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P2X and P2Y purinergic receptors on human intestinal epithelial carcinoma cells: effects of extracellular nucleotides on apoptosis and cell proliferation

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Coutinho-Silva, Robson, Lynn Stahl, Kwok-Kuen Cheung, Nathalia Enes de Campos, Carolina de Oliveira Souza, David M. Ojcius, and Geoffrey Burnstock. P2X and P2Y purinergic receptors on human intestinal epithelial carcinoma cells: effects of extracellular nucleotides on apoptosis and cell proliferation. *Am J Physiol Gastrointest Liver Physiol* 288: G1024–G1035, 2005. First published January 20, 2005; doi:10.1152/ajpgi.00211.2004.—Extracellular nucleotides interact with purinergic receptors, which regulate ion transport in a variety of epithelia. With the use of two different human epithelial carcinoma cell lines (HCT8 and Caco-2), we have shown by RT-PCR that the cells express mRNA for $P2X_1$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, and $P2Y_{12}$ receptors. Protein expression for $P2Y_1$ and $P2Y_2$ receptors was also demonstrated immunohistochemically, and P2X receptor subtype protein was present in the following decreasing order: $P2X_4 > P2X_7 > P2X_1 > P2X_3 > P2X_6 > P2X_5 >> P2X_2$. The functional presence of $P2X_7$, $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors was shown based on the effect of extracellular nucleotides on apoptosis or cell proliferation, and measurement of nucleotide-dependent calcium fluxes using a fluorometric imaging plate reader in the presence of different selective agonists and antagonists. ATP, at high concentrations, induced apoptosis through ligation of $P2X_7$ and $P2Y_1$ receptors; conversely, ATP, at lower concentrations, and UTP stimulated proliferation, probably acting via $P2Y_2$ receptors. We therefore propose that stimulation or dysfunction of purinergic receptors may contribute at least partially to modulation of epithelial carcinoma cell proliferation and apoptosis.

adenosine 5′-triphosphate; purinergic receptors

EXTRACELLULAR NUCLEOTIDES exert diverse effects on a variety of tissues and cell types via specific receptors (2). Studies of purinergic signaling in various epithelial cell types carried out to date have shown mainly that ATP and UTP modify K^+, HCO_3^-, and Cl^- secretion (46).

P2 receptors consist of P2X and P2Y families. P2X ionotropic receptors form nonselective monovalent cation channels that allow, in most cases, anion or Ca^{2+} influx from the extracellular space. Seven members of the P2X family have been cloned in mammals ($P2X_{1–7}$) (51). The P2Y receptors are coupled to trimeric G proteins and are composed of eight subtypes in mammals ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) (1, 12, 33, 39). The presence of $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors has been revealed in human airway epithelial model systems (54), and recently, it was shown that the $P2Y_6$ receptors mediate NaCl secretion in colonic epithelial cells (43).

Extracellular ATP, acting via $P2X_7$ receptors, is known to trigger release of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-1β in murine and human macrophages (19) and to induce apoptosis (14); ATP has also been implicated in the formation of multinucleated giant cells (25) and elimination of intracellular bacteria (13, 15, 24, 43, 45). $P2X_7$ receptors are also expressed on gut epithelia, and their expression has been associated with cell death (32). ATP acts as a cotransmitter in many nerves of both the peripheral and central nervous systems (8); and in some sympathetic nerves, such as those supplying the arterioles in the intestine, ATP is the principal neurotransmitter (23). ATP also can be released by epithelial cells (54).

Most studies of purinergic signaling in epithelial cells have shown that extracellular ATP and/or UTP modify Cl^- secretion in various epithelial cell types (16, 42, 54, 56, 60) and that the regulation of ion transport may be mediated by P2 receptors (42, 46) and in some cases adenosine receptors (6). P2X and P2Y channels on epithelial cells derived from the gastrointestinal system have not been fully characterized (46). In addition, the function of P2 receptors on carcinoma cells has not been clearly defined, because P2Y-dependent anti-proliferative and apoptotic effects have been described on human colorectal and esophageal carcinoma cells (34, 35, 47). Nonetheless, the P2X receptors have been associated with the inhibition of proliferation by nucleotides of prostate and skin carcinoma cells (30, 40), and the P2Y receptors have an effect on proliferation of skin carcinoma cells. Our goal in the present study was to determine whether protein and mRNA from different P2 receptors are expressed in different human intestinal epithelial carcinoma cell lines and to begin identifying a biological function for these receptors.

MATERIALS AND METHODS

Cells and reagents. The human intestinal epithelial carcinoma cell lines HCT8 and Caco-2 were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained at 37°C in an atmosphere of 5% CO_2 in RPMI 1640 supplemented with 10% heat-inactivated FBS (GIBCO-BRL), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. All nucleotides, dipyridamole, 8-(p-sulfophenyl)-theophylline (8-SPT), and MRS2179 were purchased from Sigma (Poole, UK). Fluo-4-AM and pluronic acid

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were obtained from Molecular Probes (Eugene, OR). Cell culture media and reagents were from Life Technologies (Rockville, MD). The nucleotides were prepared as 10 mM stock solutions in PBS and stored at −20°C until use.

Cell proliferation assay. The effects of a sustained application of P2-receptor agonists on proliferation of human intestinal cell lines were studied using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) colorimetric assay (Boehringer-Mannheim; Indianapolis, IN). Briefly, yellow MTT is converted to an insoluble purple formazan crystal only by metabolically active cells; thus the absorbance at 570 nm is directly proportional to the number of viable cells. The HCT8 and Caco-2 cell lines were seeded in 96-well plates (Costar; Corning, NY) at a concentration of 3×10^3 cells per well in a volume of 100 μl of cell culture medium per well. After 24 h, 20-μl aliquots of the nucleotides at the indicated concentrations were added to the adherent cells in triplicate for each concentration. The plates were kept in the CO_2 incubator at 37°C for 24 and 48 h. At the end of the second and third day, the culture medium was carefully removed and replaced by 100 μl fresh culture medium. Ten microliters of the MTT reagent were added to each well, and the plates were incubated in a CO_2 incubator at 37°C for an additional 4 h. One hundred microliters of SDS solubilizing reagent (Boehringer-Mannheim) were added to each well. The plates were kept overnight in the CO_2 incubator and read at 570 nm in a luminometer (Lucy 1, Anthos Labtec, Salzberg, Austria) at a wavelength of 570 nm.

Measurement of apoptosis by cytofluorimetry. HCT8 cells were grown in 96-well plates to 60% confluence and then incubated with ATP, ATP analogs, or other nucleotides for 24 or 48 h at 37°C in a 5% CO_2 incubator. Where indicated, cells were preincubated with the $P2X_7$ blocker, oxidized ATP (oATP), for 2 h before addition of ATP. The adenosine transport inhibitor dipyridamole, the adenosine receptor blocker 8-SPT, and the $P2Y_1$ purinergic receptor blocker MRS2179 were added 25 min before ATP/adenosine addition.

Apoptosis was measured quantitatively by cytofluorimetry using three different protocols. Nuclear condensation was measured with detergent-permeabilized propidium iodide (PI)-stained cells, as described previously (21). Both adherent cells and cells in the supernatant were collected for analysis. Briefly, adherent HCT8 cells were harvested in PBS medium containing EDTA, washed with PBS, and resuspended in 0.4 ml of a hypotonic fluorochrome solution (6 μg/ml PI in 0.1% sodium citrate + 0.1% Triton X-100). Samples were stored in the dark at 4°C until flow cytometric analysis. Data from 10,000 HCT8 cells were collected on a FACScan flow cytometer (Becton-Dickinson) with an argon laser tuned to 488 nm. Cellular debris was excluded from analysis by raising the forward scatter (FS) threshold. Apoptotic cell nuclei containing hypodiploid DNA were enumerated as a percentage of total population.

Morphological changes taking place during apoptosis were detected by cytofluorimetry using specific gates for write FS and side scatter (SS), as described (17). Briefly, apoptotic cells cause lower forward light scatter (caused by cell shrinkage) and higher SS (caused by increased granularity of the cell, presumably as a result of chromatin condensation and fragmentation) than their viable counterparts.

To discriminate between necrotic cells and apoptotic cells, we also incubated the HCT8 cells with buffer containing PI and FITC-conjugated annexin V (Boehringer-Mannheim), following the manufacturer's instructions. Early apoptosis was defined as cells staining with Annexin V but excluding PI, and late apoptotic/necrotic cells were labeled with both Annexin V and PI (50).

