ATP- and UTP-activated P2Y receptors differently regulate proliferation of human lung epithelial tumor cells

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EXTRACELLULAR NUCLEOTIDES activate epithelial cells in many organs and tissues under physiological and pathological conditions (2, 37, 46). The nucleotides ATP and UTP are released from airway epithelial cells on the apical and basolateral sides in response to cell swelling (43) or mechanical stimuli (11, 27, 48). The released ATP or UTP, in an autocrine or paracrine fashion, controls cell volume through Cl− and fluid secretion (17, 22, 41, 43), mucociliary clearance (mucin, salt, and water secretion) (28, 33), or intracellular Ca2+ oscillations (8) and coordinates responses of distant airway cells by modulating intracellular communication (12, 18) and ciliary beat frequency (35).

The effects of ATP and UTP are mediated through the metabotropic P2Y receptor family. The subtypes, P2Y1, P2Y2, P2Y4, P2Y6, and P2Y13 receptors, are expressed in human lung (37, 50). In airway epithelial cells, extracellular nucleotides regulate physiological functions via phospholipase C activation and subsequent inositol 1,4,5-trisphosphate formation, mainly through P2Y2 and P2Y1 receptors (8, 12). The P2Y2 receptor, which responds equally to extracellular UTP and ATP, is expressed at the apical side of human airway epithelial cells (26). Moreover, diadenosine polyphosphates can activate a Ca2+ increase in human lung epithelial cells via purinergic receptors (24, 25). There is no direct evidence of a role for P2X receptors in regulation of airway ion transport activities. However, it has been proposed that, in tubes and sacs of gallbladders and in the lung, tension leads to release of ATP from the lining epithelium, which then acts on P2Y2 receptor to convey information to the central nervous system (2).

Recently, extracellular nucleotides have emerged as regulators of tumor proliferation and possible effectors of neoplastic transformation. In carcinoma tissues, not only is ATP released during chemotherapy (23), it also controls proliferation of various tumor cells. Depending on the subtype(s) of P2Y receptor expressed, P2Y receptors facilitate proliferation, as in C6 glioma cells (44) and breast cancer cells (47), or inhibit cell proliferation, as reported for endometrial cells (20), colon (13), or esophageal cancer cells (31). However, the mitogenic actions of extracellular nucleotides on airway epithelial cells have not been investigated. This is especially important for understanding lung tumorigenesis. Moreover, the actions of the extracellular nucleotides, in concert with cytokines and epidermal growth factor (EGF), which are known to tightly control proliferation of tumor cells, remain to be clarified.
Extracellular nucleotides have been reported to regulate proliferation of several cancer cell lines (13, 31, 47). ATP inhibits weight loss in tumor-bearing murine hosts (38). Moreover, clinical studies have indicated that, in patients with advanced lung cancer, ATP 1) may inhibit weight loss and suppress tumor growth and 2) displays protective effects against radiation tissue damage (reviewed in Ref. 3).

We therefore examined the effect of extracellular nucleotides on proliferation of tumorigenic and nontumorigenic human lung epithelial cell lines. Proliferation of the tumorigenic A549 cells and the nontumorigenic BEAS-2B cells is stimulated by the purine nucleotide ATP and the pyrimidine nucleotides UTP and UDP through activation of the P2Y receptor subtypes P2Y<sub>2</sub> and P2Y<sub>6</sub> receptors. P2Y<sub>2</sub> receptor-induced A549 tumor cell proliferation depends on activation of phospholipase C, Ca<sup>2+</sup>/calmodulin-dependent kinase II (CaMKII), nuclear factor-κB (NF-κB), and, possibly, EGF receptor (EGFR) tyrosine kinase. This new pathway directly stimulates cell cycle progression, as indicated by the specific interference of ATP with the cytostatic actions of paclitaxel, etoposide, and cisplatin.

**MATERIALS AND METHODS**

**Cell culture and reagents.** The human lung cancer cell line A549 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), and the noncancerous human lung cell line BEAS-2B was kindly provided by Dr. F. Bühling (Otto-von-Guericke University). A549 human non-small cell lung cancer cells were cultured in DMEM (Biochrom) supplemented with 5% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a 10% CO<sub>2</sub> incubator at 37°C. The BEAS-2B cells were cultured in DMEM supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a 10% CO<sub>2</sub> incubator at 37°C.

The anticancer drugs paclitaxel, etoposide, and cisplatin were purchased from Calbiochem (Bad Soden, Germany). U-73122 and its inactive enantiomer U-73343, N-nitro-l-arginine, chelerythrine, SB-203580, wortmannin, AG-1478, NS-398, 8-cyclopentyl-1,3-dipropylxanthine, CGS-15943, alloxan, suramin, reactive blue-2 (RB-2), pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), ARL-67156, adenosine 3'-phosphate 5'-phosphate (AP5P5), Sigma), KN-62, GP-109203X, PD-98059, PP-2, methyl arachidonoyl fluorophosphate (Bio-Trend), genistein, SKF-96365, indomethacin, and NF-κB binding site of the cyclin D1 gene were dissolved according to the manufacturer's recommendations in dimethyl sulfoxide or in phosphate-buffered saline to give a stock solution of 10 or 100 mM. The cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) were obtained from R&amp;D Systems (Minneapolis, MN).

