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Expression of purinergic receptors and modulation of $P2X_7$ function by the inflammatory cytokine IFNγ in human epithelial cells

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A B S T R A C T

The cervical epithelial cell line, HeLa, is one of the oldest and most commonly used cell lines in cell biology laboratories. Although a truncated $P2X_7$ receptor has recently been identified in HeLa cells, the expression of other purinergic receptors or the function of the $P2X_7$ protein has not been characterized. We here show that HeLa cells express transcripts for most P2X and P2Y purinergic receptors. Treatment of cells with ATP or other $P2X_7$ agonists does not stimulate cell death, but can induce atypical calcium fluxes and ion currents. Cervical epithelial cells represent an important target for sexually-transmitted pathogens and are commonly exposed to pro-inflammatory cytokines such as IFNγ. Stimulation of HeLa cells with IFNγ upregulates expression of $P2X_7$ mRNA and full-length protein, modifies ATP-dependent calcium fluxes, and renders the cells sensitive to ATP-induced apoptosis, which can be blocked by a $P2X_7$ antagonist. IFNγ treatment also increased dramatically the sensitivity of the intestinal epithelial cell line, HCT8, to ATP-induced apoptosis. Significantly, IFNγ also stimulated $P2X_7$ expression on human intestinal tissues. Responses to other purinergic receptor ligands suggest that HeLa cells may also express functional $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors, which could be relevant for modulating ion homeostasis in the cells.

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1. Introduction

Extracellular nucleotides are signalling molecules that exert effects on a variety of tissues, mainly via interaction with specific membrane-bound receptors [1]. The target-cell responses are mediated by P2 receptors named either P2X or P2Y. P2X receptors are ligand-gated ion channels, and P2Y receptors are coupled to trimeric G-proteins [2]. Seven members of the P2X family in mammals have been cloned ($P2X_{1-7}$) [3], and eight P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) [4,5].

Activation of the $P2X_7$ receptors leads to opening of a plasma membrane pore, inducing the permeabilization of the cells to molecules up to 800 Da; although not all cells display pore formation in response to $P2X_7$ ligation [3]. The $P2X_7$ receptor is responsible for the release of pro-inflammatory cytokines such as IL-1β and subsequently IL-6 in murine and human macrophages and mast cells [6,7], induction of apoptosis in different cell types [6,8], and elimination of intracellular parasites in macrophages [9–12].

Although most studies on purinergic receptors have been performed with cells from the central nervous system or macrophages, various P2X and P2Y receptors have also been described recently in epithelial cells from different sources [13,14]. Thus, human intestinal epithelial 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 $P2Y_{12}$, and the functional presence of $P2X_7$, $P2Y_1$, $P2Y_2$ and $P2Y_4$ has been confirmed based on the effect of extracellular nucleotides on apoptosis, cell proliferation, and calcium fluxes [15]. The $P2X_7$ receptor is also expressed in the murine gut epithelium, where its expression is associated with cell death [16]. While parotid acinar epithelial cells do not express functional $P2X_7$, duct cells express functional receptor [17], showing a restricted distribution of some receptors even within the same organ. The $P2Y_1$ and $P2Y_2$ receptors are also involved with ATP-induced calcium signaling in airway epithelial cells [18,19], and $P2Y_2$ has been linked with inhibition of growth of human eosophageal squamous and colorectal carcinoma cells [20,21]. Finally, the $P2Y_4$ and $P2Y_6$ receptors control chloride transport in jejunum and colon epithelial cells, respectively [22,23].

Abbreviations: DMEM, Dulbecco's modified Eagle's Medium; IFNγ, interferon-γ; IL-1β, interleukin-1β; IL-6, interleukin-6; PLC, phospholipase C; TNFα, tumor necrosis factor α; α,β-meATP, α,β-methylene adenosine 5′-triphosphate