Terminal deoxythymidine transferase-mediated dUTP nick end labeling. Terminal deoxythymidine transferase-mediated dUTP nick end labeling (TUNEL) staining was performed using an in situ cell death detection kit (Boehringer-Mannheim; Meylan, France) according to the manufacturer's recommendations. Briefly, harvested cells were fixed in 4% paraformaldehyde (PFA; BDH Laboratory Supplies, Poole, UK) at room temperature for 30 min and permeabilized with a buffer containing 0.1% Triton X-100 and freshly prepared 0.1% sodium citrate. Fixed cells were labeled with FITC-dUTP using terminal deoxythymidine transferase. The FITC-stained cells were visualized with a Zeiss Axioplan microscope (Jena, Germany) coupled to a Leica DC 200 image-acquisition system (Cambridge, UK). The figures were prepared using the Adobe Photoshop 5.0 program.

Intracellular calcium measurements. HCT8 and Caco-2 cell lines were grown in 96-well plates (black well, clear bottom; BD Biosciences, NJ) until confluence. The cells were used 1, 2, 3, 5, 6, and 10 days after being plated at subconfluence. The growth medium was then aspirated and replaced with 100 μl of loading medium (PBS containing 1 mM Fluo-4-AM, 10% pluronic acid, and 2.5 mM probenicid) and incubated for 30 min at room temperature. The cells were subsequently washed three times with PBS, and 100 μl of PBS supplemented with 1 mM $CaCl_2$ or with 5 mM EGTA were added to each well. The cells were then placed in a fluorometric imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA), and changes in cellular fluorescence were recorded after the addition of 50 μl of control buffer or 50 μl of the indicated concentration of tested compounds in PBS.

Immunofluorescence. Cultures of HCT8 and Caco-2 cell lines were maintained in RPMI-1640 medium containing 10% heat-inactivated FBS, 2 g/l sodium bicarbonate, 100 U/ml penicillin, and 100 μg/ml streptomycin and were then plated in eight-well LAB-TEC slides (Nalge Nunc International, Naperville, UK) or on microscope coverslips in 12-well plates (Costar) for 24–48 h before use in functional experiments or immunostaining. Cells were postfixed for 10 min at room temperature in 4% PFA prepared in PBS. Blocking of nonspecific binding sites was achieved by incubation with normal horse serum (NHS; Harlan Sera-Lab, Poole, UK) in PBS containing 0.05% Merthiolate (Sigma) at room temperature for 20 min.

For immunostaining, an indirect method with two layers of antibodies was used. Rabbit polyclonal antibodies against human $P2X_{1-7}$ (Roche Bioscience, Palo Alto, CA), $P2Y_1$, $P2Y_2$, and $P2Y_4$ (Alomone Laboratories, Jerusalem, Israel) receptors were allowed to react with biotinylated donkey anti-rabbit IgG secondary antibody (Jackson Immunoresearch, West Grove, PA) and detected with either Oregon Green or avidin-coupled Texas Red (Sigma). Briefly, the slides were incubated overnight with primary antibodies diluted to 5 and 2.5 μg/ml (determined as optimal by previous titration) with 10% NHS in PBS containing 0.05% Merthiolate and 0.1% saponin. After being washed, the sections were incubated with biotinylated secondary antibodies diluted 1:500 in 1% NHS in PBS containing 0.05% Merthiolate and 0.1% saponin for 30 min, followed by a 1 h incubation with ExtrAvidin-Oregon Green or Texas Red both at a concentration of 1:100. In some experiments, a directly labeled, donkey anti-rabbit IgG Cy3 secondary antibody (Jackson Immunoresearch) was applied at a concentration of 1:100 for 1 h. All incubations were carried out at room temperature and separated by three 5-min washes in PBS. Control experiments were performed using an excess of the appropriate homologous peptide antigen to absorb the primary antibodies and thus confirm specific immunoreaction. The slides were visualized, and images were prepared using a Zeiss Axioplan microscope (Jena, Germany) coupled with a Leica DC 200 image-acquisition system (Leica, Cambridge, UK). The figures were prepared using the Adobe Photoshop 5.0 program.

RT-PCR analysis for purinergic receptor expression. Total RNA was extracted from HCT8 and Caco-2 cells with Trizol reagent (GIBCO-BRL) according to the manufacturer's instructions. Total RNA was reverse transcribed, and RT-PCR was performed following standard procedures. Amplification was done with the following sequences: actin A: 5′-TCACCCACACTGTGCCCATCTACGA-3′, 5′-CAGCGGAACCGCTCATTGCCAATGG-3′;$P2X_1$:5′-GCGTAATAAGAAGGTGGGCGTTA-3′, 5′-GCCGCTCGAGGTCTGGTA-3′; $P2X_2$: 5′-CAGGTTTGCCAAATACTACAAGATCA-3′, 5′-AACTTCCCGGCCTGTCCAT-3′; $P2X_3$: 5′-TCTTCACCTATGAGACCACCAAGTC-3′, 5′-GATCAGAAGCTGAACTACTCGGTTGATG-3′; $P2X_4$: 5′-CGCAGGACACGGTTTCCA-3′, 5′-GCAGTCCCAGTTGACC-

TGGAT-3′; $P2X_5$: 5′-CGCTGGGGAAGCGGTTA-3′, 5′-GCACCAGGCAAAGATCTCACA-3′; $P2X_6$: 5′-CACTGCCGCTATGAACCACAA-3′, 5′-CGAAGGTCCCTCCAGCCTT-3′; $P2X_7$: 5′-CTTTCTCAAAACAGAAGGCCAAGA-3′, 5′-CAACCTCGGTCAGAGGAACAGA-3′; P2Y1: 5′-CGGTCCGGGTTCGTCCT-3′, 5′-TAGTAAAACTGGAAGCCCGTCTTG-3′; $P2Y_2$: 5′-CTCTACTTTGTCACCACCAGCGC-3′, 5′-CCACGAAGCGGCTGAAGA-3′; $P2Y_4$: 5′-CCACCTGGCATTGTCAGACAC-3′, 5′-GGTTGTGGGCTGCATAATAGTAGATGA-3′; $P2Y_6$: 5′-CGCTTCCTCTTCTATGCCAACC-3′, 5′-CCCACGTTTGTGCCAGGG-3′; $P2Y_{11}$: 5′-CAGCGTCATCTTCATCACCTGC-3′, 5′-AGGTGGCTTCGGGCGA-3′; $P2Y_{12}$: 5′-CTGGGCATTCATGTTCTTACTC-3′, 5′-TGCCAGACTAGACCGAACTCT-3′. The following PCR cycling parameters were used: 90°C, 10 min; 40× (95°C, 45 s; 60°C, 45 s; 72°C, 45 s); 72°C, 10 min; 4°C. Peripheral blood lymphocytes were used as a positive control for expression of all the P2X receptors. Brain tissue was used as a positive control for $P2Y_1$, $P2Y_{11}$, $P2Y_{12}$, and $P2Y_{13}$ receptors; whereas spleen was used as a positive control for the $P2Y_6$ receptor, the small intestine for the $P2Y_4$ receptor, and skeletal muscle for the $P2Y_2$ receptor.

Statistical analysis. Statistical analysis was performed using the unpaired Student's *t*-test. *P* values <0.05 were considered significant.

RESULTS

Effects of extracellular nucleotides on proliferation of intestinal epithelial cell lines. HCT8 and Caco-2 cells were seeded at a low cell density and cultured for up to 3 days in cell culture medium, in the presence or absence of different nucleotides. As measured by the MTT assay 48 h after treatment, the growth of both HCT8 (Fig. 1*A*) and Caco-2 (Fig. 1*B*) cells was inhibited by treatment with 1 mM ATP. Growth inhibition was dose dependent (100 μM-1 mM ATP), beginning after 24 h of treatment and becoming pronounced after 48 h (Fig. 2, *A* and *B*). However, when cells from either cell line were incubated with ATP at a concentration of 10 μM, the effect was inverted and cells proliferated more efficiently (Fig. 2). Given the unexpected stimulatory behavior of low concentrations of ATP, we also treated the epithelial cells with other nucleotides. A 48 h treatment with the pyrimidine UTP increased proliferation of HCT8 and Caco-2 cells at all the concentrations tested (Figs. 1, *C* and *D*, and 2, *C* and *D*). UDP at 100 μM also stimulated proliferation after 48 h (Fig. 1, *C* and *D*). ADP at 100 μM, similar to ATP, had a slight inhibitory effect on HCT8 cells (Fig. 1, *C* and *D*), but no interference with cell growth was observed at lower concentrations.