**RNA extraction and RT-PCR for detection of P2Y<sub>2</sub> receptor subtypes.** Total RNA was isolated from A549 and HEK-293 cells with the RNeasy kit (Qiagen). HEK-293 cells were used to verify the usefulness of the primers chosen for PCR and to control the RT-PCR conditions, because HEK-293 cells endogenously express P2Y<sub>1</sub>, P2Y<sub>4</sub>, and P2Y<sub>6</sub> receptors. The isolation included a DNase treatment to remove contaminating DNA. RT was performed with 1 μg of total RNA using oligo(dT) primer with the Omniscript kit (Qiagen). For amplifying the P2Y receptor cDNAs, sets of specific oligonucleotide primers were synthesized on the basis of the published sequences for P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, and P2Y<sub>6</sub> receptors (see below). Because the P2Y<sub>4</sub> receptor has only one exon and the P2Y<sub>6</sub> receptor has two exons, primers were defined, flanking the intron of P2Y<sub>6</sub> (exon 1 = 156–242, exon 2 = 243–1,563). PCR without the RT step was used to control to exclude amplification of genomic DNA. Amplification was performed with 1 μl of cDNA, and the conditions were as follows: 98°C for 1 min, 94°C for 1 min, 55°C for 90 s, 72°C for 2 min, and a final extension step of 10 min at 72°C for 30 cycles.

The primers were 5'-GCC GCC GTC TCC TCG TCG TT 3' (forward, position 146–166) and 5'-CCA TTC TGC TTG AAC TCA G (reverse, position 1133–1152) with 1,006 bp for hP2Y<sub>2</sub> (accession no. U42029), 5'-CGA GGA CTT GTA CAA GTA CGT GC 3' (forward, position 329–349) and 5'-GTC CTC GCT ACC AGC CGG CG 3' (reverse, position 1338–1358) with 1,030 bp for hP2Y<sub>6</sub> (accession no. U07225), 5'-GCT GCC TGT GAG CTA TGCG CAG 3' (forward, position 110–140) and 5'-GAG CTC GAG CTC AGC CAG TGC C (reverse, position 939–960). Each cycle consisted of 98°C for 1 min, 55°C for 1 min, and 72°C for 1 min.

**Data analysis.** Proliferation data refer to the chemiluminescence values of BrdU-labeled cellular DNA content per well. Stimulation is expressed as fold stimulation over basal. Values are means ± SEM of three or four experiments.
lung tumor cells, proliferating A549 cells were pulse stimulated with ATP or UTP. Then cell proliferation was measured by monitoring BrdU incorporation into newly synthesized DNA for 12 h after addition of nucleotides. ATP and UTP induced a concentration-dependent increase in proliferation of A549 cells. The maximal stimulation, which was seen at 100 μM nucleotide, was 3.2-fold for ATP and 1.8-fold for UTP (Fig. 1A). Despite the difference in maximal stimulation, the potency of both nucleotides to stimulate proliferation was very similar, with EC50 of 26.0 ± 2.8 μM (n = 3) for ATP and 31.3 ± 4.1 μM (n = 3) for UTP.

The effect of the nucleotides on BrdU incorporation into A549 cells was time dependent. The maximal stimulatory effect by ATP and UTP was reached at 1 h after addition of the nucleotides and lasted for 3–6 h (Fig. 1B). Incorporation of BrdU into newly synthesized DNA rapidly declined to baseline values at >6 h after nucleotide addition. This indicates that extracellular nucleotides stimulate DNA synthesis by direct interaction with cell cycle progression.

Besides ATP and UTP, the diphosphate nucleotides ADP and UDP also stimulated BrdU incorporation into proliferating A549 cells with an efficacy nearly identical to that of the respective triphosphate nucleotides (Fig. 2A). At 100 μM, ADP and UDP enhanced proliferation of A549 cells 3.1- and 2.2-fold, respectively.

To determine whether the stimulatory effect of extracellular nucleotides on proliferation of A549 lung tumor cells was associated with neoplastic transformation of the cells, we also investigated the effect of nucleotides on proliferation of the nontumorigenic human epithelial lung cell line BEAS-2B. BEAS-2B and
A549 cells serve as in vitro models for bronchiolar and alveolar cells, respectively.