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Nonetheless, the presence of the family of P2 receptors has not been fully characterized on the cervical epithelial cell line, HeLa, which represents the "work horse" of many cell biology and microbiology laboratories [24]. The uterine cervical epithelium is maintained by a balance between proliferation of a basal layer of cells, and death of cells in the upper layer. They regulate lubrication of the genital tract, represent a barrier against invasion by pathogens, and provide the necessary conditions for reproduction. Recently it has been shown that extracellular ATP mediates apoptosis via $P2X_7$ in hECE and CaSki, two human cervical epithelial cell lines; and that estrogen, epinephrine, and epidermal growth factor, combined with epinephrine, inhibit $P2X_7$-receptor-mediated pore formation and apoptosis in these cells [25–27]. HeLa cells, a human cervical carcinoma cell line that is used as a model host cell for infection by pathogens that infect the cervical epithelium but also other tissues [28–32], express $P2Y_2$ and $P2Y_6$ receptors [33,34] and a truncated form of $P2X_7$ [35]. However, given the presence of the truncated $P2X_7$ [35] and the effect of a PLC inhibitor on calcium signaling, it was assumed that HeLa cells do not express functional P2X purinergic receptors [33]. In addition, the functional expression of other P2 receptors on this cell line has never been investigated fully. We therefore examined whether other P2X and P2Y receptors may be expressed in epithelial HeLa cells, have begun to identify their cellular behavior, and have re-addressed the question of whether $P2X_7$ on HeLa cells may be functional. As cervical epithelial cells are also a common site of infection with sexually-transmitted pathogens, most of which initiate a strong inflammatory response [36–38], we also investigated whether the pro-inflammatory cytokines, IFNγ and TNFα, may modulate the expression or activity of $P2X_7$ receptors on HeLa cells.

2. Materials and methods

2.1. Cells and materials

The human cervical adenocarcinoma cell line, HeLa229, was from the American Type Culture Collection (ATCC; Manassas, VA). The cells were cultured in a humidified incubator at 37 °C with 5% CO_2 in Dulbecco's modified minimal essential medium (DMEM) with Glutamax-1 (Life Technologies, Inc.; Rockville, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 25 μg/ml gentamicin. Recombinant human IFN-γ and TNFα were from BD Biosciences Pharmingen (San Jose, CA). All nucleotides were purchased from Sigma-Aldrich (St. Louis, MO). Fura-2 was obtained from Molecular Probes (Eugene, OR). Unless specified otherwise, all other reagents were purchased from Sigma. The nucleotides were prepared as 100 mM stock solution in PBS and stored at −20 °C until use.