We next investigated the effect of Zn^{2+} in proliferation assays in the presence of nucleotides. We observed that the antiproliferative response of 1 mM or 100 μM ATP or 100 μM ADP was potentiated in the presence of 50 μM Zn^{2+} for ATP and was slightly potentiated or not at all for ADP (Fig. 1*E*). The zinc treatment had no effect on UTP-induced proliferation of HCT8 cells (data not shown). At higher

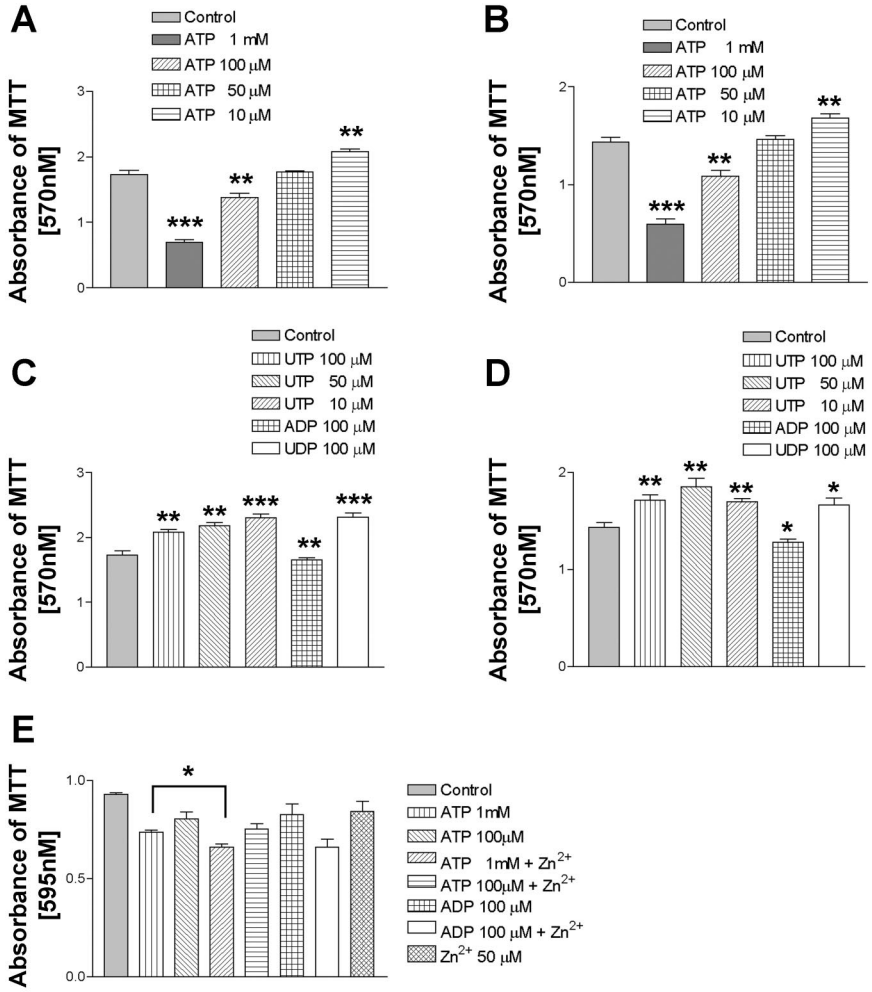

Fig. 1. Effects of nucleotides on growth of human epithelial cell lines. HCT8 and Caco-2 cells were seeded in 96-well plates at a concentration of 3×10^3 cells/well, and proliferation was studied using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) colorimetric assay after 48 h, as described in MATERIALS AND METHODS. *A* and *B*: Dose dependence of ATP effects on HCT8 and Caco-2 cell growth after 48 h incubation in *A* and *B*, respectively. *C* and *D*: dose dependence of UTP, ADP, and UDP effects on proliferation of HCT8 and Caco-2 cells, respectively. *E*: effect of 50 μM zinc on ATP- and ADP-dependent proliferation. The data represent the means ± SE of 3 independent experiments in quadruplicate. Significance between control and stimulated conditions: $*P < 0.05$; $**P < 0.001$; $***P < 0.0001$.

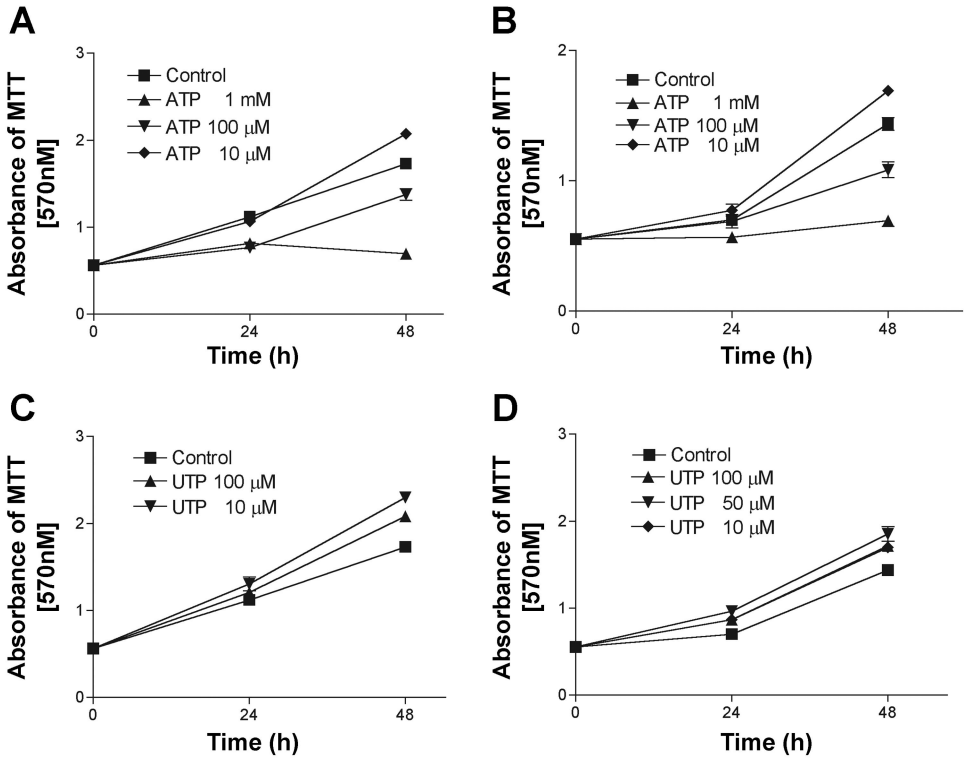

Fig. 2. Time course of ATP and UTP on growth of human epithelial cell lines. HCT8 cells (*A* and *C*) and Caco-2 cells (*B* and *D*) were incubated with nucleotides for 24 or 48 h, and proliferation was assayed using MTT. Note that the ATP and UTP effects start before 24 h and became larger after 48 h. The data represent the means ± SE of 3 independent experiments done in triplicate.

concentrations (>100 μM), zinc by itself inhibited proliferation.

Effects of extracellular nucleotides on apoptosis. The decrease in the number of cells following treatment with ATP could be due to either a lower rate of proliferation or constant proliferation but a higher level of cell death. To distinguish between the two possibilities, we measured the effects of ATP on apoptosis of HCT8 cells. Forty-eight hours after incubation with 2 mM ATP, ~40% of the cells were undergoing apoptosis, as assayed by cytofluorimetric measurement of nuclear condensation (Fig. 3*A*). There was no apoptosis following treatment with 10 μM ATP or 500 μM UTP at any concentration (shown for 500 μM in Fig. 3*A*), consistent with the observation that the nucleotides at these concentrations enhance the rate of proliferation. Thus there is apoptosis following incubation with a concentration of ATP that decreases the number of cells, but no apoptosis under conditions in which the extracellular nucleotides stimulate proliferation or have no effect on cell growth.