Enhancement of proliferation by extracellular nucleotides was even stronger in the BEAS-2B cells than in the A549 cells. Here, 100 μM ATP and 100 μM ADP stimulated BrdU incorporation into newly synthesized DNA 7.1- and 6.5-fold, respectively (Fig. 2B). The pyrimidine nucleotides UDP and UDP exhibited identical efficacy and induced a 3.2- and 3.4-fold stimulation, respectively (Fig. 2B). Thus, purine and pyrimidine nucleotides may regulate human lung epithelial cell proliferation in vivo. Moreover, control of proliferation by nucleotides is not due to transformation of lung epithelial cells, because proliferation of tumorigenic and nontumorigenic airway cells is equally affected.

To evaluate whether the trophic actions of the extracellular nucleotides on the human lung cells were mediated by the P2Y or the P2X receptors, proliferative regulation of A549 and BEAS-2B cells was investigated by using different nucleotides, nucleotide analogs, and P2 receptor antagonists. Characterization of A549 cell proliferation revealed that only ATP, ADP, UTP, and UDP, which stimulate different P2Y receptor subtypes, were active, but the P2Y1 receptor-selective antagonists 2-methylthioadenosine 5′-diphosphate (2-MeS-ADP) and ATPαS and the P2X receptor-activating nucleotides α,β-methylene adenosine 5′-triphosphate (α,β-MeATP) and 2′,3′-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate (BzATP) were inactive (Fig. 2). AMP and UMP at up to 100 μM also had no effect on proliferation (data not shown).

The efficacy of the different nucleotide ligands was as follows: ATP > ADP > UDP > UTP > 2-MeS-ADP, whereas α,β-MeATP, BzATP, AMP, UMP, and ATPαS were inactive. This clearly shows that stimulation of A549 cell proliferation is mediated by P2Y, but not by P2X, receptors, inasmuch as α,β-MeATP and BzATP, which, in addition to ATP, can activate P2X receptors (P2X1, P2X2, P2X3, P2X7) (21), have no influence on proliferation (Fig. 2). In addition, the lack of activity of 2-MeS-ADP and ATPαS indicates that in A549 cells no functional P2Y1 receptors are involved. However, the pharmacological profile suggests involvement of more than one P2Y receptor in stimulation of A549 cell proliferation, because the profile is not consistent with the properties of a single P2Y receptor subtype (46).

We further attempted to identify the P2Y receptor subtypes involved by applying the P2 receptor-selective antagonists PPADS, suramin, RB-2, and ARL-67156. Preincubation of A549 cells with 100 μM suramin or RB-2 inhibited ATP-stimulated proliferation to 37 ± 8.4% (n = 4) and 45 ± 4.5% (n = 3), respectively (Table 1). There was no significant inhibition of ATP-stimulated proliferation by PPADS, which predominantly inhibits the human P2Y1 and P2Y4 receptors or the P2Y1 receptor-selective antagonists ARL-67156 and A3P5P (Table 1). Thus, this approach also did not identify a single P2Y receptor subtype (see DISCUSSION).

Adenosine did not mimic ATP-stimulated proliferation. At 100 μM, where ATP is maximally effective, adenosine enhanced proliferation only marginally by 35 ± 4.3% and 57 ± 12.1% in A549 and BEAS-2B cells, respectively (Fig. 2), excluding the possibility that the stimulatory effect of ATP was caused by the metabolic breakdown product adenosine. Furthermore, to rule out the possibility that adenosine-mediated signaling partly contributed to the ATP-stimulated proliferative response, the effect of different adenosine receptor antagonists (i.e., P1 antagonists) on ATP-stimulated proliferation of A549 cells was investigated (Table 1). Neither the nonselective adenosine receptor antagonist CGS-15943 nor the A1 and A2B receptor-selective antagonists 8-cyclopentyl-1,3-dipropylxanthine and alloxazine, respectively, inhibited ATP-stimulated proliferation. We did not test specific inhibitors of A3 receptors, inasmuch as this receptor subtype is not expressed in A549 cells (42). Thus, clearly, P1 receptors are not contributing to the signaling of extracellular nucleotides in the proliferative response of A549 cells. Therefore, characterization of A549 cell proliferation using agonists and antagonists of P2 and P1 receptors reveals that only P2Y receptors but not P2X or P1 receptors, mediate stimulation of lung epithelial cell proliferation.

Furthermore, we examined expression of different P2Y receptor subtypes (P2Y1, P2Y2, P2Y4, and P2Y6 receptors) by RT-PCR. In A549 cells, only mRNA specific for the P2Y2 receptor, which is activated with identical potency by ATP and UTP, and the UDP-selective P2Y6 receptor was detected by RT-PCR, whereas no transcripts for the P2Y1 or P2Y4 receptor could be found (Fig. 3). In the same experiment, we employed HEK-293 cells to control the RT-PCR conditions. HEK-293 cells endogenously express P2Y1, P2Y2, and P2Y4 receptors. These results indicate that primarily the P2Y2 and P2Y6 receptors mediate nucleotide-stimulated proliferation of A549 cells.