2.2. RT-PCR detection of nucleotide receptors

RNA from HeLa cells was isolated using an RNeasy kit (Qiagen), following the manufacturer's instructions. Total RNA was converted into cDNA by standard reverse transcription with Superscript II Rnase H^- Reverse Transcriptase in the manufacturer's buffer (Invitrogen, Life Technologies; Carlsbad, CA). cDNAs were amplified using the iCycler (BIO-RAD laboratories, Richmond, CA) in a 50 μl reaction mixture containing one-fifteenth of the cDNA generated from reverse transcription reaction, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM (each) dNTPs, 0.5 μM forward and reverse primers, and 1 U HotGoldstar DNA polymerase (Eurogentec, Seraing, Belgium). Amplification was done with the following sequences: GAPDH: 5′-AACGGATTTGGTCG-TATTGGGC-3′, 5′-CTTGACGGTGCCATGGAATTTG-3′; $P2X_1$: 5′-GCTGGTGCGTAATAAGAAGGTG-3′, 5′-ATGAGGCCGCTCGAGGTCTG-3′; $P2X_2$: 5′-AGGTTTGCCAAATACTACAAGATC-3′, 5′-GCTGA-ACTTCCCGGCCTGTC-3′; $P2X_3$: 5′-CTTCACCTATGAGACCACCAAG-3′, 5′-CGGTATTTCTCCTCACTCTCTG-3′; $P2X_4$: 5′-GATACCAGCTCAGGAG-GAAAAC-3′, 5′-GCATCATAAATGCACGACTTGAG-3′; $P2X_5$: 5′-GGCA-TTCCTGATGGCGCGTG-3′, 5′-GGCACCAGGCAAAGATCTCAC-3′; $P2X_6$: 5′-AGCACTGCCGCTATGAACCAC-3′, 5′-AGTGAGGCCAGCAGCCAGAG-3′; $P2X_7$: 5′-TGATAAAAGTCTTCGGGATCCGT-3′, 5′-TGGACA-AATCTGTGAAGTCCATC-3′; $P2Y_1$: 5′-CTTGGTGCTGATTCTGGGCTG-3′, 5′-GCTCGGGAGAGTCTCCTTCTG-3′; $P2Y_2$: 5′-CCGCTCGCTGGACCT-CAGCTG-3′, 5′-CTCACTGCTGCCCAACACATC-3′; $P2Y_4$: 5′-CGCTGCCCACCCTCATCTAC-3′, 5′-CCGAGTGGTGTCATGGCACAG-3′; $P2Y_5$: 5′-TGTTCAGTCTACCCACTCTCAG-3′, 5′-CTTACTGCTGCCACTACT-GAGC-3′; $P2Y_6$: 5′-AACCTTGCTCTGGCTGACCTG-3′, 5′-GCAGG-CACTGGGTTGTCACG-3′; $P2Y_8$: 5′-CTCCTGGCGCACATCGTGAG-3′, 5′-GGAGCGCACGGACGTGGTC-3′; $P2Y_{10}$: 5′-GGTGTTCATGTGTGCTG-CAGTC-3′, 5′-ATGGCGGGATAGTTGGTCACG-3′; $P2Y_{11}$: 5′-GAGGCCTG-CATCAAGTGTCTG-3′, 5′-ACGTTGAGCACCCGCATGATG-3′; $P2Y_{12}$: 5′-ACCGGTCATACGTAAGAACGAG-3′, 5′-GCAGAATTGGGGCACTTCAGC-3′; $P2Y_{13}$: 5′-GGAAGCAACACCATCGTCTGTG-3′, 5′-GACTGTGAGTA-TATGGAACTCTG-3′; and $P2Y_{14}$: 5′-CCGCAACATATTCAGCATCGTG-3′, 5′-ACATTTGCAGCAGATAGTAGCAG-3′. The PCR cycling protocol for all primers was 94 °C at 45 s, 60 °C at 45 s, 72 °C at 45 s. The protocol was conducted for 40 cycles and included an initial 10-min enzyme activation step at 95 °C and a final 10-min extension step at 72 °C. PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. Peripheral blood lymphocytes were used as a positive control for expression of all the P2X receptors. The human laryngeal epithelial carcinoma cell line Hep2 was used to verify expression of all P2Y receptors.

2.3. Real-time PCR for $P2X_7$ mRNA from HeLa cells

RNA from HeLa cells was isolated using an RNeasy kit (Qiagen) following the manufacturer's instructions. Total RNA was converted into cDNA by standard reverse transcription with Superscript II Rnase H^- Reverse Transcriptase in the manufacturer's buffer (Invitrogen). Quantitative PCR was performed with one-fifteenth of the cDNA preparation in an Mx3000P (Stratagene; La Jolla, CA) in 25 μl final volume with Brilliant QPCR Master Mix (Stratagene). cDNA was amplified using 300 nM of $P2X_7$ forward primer, 100 nM of $P2X_7$ reverse primer or 400 nM of GAPDH forward and reverse primers. We also used 300 nM of the fluorogenic oligonucleotides specific for the gene segments in which a reporter fluorescent dye on 5′ (FAM®) and a quencher dye on 3′ (TAMRA®) were attached. Real-time PCR was carried out at 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s, 60 °C for 1 min. The specific activities of cDNA from $P2X_7$ were compared with GAPDH and normalized to untreated control HeLa cells by the comparative C_T method as described by the manufacturer. For human GAPDH, the primers used were as follows: sense primer, 5-GAGAAGGCTGGGGCTCAT-3′; antisense primer, 5′-TGCTGATGATCTT-GAGGCTG-3′; probe, 5′-CTCTGCTGATGCCCCCATGTTCGT-3′. For $P2X_7$, the primers used were as follows: sense primer, 5′-CTTTCTCAAAA-CAGAAGGCCAAGA-3′; antisense primer, 5′-CAACCTCGGTCAGAGGAA-CAGA-3′; probe, 5′-TGTGTCCCGAGTATCCCACCCGC-3′.