Effects of specific inhibitors on nucleotide-induced apoptosis. We also pretreated HCT8 cells with the irreversible $P2X_7$ receptor antagonist oATP (18, 48) before incubation with 2 mM ATP. Pretreatment with oATP inhibited the proapoptotic effect of ATP by ~50%, suggesting that $P2X_7$ stimulation is

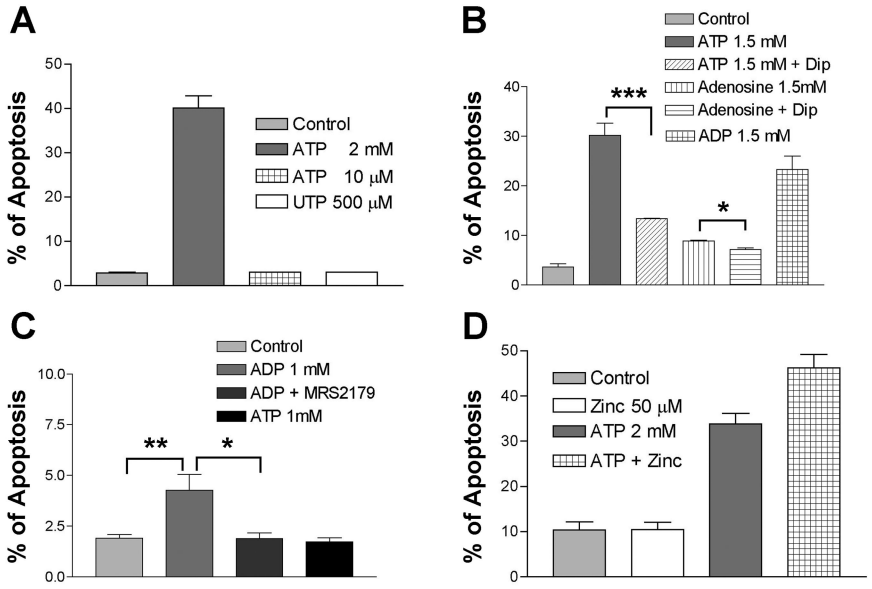

Fig. 3. Apoptotic effects of extracellular nucleotides. *A*: apoptosis was quantified by cytofluorimetry based on measurement of hypodiploid HCT8 cells after 48 h of incubation with nucleotides at different concentrations, as described in MATERIALS AND METHODS. *B*: HCT8 cells were preincubated with 10 μM dipyridamole (Dip), an adenosine-transport inhibitor, for 20 min before nucleotide incubation and then treated with ATP, ADP, or adenosine for 48 h. *C*: cells were preincubated for 15 min with 30 μM MRS 2179, a known $P2Y_1$ antagonist, and then treated with 1 mM ADP for 24 h. *D*: cells were preincubated for 5 min with 50 μM zinc and then treated with 2 mM ATP for 48 h. Dip, MRS2197, and zinc alone have no effect on apoptosis. Apoptosis was quantified by cytofluorimetry by measuring light scattering and cells that are positive for annexin staining but negative for propidium iodide (PI) staining. Significant differences from cells treated with ATP or adenosine alone: $^*P < 0.05$ and $^{**}P < 0.001$.

involved in about one-half of the ATP-dependent apoptosis but that other purinergic receptors may also contribute to apoptosis of the intestinal epithelial cells.

Cell death was also quantified by measuring typical morphological changes associated with apoptosis (17). Two days after treatment with 1.5 mM ATP, nearly one-third of the HCT8 cells had apoptotic morphology (Fig. 3*B*), in line with the results obtained on nuclear condensation. ADP at 1.5 mM, a concentration that inhibits cell growth, also induced apoptosis. Given the possibility that some of the effects of ATP or ADP may be due to adenosine, which is produced following spontaneous hydrolysis of ATP or ADP or their catabolism by ectonucleotidases, HCT8 cells were also incubated with 1.5 mM adenosine, which caused a measurable level of apoptosis (Fig. 3*B*). Significantly, because adenosine receptors are stimulated by 10-fold lower concentrations of ligand than those required to stimulate ATP- or ADP-specific receptors, adenosine had no effect on apoptosis at lower concentrations ($<$500 μM). The effects of adenosine could involve either activation of extracellular receptors or direct intracellular action, but 8-SPT, a broad-spectrum blocker of adenosine receptors (51), had no effect on adenosine-induced apoptosis.

ATP can interact with purinergic receptors but could, in addition, modify the concentration of other intracellular nucleotides (11, 59). This possibility was tested by coincubating cells with dipyridamole, an inhibitor of adenosine transport (59). Dipyridamole inhibited ATP-mediated apoptosis by $>$50%, suggesting that part of the effect of ATP may be due to transport of adenosine across the cell membrane. Taken together, the results obtained with dipyridamole and theophylline suggest that cell death induced by adenosine, and partially by ATP, is mediated by a direct toxic effect of adenosine on the epithelial cells, rather than through adenosine receptors, as previously shown for human leukemia HL-60 cells (59). The observation that only adenosine concentrations $>$1 mM are able to induce apoptosis reinforces this view.

Two types of ADP receptors are known to be involved in platelet aggregation, the $P2Y_1$ and $P2Y_{12}$ receptors (33). To distinguish between the two possibilities for HCT8 cells, apoptosis was induced by incubation with ADP, with or without cotreatment with MRS2179, an antagonist of the $P2Y_1$ receptor (51). MRS2179 blocked all of the cell death when measured 24 h after incubation with 1 mM ADP (Fig. 3*C*), suggesting that ADP triggers apoptosis in intestinal epithelial cells due to ligation of $P2Y_1$. However, the level of ADP-mediated apoptosis was low after 24 h. A higher level of ADP-dependent apoptosis was observed after 48 h, but this cell death was no longer inhibited by MRS2179.

We then tested the effect of Zn^{2+} in the apoptosis assay. At low concentraions, zinc is an antagonist of the $P2X_7$ and $P2X_1$ receptors but potentiates ATP effects on PX_2-$P2X_6$ (49). We observed that in the presence of 50 μM Zn^{2+}, the apoptotic response to 2 mM ATP was enhanced, being 37% higher than in the presence of ATP alone (Fig. 3*D*).

To confirm that the nuclear condensation measured by cytofluorimetry corresponds to apoptosis, fragmentation of nuclear DNA was also assessed by the TUNEL technique, which identifies cells with apoptosis-dependent DNA breaks. Two days after treatment with 2 mM ATP, more than one-third of the cells had apoptotic nuclei (Fig. 4*A*). UTP at the same concentration had no effect on DNA fragmentation of HCT8 cells (Fig. 4*B*).

Expression of P2X and P2Y mRNA and protein. To determine which P2X and P2Y isoforms are expressed by the intestinal epithelial cells, mRNA was isolated from HCT8 and Caco-2 cells and was analyzed by RT-PCR. Amplified PCR products of the expected sizes were obtained for $P2X_1$ (109 bp), $P2X_3$ (83 bp), $P2X_4$ (393 bp), $P2X_5$ (99 bp), $P2X_6$ (84 bp), and $P2X_7$ (84 bp) receptors (Fig. 5*A*). Although the expression was faint, $P2X_7$ receptor mRNA was found in HCT8 and Caco-2 cells, whereas $P2X_2$ receptor mRNA was absent from both cell types. RT-PCR for the housekeeping gene, β-actin, was performed to control for the quantity of cDNA loaded on the gel (data not shown). Peripheral blood lymphocytes, used as a positive control for the expression of all the P2X receptors,