Signal transduction pathways of nucleotide-stimulated proliferation of A549 cells. We next investigated the signaling pathways involved in P2Y receptor-stimulated proliferation. A549 or BEAS-2B cells were pre-

Table 1. Effect of P2 and P1 receptor antagonists on ATP-stimulated proliferation of A549 lung tumor cells

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Stimulation, %</th>
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<tbody>
<tr>
<td><strong>P2 receptor</strong></td>
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</tr>
<tr>
<td>Suramin (100 μM)</td>
<td>37.2 ± 8.4</td>
</tr>
<tr>
<td>RB-2 (100 μM)</td>
<td>45.2 ± 4.5</td>
</tr>
<tr>
<td>PPADS (300 μM)</td>
<td>96.5 ± 3.8</td>
</tr>
<tr>
<td>ARL-67156 (100 μM)</td>
<td>97.6 ± 4.1</td>
</tr>
<tr>
<td>A3P5P (100 μM)</td>
<td>93.8 ± 5.7</td>
</tr>
<tr>
<td><strong>P1 receptor</strong></td>
<td></td>
</tr>
<tr>
<td>DPCPX (25 μM)</td>
<td>112.7 ± 5.9</td>
</tr>
<tr>
<td>CGS-15943 (1 μM)</td>
<td>110.2 ± 3.1</td>
</tr>
<tr>
<td>Alloxazine (10 μM)</td>
<td>94.3 ± 2.3</td>
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</tbody>
</table>

Values (means ± SD of 3-5 independent experiments run in triplicate) are expressed as percentage of ATP-stimulated proliferation (=100%). RB-2, reactive blue-2; PPADS, pyridoxal phosphate-6-azophenyl-2,4′-disulfonic acid; A3P5P, adenosine 3′-phosphate 5′-phosphate; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine.
treated with different inhibitors of signaling cascades that mediate proliferation of other cell types. Basal DNA synthesis of proliferating cells was not blocked by these inhibitors at the concentrations used. ATP-stimulated proliferation of A549 cells was almost completely attenuated by preincubation for 1 h with U-73122 (10 μM), an inhibitor of phospholipase C, whereas the inactive control derivative U-73343 was not effective (Fig. 4A). Also, inhibition of CaMKII activity with KN-62 (10 μM) and of the influx of extracellular Ca<sup>2+</sup> through receptor-operated Ca<sup>2+</sup> channels with SKF-96365 (50 μM) strongly decreased ATP-stimulated BrdU incorporation to 34.6 ± 8.7% (n = 6) and 33.3 ± 5.2% (n = 5), respectively (Fig. 4A). However, the following drugs did not influence the ability of ATP or UTP to stimulate proliferation of A549 cells (Table 2): chelerythrine (20 μM) and GF-109203X (1 μM), which inhibit different protein kinase C (PKC) isoforms, the mitogen-activated protein kinase (MAPK) kinase (MEK1/2) inhibitor PD-98059 (20 μM), the p38 kinase inhibitor SB-203580 (20 μM), the phosphatidylinositol 3-kinase pathway inhibitors wortmannin (0.1 μM) and LY-294002 (25 μM), and N<sup>ω</sup>-nitro-L-arginine (10 μM), which blocks generation of nitric oxide.

To identify components of the signal transduction pathway resulting in DNA synthesis, we evaluated pathways that can be activated by CaMKII or tyrosine kinases. Pretreatment of A549 cells with an inhibitory peptide specific for the p50 form of NF-κB (NF-κB SN-50; 20 μM) or with AG-1478 (500 nM), specifically inhibiting EGFR tyrosine kinase activity, significantly reduced ATP stimulation of DNA synthesis to 42.8 ± 5.9% and 43.0 ± 4.6%, respectively (Fig. 4B, Table 2). Inhibition of Src kinase, which can also be activated by G protein-coupled receptors, with PP-2 (500 nM), inhibition of general tyrosine kinase activity (genistein at 100 μM), inhibition of cytosolic phospholipase A<sub>2</sub> (methyl arachidonyl fluorophosphonate, 10 μM), or inhibition of cyclooxygenases with indomethacin (20 μM) or NS-398 (10 μM) did not abolish the effect of ATP and UTP on proliferation of A549 cells (Fig. 4B, Table 2).
Interestingly, similar results were derived for BEAS-2B cells. In the analysis of signal transduction, we obtained identical inhibition by the various drugs for the bronchial epithelial BEAS-2B cells, except for genistein, which only in BEAS-2B cells decreased ATP- and UTP-stimulated proliferation to 60 and 68%, respectively (Table 2). This indicates that, in the immortalized bronchial BEAS-2B cells, additional tyrosine kinases are involved in the proliferative regulation.

