undergo desensitization upon repeated agonist exposure (Wilkinson et al., 1994). Although the mechanism of homologous desensitization has been well established for adrenergic receptors (Hausdorff et al., 1990), the pathways for the desensitization of P2Y2 receptors are not well characterized. Brown et al. (1991) have shown that incubation of airway epithelial cells with UTP for 5 min or longer decreases the total inositol phosphate turnover, suggesting that agonist induced desensitization is a characteristic of its receptor. In the present study, we have determined the effects of short and prolonged, repeated exposures to UTP on the increase in intracellular Ca2+ in response to de novo UTP stimulation. The results show that UTP mediated increases in [Ca2+]i, were enhanced when the cells were exposed to UTP for 10 and 30 min. Although we did not specifically investigate the mechanisms underlying the increased response, possible mechanisms include an up-regulation of the receptor binding affinity or the activation of a tyrosine kinase pathway and of the smooth reticulum-ATPase Ca2+ pump activity (Kito et al., 2001; Carbajal et al., 2005). The former seems less likely since we have used concentrations of UTP that are close to maximal.

The data also show that Ca2+ release was decreased in response to both UTP and ATP after longer and repeated exposure to UTP (once a day for 3 days). The response to histamine was not affected by preexposure to UTP, suggesting that desensitization was confined to the P2 receptors. These data are consistent with studies by Clarke et al. (1999), who showed that in epithelial cells, the desensitization of P2Y2 receptors depends on the duration of exposure to UTP. Although we have not addressed the mechanism of desensitization of the receptors, work from other authors suggests that uncoupling and sequestration of the P2Y2 receptors, caused by phosphorylation of the carboxy terminus of the receptor by a G protein-coupled receptor kinase (Gar- rad et al., 1998), and agonist-promoted internalization are likely mechanisms (Sromek and Harden, 1998).

It has been reported that P2Y2 receptors are up-regulated in proliferating cells and that cell culture conditions can modulate the smooth muscle cell phenotype from contractile to synthetic (for review, see Hirst et al., 2000b). Although we cannot rule out this possibility in our experimental conditions, the results we obtained show that the presence of the P2Y receptors on HASM is not restricted to cultured cells of the synthetic/proliferative phenotype because the cells also contracted in the presence of ATP and UTP. In addition, phenotypic heterogeneity of smooth muscle cells has been described in blood vessels and in airways. Indeed, using flow cytometric analysis, Halayko et al. (1997, 2005) described subtypes of smooth muscle cell populations in freshly dissociated tracheal muscle. Based on DNA content analysis, 87% of the cells were diploid and 13% tetraploid. In addition, groups of cells with high or low content in myosin and α-actin could be found. In vascular smooth muscle, extracellular nucleotides have different effects, depending on the phenotype of the cell. Stimulation of P2Y receptors produces a contraction of the contractile phenotype and mitogenesis of the synthetic phenotype and is implicated in vessel wall remodeling (Erllinge, 2004). Analogous mechanisms are probable in airway tissue.

In conclusion, these data show that several subtypes of P2Y receptors are present in HASM cells. Their stimulation produces an increase in [Ca2+]i, and cellular contraction. In vivo stimulation of the P2Y receptors present on HASM cells, whether of the synthetic or of the contractile phenotype, could contribute to airway function and also to airway remodeling. Considering that the P2Y2 receptor ligands ATP and UTP are endogenous mediators, their role in physiological and pathophysiological processes in the airways warrants further investigation.

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