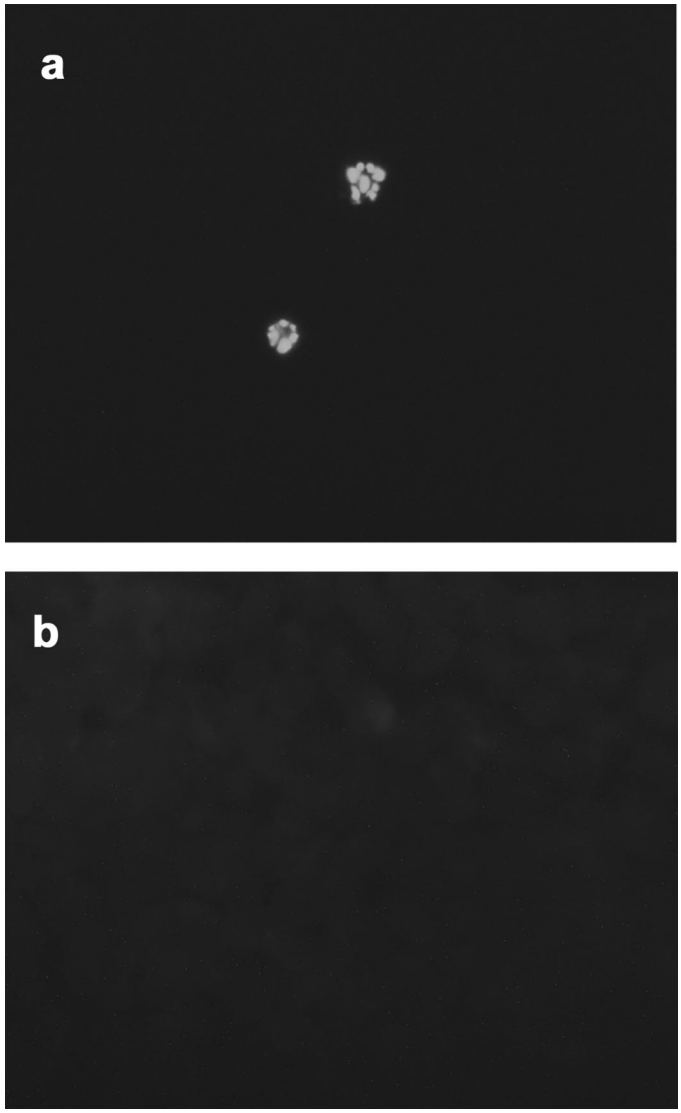

Fig. 4. Induction of nucleotide-dependent apoptosis measured by the terminal deoxythymidine transferase-mediated dUTP nick end labeling (TUNEL) assay. HCT8 cells were treated for 48 h with ATP or UTP and prepared for labeling with the TUNEL technique. *a*: Fluorescence micrograph of cells incubated with 2 mM ATP. Note typical apoptotic morphological changes (nuclear fragmentation) induced by treatment with the nucleotide. *b*: Fluorescence micrograph of cells incubated with 2 mM UTP. *a* and *b*: magnification, ×400.

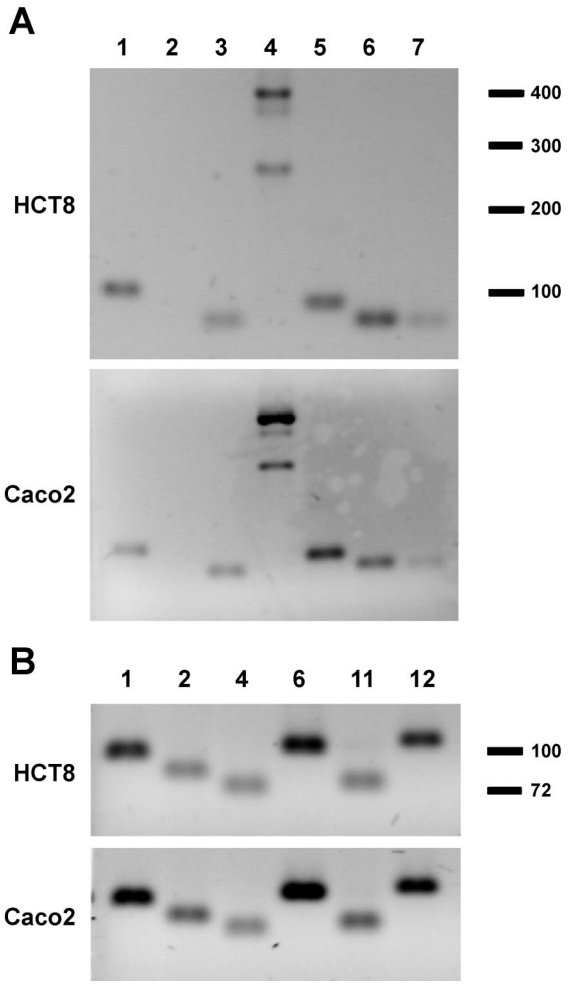

Fig. 5. P2X and P2Y mRNA are expressed in human epithelial cells. *A*: P2X RT-PCR products from HCT8 and Caco-2 cells. *B*: P2Y RT-PCR products from HCT8 and Caco-2 cells. All PCR amplifications were performed at least 3 times. Peripheral blood lymphocytes were used as positive controls for expression of all the P2X receptors. Tissues known to express different P2Y receptors were used as controls for P2Y expression, as described in MATERIALS AND METHODS.

showed expression of the $P2X_2$ receptor, confirming the quality of the $P2X_2$ receptor PCR probes used in the epithelial cells. Similarly, amplified PCR products of the expected sizes were obtained for the $P2Y_1$ (103 bp), $P2Y_2$ (85 bp), $P2Y_4$ (73 bp), $P2Y_6$ (110 bp), $P2Y_{11}$ (77 bp), and $P2Y_{12}$ (117 bp) receptors in both HCT8 and Caco-2 cells (Fig. 5*B*). The amplified PCR product of the expected size was obtained for $P2Y_{13}$ (576 bp) in Caco-2 cells. The expected band was sometimes, but not always, present in HCT8 cells.

The synthesis of P2X receptor protein was then evaluated by immunofluorescence labeling, using polyclonal antibodies against $P2X_{1-7}$. As shown in Table 1 and Fig. 6, *A-D*, receptor protein expression was observed for most of the P2X receptors tested. In most experiments, $P2X_4$ and $P2X_7$ receptor immunostaining was stronger than for the other P2X subtypes. Thus, despite the low level of $P2X_7$ mRNA, a high level of $P2X_7$ receptor protein was found on HCT8 and Caco-2 cells. Antibody specificity was determined by preincubating cells with an excess of the peptide recognized by the polyclonal antibodies (Fig. 6, *insets*). In general, we observed the following order of staining intensity: $P2X_4 > P2X_7 > P2X_1 > P2X_3 > P2X_6 > P2X_5 >> P2X_2$.

Likewise, the expression of the P2Y receptor subtypes was studied by immunofluorescence, using polyclonal antibodies against the $P2Y_1$ and $P2Y_2$ receptors. As shown in Fig. 6, *E-H*, HCT8 cells expressed protein for both of the P2Y receptors tested. Consistently, immunolabeling was strongest for $P2Y_1$ on the cell surface in both cell lines. In contrast, there was intense intracellular immunostaining for $P2X_5$ and $P2X_6$ receptors (data not shown).

Taken together, these data agree with the functional data on nucleotide-mediated apoptosis, which revealed the presence of at least $P2X_4$ and $P2X_7$ and $P2Y_1$ and $P2Y_2$ receptors on the epithelial cell surface.

Intracellular calcium concentration changes due to treatment with extracellular nucleotides. We measured calcium concentration changes in HCT8 and Caco-2 cells. Cells in 96-well plates maintained a stable, basal intracellular calcium concentration, but exposure to 1 mM ATP resulted in a rapid rise in the calcium concentration, which subsided to basal levels within 2 min (Fig. 7*A*). However, larger increases were observed reproducibly following treatment with 1 mM UTP. UDP and ADP also triggered calcium increases, with UDP being twice as effective as ADP. The agonist for $P2X_1$, $P2X_3$, and $P2X_4$ receptors, α,β-meATP, had no effect on intracellular calcium levels (Fig. 7*A*).

It should be noted that there was some variability in the calcium responses to ATP in cells that remained on the 96-well plates for <5 days. The sensitivity of young cells to ATP observed at 100 μM was always less than that at 1 mM, whereas cells that had been cultured for >6 days had similar responses at 100 μM and 1 mM ATP. Although the basis for this age-dependent sensitivity is not clear, it is consistent with a previous report that the nucleotide effects on Caco-2 cells depend on the length of the cell culture period (38). Accordingly, we analyzed dose-response curves for different nucleotides obtained only from calcium flux measurements on cell cultures that had been plated for >6 days (Fig. 7*B*). The concentrations of nucleotides that give half-maximal calcium responses (EC_{50}) were the following: 1.1 μM for UTP, 5.3 μM for ATP, 11 μM for UDP, and 82 μM for ADP.

The presence of functional $P2X_7$ receptors was confirmed by incubating epithelial cells with BzATP, an agonist of the $P2X_7$ receptor (26), which efficiently triggers calcium fluxes in HCT8 cells (Fig. 7*C*). Preincubation with the irreversible $P2X_7$ receptor antagonist oATP (48) blocked all the BzATP-dependent calcium changes, reinforcing the interpretation that $P2X_7$ is expressed and is functional on intestinal epithelial cells.