Modulation of nucleotide-regulated proliferation of lung tumor cells by cytokines. Airway epithelial cells are a major source of proinflammatory cytokines and, thus, play an important role in the pathophysiology of lung diseases. Therefore, we investigated the effect of cytokines on nucleotide-regulated proliferation. Moreover, cytokines had also been found to upregulate P2Y2 receptors in vascular smooth muscle cells (15). Preincubation of A549 cells for 24 h with IL-1β (10 ng/ml) or IL-6 (50 ng/ml), at concentrations that are detected under inflammatory conditions, attenuated UTP-stimulated proliferation to 59.7 ± 10.3% and 34.7 ± 12.1%, respectively, compared with the untreated stimulation (Fig. 5, Table 3). However, proliferation of A549 cells induced by the purine nucleotide ATP was clearly not affected by pretreatment with the cytokines. TNF-α had no effect at concentrations up to 50 ng/ml. Moreover, pretreatment of BEAS-2B cells with the cytokines IL-1β, IL-6, and TNF-α did not influence ATP- or UTP-stimulated proliferation (Table 3).

Interaction of anticancer drugs with nucleotide-stimulated proliferation of lung tumor cells. Inasmuch as control of proliferation is a key target in cancer therapy, we also examined the effect of anticancer drugs on the nucleotide-regulated proliferation. BrdU incorporation into proliferating A549 cells was inhibited dose dependently by the anticancer drugs cisplatin, etoposide, and paclitaxel, with IC_{50} of 2.7 ± 1.1 μM (n = 5), 19.8 ± 2.2 μM (n = 5), and 17.6 ± 3.8 nM (n = 4), respectively (Fig. 6). The simultaneous addition of 100 μM ATP enhanced the antiproliferative potency of cisplatin 3.6-fold, shown by reduction of IC_{50} to 0.75 μM (Fig. 6). The effect of ATP was most obvious at low concentrations of the anticancer drugs. At 1 μM, cisplatin was twice as effective in reducing proliferation in the presence of ATP than in its absence.

The antiproliferative activity of etoposide and paclitaxel was antagonized by the simultaneous presence of 100 μM ATP, resulting in 1.6-fold (IC_{50} = 31 ± 2.9 μM, n = 5) and 2.6-fold (IC_{50} = 46.2 ± 4.8 nM, n = 4) lower proliferation compared with the untreated stimulation (Table 3).

Interaction of anticancer drugs with nucleotide-stimulated proliferation of lung tumor cells. Table 2. Effect of signal transduction pathway inhibitors on nucleotide-induced proliferation

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>A549 Cells</th>
<th>BEAS-2B Cells</th>
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<tbody>
<tr>
<td></td>
<td>ATP (nM)</td>
<td>UTP (nM)</td>
</tr>
<tr>
<td>U-73122 (10 μM)</td>
<td>19.1 ± 7.0</td>
<td>17.5 ± 6.9</td>
</tr>
<tr>
<td>U-73343 (10 μM)</td>
<td>101 ± 6.4</td>
<td>94.9 ± 3.2</td>
</tr>
<tr>
<td>KN-62 (10 μM)</td>
<td>34.6 ± 8.7</td>
<td>26.2 ± 3.9</td>
</tr>
<tr>
<td>SKF-96365 (50 μM)</td>
<td>33.3 ± 5.2</td>
<td>35.4 ± 6.5</td>
</tr>
<tr>
<td>Cheletrixine (20 μM)</td>
<td>102 ± 5.1</td>
<td>103 ± 3.6</td>
</tr>
<tr>
<td>GF109203X (1 μM)</td>
<td>92.0 ± 1.3</td>
<td>93.6 ± 4.2</td>
</tr>
<tr>
<td>PD98059 (20 μM)</td>
<td>96.1 ± 4.7</td>
<td>91.2 ± 3.3</td>
</tr>
<tr>
<td>SB203580 (20 μM)</td>
<td>95.1 ± 2.9</td>
<td>99.2 ± 5.1</td>
</tr>
<tr>
<td>Wortmannin (0.1 μM)</td>
<td>96.1 ± 4.7</td>
<td>91.2 ± 3.3</td>
</tr>
<tr>
<td>LY294002 (25 μM)</td>
<td>118 ± 5.2</td>
<td>93.3 ± 2.3</td>
</tr>
<tr>
<td>li-NNA (10 μM)</td>
<td>95.2 ± 4.7</td>
<td>93.8 ± 3.3</td>
</tr>
<tr>
<td>NF-κB SN50 (20 μM)</td>
<td>42.8 ± 5.9</td>
<td>38.3 ± 4.1</td>
</tr>
<tr>
<td>AG-1478 (500 nM)</td>
<td>43.0 ± 4.6</td>
<td>31.7 ± 2.9</td>
</tr>
<tr>
<td>PP-2 (0.5 μM)</td>
<td>99.8 ± 1.4</td>
<td>97.1 ± 2.9</td>
</tr>
<tr>
<td>Genistein (100 μM)</td>
<td>91.3 ± 7.6</td>
<td>87.0 ± 6.7</td>
</tr>
<tr>
<td>MAFP (20 μM)</td>
<td>84.8 ± 6.9</td>
<td>76.3 ± 5.7</td>
</tr>
<tr>
<td>Indomethacin (20 μM)</td>
<td>100 ± 3.4</td>
<td>97.3 ± 3.6</td>
</tr>
<tr>
<td>NS-398 (10 μM)</td>
<td>101 ± 4.4</td>
<td>102 ± 2.7</td>
</tr>
</tbody>
</table>