To begin identifying the purinergic receptors that could mediate the calcium fluxes, HCT8 cells were incubated with ATP, UTP, or ADP in the presence or absence of different P2

Table 1. *Summary of P2X and P2Y receptor subtypes identified on intestinal epithelial cells by immunostaining*

	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$	$P2X_7$	$P2Y_1$	$P2Y_2$
HCT8	+*	±	+*	+	+	+	+	+	+
Caco-2	+*	±	+*	+	+	+	+	+	+

+, present; +, weakly present or absent; *, negative controls used for the $P2X_1$ and $P2X_3$ receptors were secondary antibody without primary antibody.

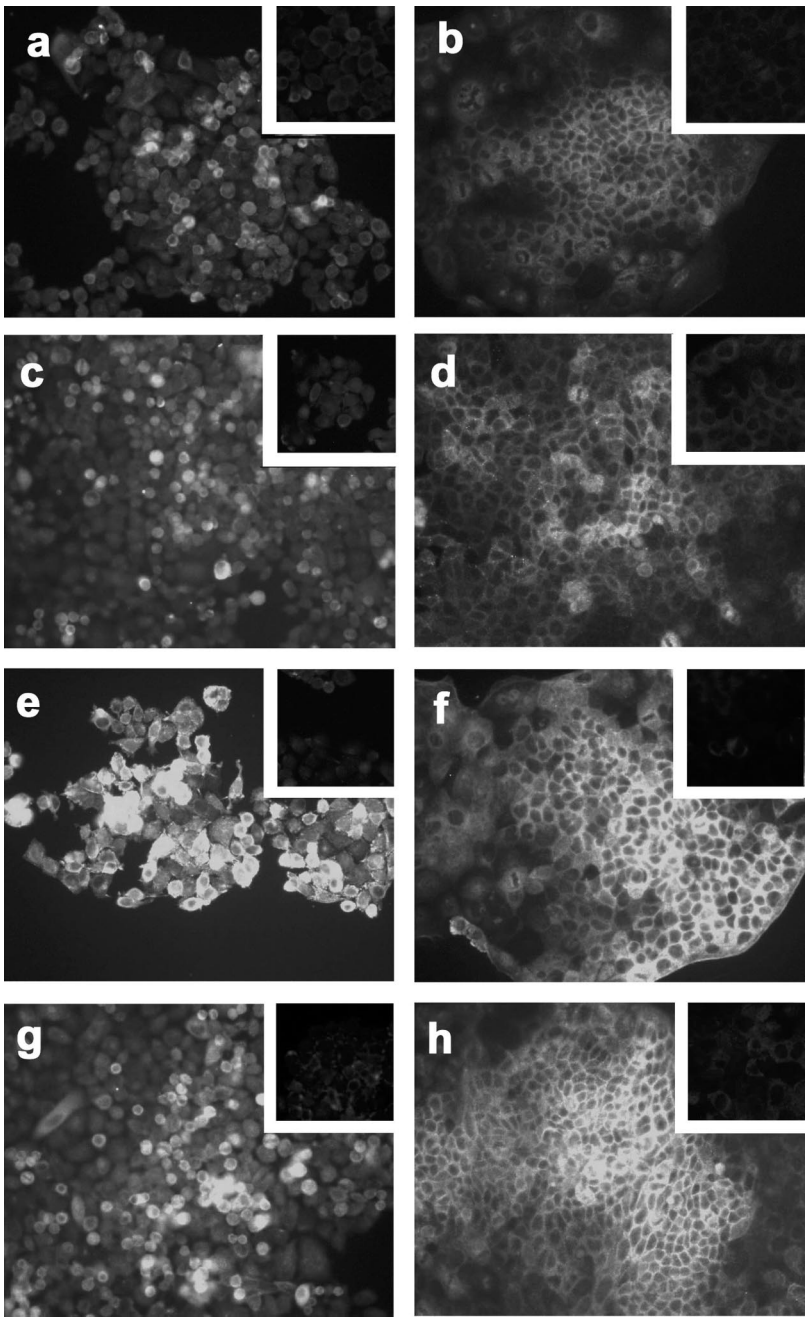

Fig. 6. P2X and P2Y immunolabeling on HCT8 and Caco-2 cells. Immunofluorescent staining of $P2X_4$ (*a* and *b*). $P2X_7$ (*c* and *d*) $P2Y_1$ (*e* and *f*) and $P2Y_2$ (*g* and *h*) on HCT8 and Caco-2 cells, respectively. The *insets* show the antibody specificity of cells that had been preincubated with an excess of the peptide recognized by the polyclonal antibodies *a*–*h*. Magnification, ×200.

inhibitors. Exposure of cells to 1 mM or 100 μM ATP induced similar levels of calcium increases, as shown in the histogram in Fig. 8*A*, which shows the maximal fluorescence increase over basal levels, corresponding to the maximal calcium concentration changes. Nearly one-half of the calcium concentration increases due to 1 mM or 100 μM ATP was blocked by the $P2X_7$ inhibitor KN-62 (27). Because the effect was not complete, though, the results suggest that other ATP-dependent purinergic receptor(s) can also trigger calcium fluxes. As expected, KN-62 had no effect on calcium changes following treatment with 10 μM ATP, a concentration at which ATP does not stimulate $P2X_7$.

Similarly, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), which inhibits preferentially P2X receptors, although not all of them (51), inhibited more than one-half of the calcium increases following treatment with 1 mM or 100 μM ATP (Fig. 8*B*). These results confirm the presence of functional $P2X_7$ on intestinal epithelial cells. However, the inhibition by PPADS was not complete, suggesting that the $P2X_4$, $P2X_6$, $P2Y_2$, and/or $P2Y_{11}$ receptors may also be present.

Calcium concentration changes were also induced weakly by 1 μM UTP and strongly by 100 μM or 1 mM UTP (Fig. 8*C*). UTP could trigger calcium fluxes through ligation of either the $P2Y_2$ or $P2Y_4$ receptor. To distinguish between the two possibilities, epithelial cells were incubated with suramin, which inhibits the $P2Y_2$ receptor. Suramin decreases calcium fluxes by about one-half those at 1 mM UTP, implying that the $P2Y_2$ receptor is activated by UTP. However, the inhibition was not

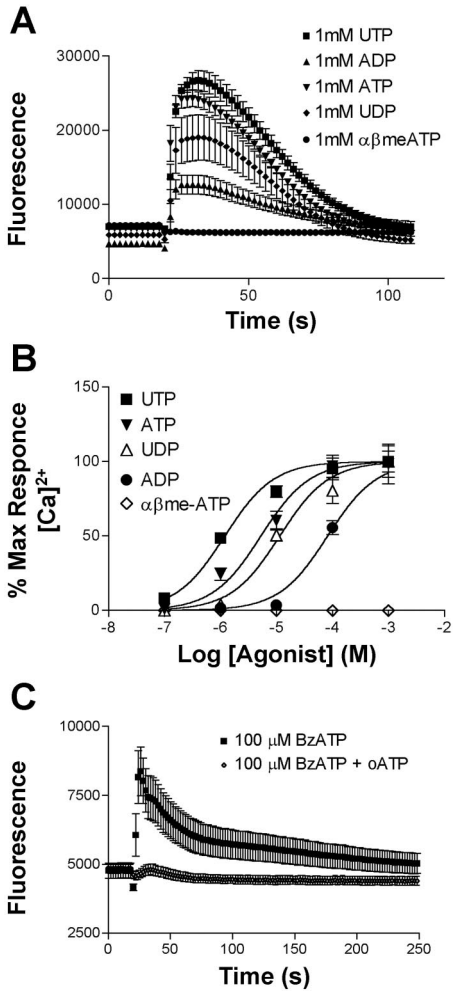

Fig. 7. Effect of different nucleotides on induction of intracellular Ca^{2+} responses. *A*: changes in $(Ca^{2+})_i$ in response to 1 mM of the indicated nucleotides were measured in fluo-4-AM-loaded HCT8 epithelial cells using the fluorometric imaging plate reader (FLIPR) assay. The trace is from a representative experiment from 6 independent experiments done in quadruplicate. *B*: intracellular calcium dose-response curves were plotted as means ± SE of 4 independent experiments done in triplicate using the PRISM program. Ca^{2+} mobilization in response to increasing concentrations of each compound was measured by the FLIPR assay. *C*: HCT8 cells were preincubated for 2 h with 400 μM oxidized ATP (oATP) and then stimulated with 100 μM BzATP. The trace shows a representative experiment from 3 independent experiments done in triplicate.

complete, leaving open the possibility that the $P2Y_4$ receptor may also be activated.

Finally, ADP triggers calcium fluxes, which could take place through the $P2Y_1$ and/or $P2Y_{11}$ receptor. MRS2179, an antagonist of the $P2Y_1$ receptor, weakly inhibits ADP-induced calcium fluxes (Fig. 8*D*), suggesting that most of the ADP-dependent calcium changes are most likely due to $P2Y_{11}$ receptor stimulation, although smaller fluxes may be due to the $P2Y_1$ receptor.

Cross-desensitization between P2 agonists. There is a large rundown in the amplitude of inward currents following repeated applications of BzATP, due to phosphorylation of the $P2X_7$ receptor following each application of the agonist (41). To investigate possible synergy or cross-desensitization between different agonists in nucleotide-promoted calcium responses, HCT8 and Caco-2 cells were first exposed to a known concentration of agonist. When the calcium concentration had returned to near-basal levels, the same cells were treated with 100 μM of a second agonist in the continued presence of the first agonist.

It has been shown that ATP and BzATP are antagonists of $P2Y_1$ receptors (61, 62). A prepulse of HCT8 cells with 100 μM ATP (Fig. 9*A*) or BzATP completely abolished a subsequent calcium flux due to treatment with 100 μM ADP. However, a prepulse with ADP reduced the subsequent ATP-mediated flux by only 22 + 3% (Fig. 9*B*).

Pretreatment with 100 μM ATP reduced the UTP-mediated calcium response by 23 ± 5% (Fig. 9*C*), whereas pretreatment with 100 μM UTP decreased markedly, but not completely (by 48 ± 6%), the ATP response (Fig. 9*D*). Interestingly, pretreatment with BzATP did not have a significant effect on UTP-induced calcium concentration changes (Fig. 9, *E* and *F*), suggesting that the two $P2X_7$ agonists, ATP and BzATP, may act on different receptors expressed on intestinal epithelial cells.

DISCUSSION

Intestinal epithelial cells represent one of the organism's first lines of defense against pathogens that invade the gastrointestinal tract. The intestinal cells are renewed rapidly and are both targets for microbial infection and a source of early inflammatory cytokines secreted by the organ. ATP release is expected to occur in different tissues during inflammation (2), and it has been proposed that purinergic signaling may play a role in regulating intestinal physiology (3, 4). Recently $P2Y_6$ receptors have been implicated with rat colonic Cl^- secretion (43). $P2X_7$ and $P2X_5$ receptors are also expressed on gut epithelia, and their expression has been associated with cellular differentiation and cell death (32). With regard to cancer, $P2Y_2$ receptors have been extensively described as being functional on carcinoma cells from different tissues (34, 35, 47); and recently, $P2X_5$, $P2X_7$,$P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors have been identified by immunohistochemical analysis in human basal and squamous cell carcinomas (30). Nonetheless, none of the studies until now has examined in detail the presence and function of different P2X and P2Y receptor subtypes on human intestinal carcinoma epithelial cells. We have therefore characterized, through molecular and functional assays, the activity and expression of purinergic receptors in two unrelated human intestinal carcinoma epithelial cell lines.

We observed a paradoxical effect of ATP on proliferation of the HCT8 and Caco-2 cell lines. At higher concentrations that normally induce activation of the $P2X_7$ receptor in other systems, ATP inhibits proliferation, but at 10 μM, a suboptimal concentration for $P2X_7$ receptor activation, ATP stimulates proliferation of the same cells. Inhibition of proliferation was most likely due to apoptosis of the epithelial cells, but according to our data, more than one purinergic receptor is involved in this process. Although the identity of all the receptors is not yet known, one of the receptors could be $P2X_7$, because a known $P2X_7$ receptor agonist, oATP, partially blocked ATP-mediated apoptosis. It was found that the BzATP calcium response was weaker than that for ATP. This can be explained in two ways: first, we performed the calcium experiments at room temperature, while $P2X_7$ receptor pore forma-

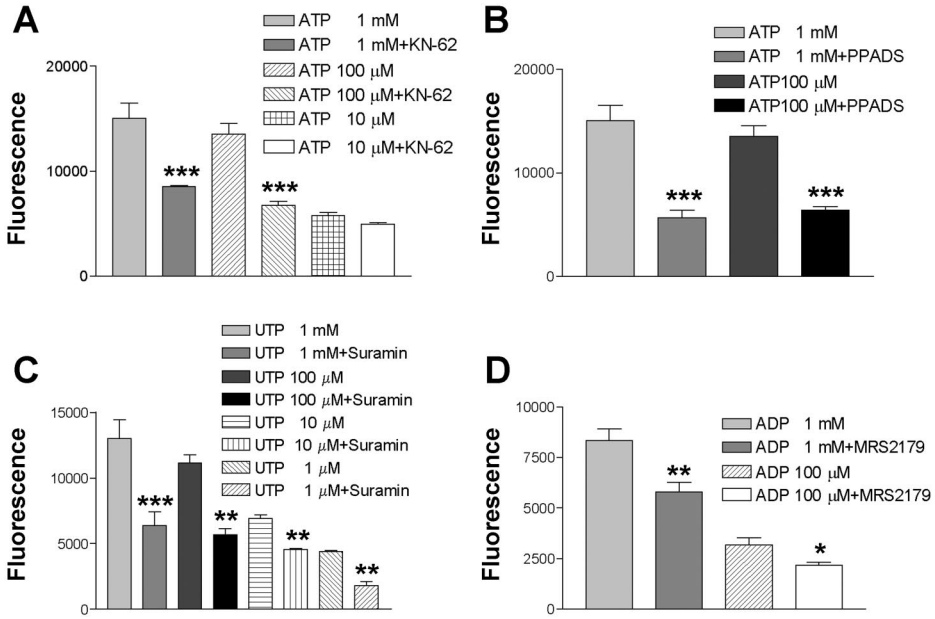

Fig. 8. Comparison of the effects of KN-62, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), suramin, and MRS2179 on ATP and UTP-induced Ca^{2+} responses. The figure shows the difference of the maximal response minus the basal level (~5,000 fluorescence units). All nucleotides were injected after 20 s of acquisition. The cells were treated with KN-62 (5 μM), PPADS (100 μM), suramin (100 μM), or MRS2179 (30 μM) 15 min before adding increasing concentrations of nucleotides. *A* and *B*: KN-62 and PPADS effects on ATP responses. *C*: suramin effects on UTP responses. *D*: MRS2179 effects on ADP responses. The effect of blockers on Ca^{2+} responses due to treatment with increasing concentrations of nucleotides was measured by the FLIPR assay. The data are from a representative experiment of 4 independent experiments done in triplicate. $*P < 0.05$, $**P < 0.001$, $***P < 0.0001$.

tion is known to be temperature-dependent. Secondly, the ATP response was due to activation of more than one P2 receptor, particularly $P2Y_2$ receptors, which, similar to $P2X_7$ receptors, were always present. On the other hand, given the results with BzATP, we favor activation of the $P2X_7$ receptor but not the $P2Y_2$ receptor. Considering the temperature dependence of $P2X_7$-induced pore opening: at room temperature (the condition of our calcium measurement), only the $P2X_7$ ion channel should open, but at 37°C (the condition of our proliferation and apoptosis assays), $P2X_7$-induced pore opening and calcium fluxes are maintained at high levels for a long period of time.

In HCT8 cells, we did not detect upregulation of $P2X_7$ receptors on cells. In fact, the time course of apoptosis induced by ATP was much longer (24–48 h in HCT8 cells) compared with other cells that express $P2X_7$ receptors at high levels, such as macrophages and dendritic cells (6 h) (13, 14). The difference could be explained by the number of $P2X_7$ receptors present on the membrane or by the presence of ectoATPase on the plasma membrane of epithelial cells.

The Zn^{2+} results suggest that the main contribution is from $P2X_7$ receptors with a contribution from other P2 receptors, possibly $P2X_4$ or $P2X_5$, on the antiproliferative and apoptotic effects of ATP. The effects of $P2X_4$ and $P2X_5$ receptors are potentiated by Zn^{2+} (49), and we have found mRNA for both of these receptors in the epithelial cells studied. In particular, $P2X_5$ receptors have been associated with inhibition of cell proliferation of satellite cells, human keratinocytes, and skin cancer cells (30, 31, 53), but in all cases, they were associated with cell differentiation. The $P2Y_1$ receptor is also likely to be involved, because ADP inhibited proliferation and induced apoptosis in both cell lines and the selective $P2Y_1$ receptor antagonist, MRS2179, inhibited apoptosis. Consistent with this interpretation, the $P2Y_1$ receptor has already been linked with apoptosis in human astrocytoma cells (53). It is not clear why MRS2179 had no effect at the longer time of incubation with ADP, but one possibility may be that adenosine-mediated apoptosis may be taking place due to adenosine production following ADP hydrolysis.