Values (means ± SD of 6–8 independent experiments run in triplicate) are expressed as percentage of maximal ATP- or UTP-stimulated proliferation (% 100%) after preincubation with inhibitor. ND, not determined; l-NNA, Nω-nitro-L-arginine; NF-κB, nuclear factor-κB. MAFP, methyl arachidonyl fluorophosphonate.

Fig. 5. Cytokines modulate nucleotide-regulated proliferation of A549 lung tumor cells. Proliferating A549 cells were preincubated with vehicle (control) or interleukin (IL)-1β (10 ng/ml), IL-6 (50 ng/ml), or tumor necrosis factor (TNF)-α (50 ng/ml) for 24 h and then stimulated with 100 μM ATP or 100 μM UTP. Values (means ± SD of 3–4 independent experiments run in triplicate) are expressed as percentage of maximal stimulation of BrdU incorporation induced by ATP (3.22-fold) or UTP (1.98-fold). *P < 0.001 (Student’s t-test).
inhibitory potency, respectively (Fig. 6). ATP still stimulated proliferation 80% above control in the presence of 1 μM etoposide. Addition of 100 μM UTP did not change the potency of the three anticancer drugs (data not shown). These results support the notion that ATP regulates proliferation of human lung tumor cells by direct control of the cell cycle.

**DISCUSSION**

This is the first study showing that extracellular nucleotides stimulate proliferation of human lung epithelial cells by activation of the P2Y2 and P2Y6 receptors, which are detected by RT-PCR to be expressed in A549 cells. The identical EC50 values for ATP- and UTP-stimulated proliferation of A549 cells indicate that both nucleotides stimulate A549 cell proliferation through activation of P2Y2 receptors, because this receptor is equally sensitive to ATP and UTP. This conclusion is also supported by the activity profile of the P2 receptor antagonists. The significant, but not complete, inhibition of ATP- and UTP-stimulated proliferation by 100 μM suramin or 100 μM RB-2 and the lack of activity of 100 μM PPADS are characteristic of the human P2Y2 receptor (46).

Furthermore, our results showing that UTP and UDP stimulate BrdU incorporation in A549 cells with equal efficacy indicate that, in addition to the P2Y2 receptor, the P2Y6 receptor can also mediate stimulation of proliferation. At the human P2Y6 receptor, UDP is ~100-fold more potent than UTP (46). Thus, as in vascular smooth muscle cells, where for the first time UDP-stimulated growth via the P2Y6 receptor had been reported (14), the P2Y6 receptor is likely to be involved in the control of proliferation in lung epithelial cells.

However, the efficacy profile (ATP = ADP > UDP ≥ UTP) found for A549 cells indicates involvement of some additional receptor, other than the P2Y2 or P2Y6 receptor subtypes (46). ADP activated proliferation of A549 cells with a potency similar to that of ATP. The identity of the receptor, which mediates the action of ADP, is not known. ADP has been found to be considerably less active at the P2Y2 and P2Y6 receptors than the preferred agonists ATP/UTP and UDP, respectively (46). ADP preferentially activates the P2Y1 receptor, which, however, is not expressed in A549 cells. The absence of P2Y1 receptor expression in A549 cells is also pharmacologically underlined by the inactivity of 2-MeS-ADP. 2-MeS-ADP activates the P2Y1 receptor to an even greater degree than ADP.

An alternative possibility explaining the effectiveness of ADP implies that ADP may be converted to ATP by an ectonucleoside diphosphokinase or by an extracellular adenylate kinase, similar to cardiac endothelial cells (4) and nasal airway cells (7), thereby activat-

### Table 3. Cytokines modulate nucleotide-regulated proliferation of lung epithelial cells

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>A549 Cells</th>
<th>BEAS-2B Cells</th>
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<tbody>
<tr>
<td>IL-1β (10 ng/ml)</td>
<td>ATP: 99.0 ± 4.6, UTP: 59.7 ± 10.3</td>
<td>ATP: 87.5 ± 8.4, UTP: 87.9 ± 7.8</td>
</tr>
<tr>
<td>IL-6 (50 ng/ml)</td>
<td>ATP: 100.7 ± 2.9, UTP: 34.7 ± 12.1</td>
<td>ATP: 94.7 ± 6.2, UTP: 90.3 ± 5.1</td>
</tr>
<tr>
<td>TNF-α (50 ng/ml)</td>
<td>ATP: 95.2 ± 6.0, UTP: 96.1 ± 5.8</td>
<td>ATP: 98.1 ± 7.1, UTP: 92.2 ± 4.0</td>
</tr>
</tbody>
</table>