In addition, we have shown that mRNA and protein for both receptors are expressed in the intestinal epithelial cells. Finally, the intracellular calcium fluxes and their dependence on different agonists and antagonists of purinergic receptors strongly suggest that the functional receptors are present.

An alternative mechanism should also be considered for the induction of apoptosis. Because adenosine could be generated through ATP hydrolysis and epithelial cells express ectoATPases on their surface (20, 54, 58), it is possible that ATP could have some of its effects indirectly through adenosine. In line with this possibility, pure adenosine is directly toxic for HCT8 cells. These results are in agreement with a suggestion that millimolar concentrations of adenosine induce apoptosis (59). Thus ATP-induced apoptosis may be due partially to adenosine, which acts directly within cells. Whichever mechanisms may be responsible for nucleotide-mediated epithelial cell death, it is interesting to note that loss-of-function mutations in an intracellular "peptidoglycan receptor," Nod2, increase the risk of developing Crohn's disease (37), and it has been proposed that the Nod2 mutations may lead to autoimmune disease due to dysregulation of apoptosis and, consequently, increased inflammation (5). Whereas much remains to be learned about the role that extracellular nucleotides may play in the physiology of the intestinal tract, it is tempting to speculate that they may contribute to autoimmune disease through their effect on epithelial cell apoptosis.

Regarding proliferation, the only nucleotide that was active besides ATP was UTP, suggesting that the $P2Y_2$ receptor may also be present and may be involved in cell proliferation. The $P2Y_2$ receptor has been previously reported to stimulate proliferation of smooth muscle cells (36) and a human cutaneous squamous cell carcinoma cell line (A431) (30), but antiproliferative and apoptotic effects of $P2Y_2$ receptors have also been described for two human epithelial carcinoma cells, colorectal and esophageal cancer cells (34, 35, 47). These conflicting findings regarding the possible function of $P2Y_2$ receptors on carcinoma cells highlight the need to be careful before proposing $P2Y_2$ receptors as targets for innovative

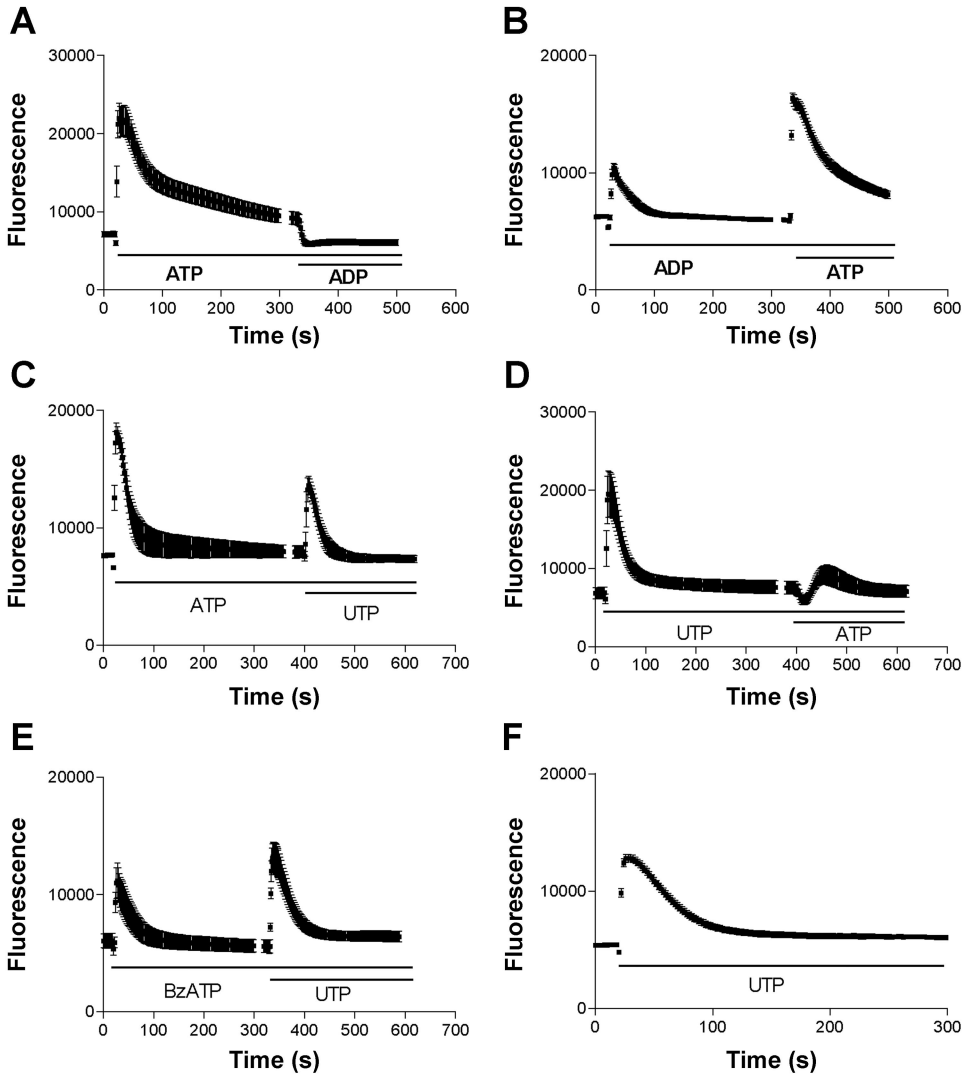

Fig. 9. The effects of nucleotides on cross-desensitization of Ca^{2+} responses. *A*: addition of ADP (100 μM) during prolonged ATP stimulation (100 μM), showing that ATP blocks ADP-induced calcium responses. *B*: addition of ATP (100 μM) during prolonged 100 μM ADP stimulation, showing that the ATP response was almost unchanged by pretreatment with ADP. *C*: addition of UTP (100 μM) in the presence of ATP (100 μM). *D*: addition of UTP (100 μM) during prolonged stimulation with ATP (100 μM). *E*: addition of UTP (100 μM) during prolonged stimulation with BzATP (100 μM). *F*: UTP response control for untreated cells shown in *E*. Representative tracings from 4 independent experiments are shown.

treatment strategies of cancer. As for $P2X_4$, $P2X_5$, $P2X_7$, and $P2Y_1$ receptors, we also confirmed that $P2Y_2$ receptor mRNA and protein are expressed in both cell lines and that a functional receptor can be activated by ATP and UTP. Furthermore, on the basis of our measurements of intracellular calcium concentration changes and the RT-PCR data, we propose that the $P2Y_4$ receptor is present and functional on human epithelial carcinoma cells. $P2Y_4$ mRNA has also been previously detected in murine stomach, intestines, and liver (57), and the protein has been shown to be present in basal cell carcinomas (30). With the use of $P2Y_4$-null mice, the $P2Y_4$ receptor has been shown to play a role in epithelial chloride transport in the jejunum (22, 52).

The main unexpected finding from this study was the expression of $P2X_3$ receptors on intestinal epithelial cells. This receptor is expressed mainly on nociceptive sensory neurons of the dorsal root and trigeminal and nodose ganglia (9, 10), and its activation was associated with the sensation of pain (7). However, recently $P2X_3$ receptors have also been found in murine endothelial and epithelial cells (28, 29). We showed that both mRNA and protein for the $P2X_3$ receptor are expressed on Caco-2 and HCT8 cells, which may be functionally relevant considering that the activity of the $P2X_3$ receptor may be modulated in the human gut during inflammatory disease (63). Nonetheless, our intracellular calcium measurements did not confirm the functional expression of the $P2X_3$ receptor on HCT8 cells. Both constitutively and under conditions of stress, epithelial cells release ATP (unpublished data; see also Ref. 54), and it may be possible that we failed to detect $P2X_3$ function if its activity was rapidly desensitized. In preliminary experiments using apyrase to degrade released ATP, we observed a small response to αβMeATP, making this explanation plausible. Thus, although we await further experiments to confirm the functional presence of the $P2X_3$ receptor, intestinal epithelial carcinoma cells clearly express functional protein for at least the $P2X_7$, $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors.

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