Values (means ± SD of 3–4 independent experiments run in triplicate) are expressed as percentage of ATP- or UTP-stimulated bromodeoxyuridine (BrdU) incorporation (≤100%). Proliferating A549 or BEAS-2B cells were preincubated with vehicle for control value or IL-1β, IL-6, or TNF-α for 24 h and then stimulated with 100 μM ATP or 100 μM UTP, and BrdU incorporation was determined. Proliferation of cytokine-treated A549 or BEAS-2B cells was not different from untreated control cells.

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![Fig. 6. Interaction of ATP and anticancer drugs in control of proliferation of A549 lung tumor cells. BrdU incorporation into proliferating A549 cells was measured in the presence of 100 μM ATP or its absence, and cells were exposed to different concentrations of the anticancer drugs cisplatin or etoposide (A) or paclitaxel (B). Values (means ± SD of 4–5 independent experiments run in triplicate) are expressed as percentage of BrdU incorporation of untreated cells (basal control) = 100%].

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ing the P2Y₂ receptor. Both kinases are secreted from human nasal epithelium and are suggested to play an important role in the interconversion and metabolism of extracellular nucleotides on airway surfaces (7). The mechanism in the proliferative response of ADP in A549 cells remains to be examined.

The two lung cell lines used in our study, bronchiolar BEAS-2B cells and alveolar A549 epithelial airway cells, originate from two distinct lung regions. These regions respond distinctly to different stimuli, such as steroids, inflammatory cytokines, or chemotherapy. Nevertheless, ATP/UTP and UDP stimulated proliferation of both cell lines, suggesting a common mechanism of action in the physiological responses of human lung cells to extracellular nucleotides. The maximal concentrations of extracellular nucleotides achieved on stimulation of epithelial cells were calculated to be 5–10 μM (see Ref. 40 and references therein). These concentrations, determined for the release of ATP from resting cells, appear to be sufficient to stimulate proliferating epithelial cells, as detected in our experiments. It is conceivable that even higher ATP concentrations, i.e., 50–100 μM, can be reached under conditions where the epithelial cell layer is disrupted (ventilation injury) or under inflammatory conditions, where proliferation of these cells is necessary to regain an intact barrier. BEAS-2B and A549 cells serve as in vitro models for bronchiolar and alveolar cells, respectively. Therefore, a further in-depth analysis, similarly studying the regulation of BEAS-2B cell proliferation by extracellular nucleotides, will be important for understanding bronchiolar physiology.

The P2Y₂ receptor has been described to have an important role in control of proliferation of different cell types. Interestingly, activation of the P2Y₂ receptor has been reported to exert contrasting effects. It can lead to proliferation, as in MCF-7 breast cancer cells (47) and C6 glioma cells (44), or to apoptosis, as in colon carcinoma cells (13), endometrial cells (20), and esophageal cancer cells (31). In these cells, the P2Y₂ receptor signals through the MAPK pathway.

Our results indicate that, in A549 and BEAS-2B human lung cells, the G₉-protein-initiated signaling by nucleotides eliciting proliferation is via NF-κB activation and does not involve the classical Ras-Raf-MAPK pathway, as in astrocytes or C6 glioma cells (36, 44), because the specific MEK1/2 inhibitor PD-98059 did not block nucleotide-mediated proliferation. In addition, inhibition of the phosphatidylinositol 3-kinase pathway with wortmannin or LY-294002 also did not affect nucleotide-stimulated lung cell proliferation. This pathway had been shown to play an important role in P2Y₂ receptor-mediated activation of MAPK in human monocytes (39) and renal mesangial cells (16).

Furthermore, in A549 cells, P2Y₂ receptor-mediated proliferation is not dependent on PKC activation and general tyrosine kinase activity, because the PKC inhibitors chelerythrine and GF-109203X and the tyrosine phosphorylation inhibitor genistein were ineffective. In A549 cells, a Ca²⁺-dependent activation of NF-κB or CaMKII-triggered phosphorylation of the EGFR may be one of the main signaling pathways. Alternatively, CaMKII may directly translocate to the nucleus, thereby acting as a trigger for centrosome duplication or coordinating centrosome formation and DNA replication (34). CaMKII and NF-κB activation in A549 cells, however, do not involve PKC activation or an intermediate tyrosine kinase, which has been found for the P2Y₂ receptor-stimulated proliferation of C6 glioma cells (44).

Signaling of the P2Y receptor through activation of NF-κB or CaMKII during regulation of proliferation has not been reported. CaMKII has only been found to be involved in the P2Y receptor-mediated enhancement of lipopolysaccharide-induced activation of IκB kinase in macrophages (5). Involvement of NF-κB in P2Y receptor signaling was detected in UTP-induced activation of nuclear factor of activated T cells, initiating IL-6 expression in vascular smooth muscle cells (1), and in UTP-initiated prostaglandin E₂ release from macrophages (5). NF-κB activation was found for P2X₇ receptor-mediated apoptosis of endothelial cells (45) and microglia cells (9). Thus the extracellular nucleotides ATP and UTP stimulate proliferation via a novel pathway in A549 and BEAS-2B cells. EGFR transactivation, suggested by the inhibitory effect of AG-1478, may also contribute to ATP-stimulated proliferation by activation of NF-κB. It is important to note that our results were obtained with proliferating cells. Whether this mechanism is also active in resting cells remains to be examined.

To further understand the mechanism of control of proliferation of lung epithelial cells by nucleotide receptors, we investigated the effects of the cytostatic drugs cisplatin, etoposide, and paclitaxel. These substances inhibit cell cycle progression and form the basis of current chemotherapeutic regimens in lung cancer treatment. The concentrations of the three anticancer drugs used in our study match those used for investigation of cell cycle inhibition of A549 cells (6). Paclitaxel and etoposide cause cell cycle arrest in A549 cells at the G2/M phase through interference with disassembly of microtubules (paclitaxel) or inhibition of spindle formation and topoisomerase II (etoposide) (6, 30). A consistent block in 40% of the cells at the G2/M phase after 24 h of application was found with 10–30 nM paclitaxel, and an induction of apoptosis was found in 20% of the cells with the higher concentration (30). Etoposide at 5–30 μM exerted a block at the G2 phase in A549 cells without significant apoptosis (6). In contrast, cisplatin does not induce G₂ arrest in A549 cells but cross-links with DNA, forming intra- and interstrand adducts (6). Cell death is believed to be mediated by recognition of the cisplatin-DNA adducts by cellular proteins (19).

We found a specific interaction of ATP, but not UTP, with control of proliferation by the anticancer drugs in A549 cells. ATP selectively potentiated the effect of cisplatin and most effectively antagonized the cell cycle-blocking activity at low paclitaxel and etoposide concentrations, where no significant apoptotic or cytotoxic activity of the anticancer drugs at A549 cells was
found (6, 30). The antagonistic effects of ATP on the action of paclitaxel/etoposide are consistent with the assumption that ATP stimulates growth by directly promoting cell cycle progression from the G1/S phase to beyond the G2/M phase. Furthermore, stimulation of cells to progress into the S phase leads to more DNA in the uncoiled state, which then forms adducts with cisplatin. This might explain enhancement of the antiproliferative activity of cisplatin by ATP. The present results using these drugs are supportive but do not provide definitive evidence for direct control of cell cycle progression by extracellular nucleotides in A549 cells. The molecular mechanisms or the proteins mediating the action of ATP are not known. Possible candidates, which are known to be regulated in A549 cells by low concentrations of paclitaxel or etoposide, include proteins regulated by the tumor suppressor protein p53 and c-jun (p21WAF1 and cyclin B1) and the metastasis suppressor gene KAI1 (6, 32).

Cytokines influence UTP-stimulated, but not ATP-stimulated, proliferation of A549 cells. Thus pyrimidine nucleotides preferentially interact with the signal transduction of inflammatory cytokines. Different interactions of extracellular nucleotides with cytokines have been described for macrophages, monocytes, and dendritic cells, where cytokines regulate inflammatory or immune responses. In other cells, such as fetal human astrocytes, P2 receptors modulate IL-1β- and TNF-α-stimulated activation of NF-κB and activator protein-1 (29), supporting a regulatory role for the P2 receptors in inflammatory reactions in the human central nervous system. Moreover, in human bronchial epithelia, activation of Cl− channels by UTP is enhanced by IL-4 (10), and in vascular smooth muscle cells, IL-1β induces upregulation of the P2Y2 receptor (15).

In conclusion, our results, for the first time, show that extracellular nucleotides exert mitogenic effects on human lung epithelial cells through ATP/UTP-mediated activation of the P2Y2 receptor and the UDP-activated P2Y6 receptor. Stimulation of the P2Y2 receptor leads to activation of CaMKII and NF-κB. This novel pathway is modulated by extracellular Ca2+ and, possibly, involves EGFR transactivation. Understanding the mechanisms of interactions of extracellular nucleotides with anticancer drugs, growth factors, and cytokines will be an important task, because non-small cell lung cancer cells, such as A549 cells, rapidly develop resistance to conventional chemotherapy.

DISCLOSURES

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REFERENCES