2.4. Measurement of cell death by cytofluorimetry

HeLa cells were incubated in the presence or absence of IFNγ (0.5, 1, or 2 ng/ml) for 3 days. HeLa cells were then treated with the indicated concentration of ATP (3 or 5 mM) in serum-free DMEM medium for 2 h, after which the medium was replaced with DMEM medium containing 10% heat-inactivated FCS, 25 μg/ml gentamicin, in the absence of nucleotides. After an additional 24 h in the incubator at 37 °C, both adherent cells and cells in suspension were collected, washed twice in PBS, and analyzed by cytofluorimetry. Phosphatidylserine exposure on dying cells was measured by labeling with Annexin V-FITC (R&D Systems; Minneapolis, MN), and loss of plasma membrane integrity was measured by PI staining, as described [39]. Data from 10,000 HeLa cells were collected on a CyAn LX flow cytometer (Dako Cytomation) with a laser tuned to 488 nm.

In addition to analysis of phosphatidylserine exposure, in some experiments the degree of apoptosis was determined by counting the number of hypo-diploid nuclei after ATP treatment. In brief, HeLa and HCT8 cells were treated as described above. The cells were incubated with 300 μM oATP for 2 h before ATP treatment. After the final 24 h in the incubator at 37 °C, both adherent cells and cells in suspension were collected, washed twice in PBS, spun for 10 min (200 ×*g*), and resuspended in an apoptotic buffer containing 50 μg/ml ethidium bromide, 0.01 g sodium citrate, and 0.1% Triton X-100. The samples were transferred into 12×75 mm FALCON 2052 FACS tubes (Becton Dickinson, San Jose, CA) and kept on ice until use. Data from 10,000 cells were collected on a FACScan flow cytometer (Becton Dickinson) equipped with a 488 nm argon laser. The cells acquired for analysis were gated to eliminate cell aggregates using WinMDI software. The cells with sub-diploid amounts of DNA were considered apoptotic.

2.5. Western blot analysis

HeLa cells were incubated in the presence or absence of IFNγ (0.2, 0.5, 2 or 5 ng/ml), or in a combination of IFNγ (0.5 or 2 ng/ml) plus TNFα (10 or 100 ng/ml) for 2 days. After an additional 24 h in the incubator at 37 °C in the absence of any cytokines, the cells were collected, transferred to microfuge tubes, and washed once in cold PBS, and the pellets were stored at −80 °C until use. Samples were lysed in buffer containing 50 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and protease inhibitor cocktail (5 μl/ml, Sigma), and left for 30 min on ice. The protein concentration was determined using the micro BCA protein assay (Pierce), and 35 μg of protein were separated on a 10% SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences; Piscataway, NJ). After blocking in Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% BSA for 1 h, the membranes were washed extensively in TBST and immunostained with a rabbit polyclonal anti-$P2X_7$ receptor antibody (Calbiochem) diluted at 1:5000 in blocking buffer. Following three washes with TBST, membranes were incubated with an anti-rabbit polyclonal antibody conjugated to HRP diluted at 1:10,000 (Amersham Biosciences) in TBST supplemented with 5% nonfat dry milk. After subsequent washes, specific bands were visualized by enhanced chemiluminescence (Amersham Biosciences).

2.6. Statistics

The effect of ATP on the viability of HeLa cells was statistically evaluated using the Student *t* test. Effects of IFNγ on $P2X_7$ expression in the normal colonic epithelium were analyzed as the difference of expression using the Wilcoxon signed rank test.

2.7. Organ culture and immunohistochemistry

Colonic mucosal explants were cultured for 24 h at 37 °C with 95% O_2 and 5% CO_2 by standard procedures. Briefly, biopsy specimens were placed on iron grids in an organ culture dish in the presence of medium alone, consisting of RPMI 1640 medium (Life Technologies), 10% fetal calf serum, and penicillin and streptomycin (100 U/ml), or with the addition of IFNγ (2 ng/ml). After 24 h, mucosal explants were immediately embedded in Tissue-Tek O.C.T. compound (Miles Scientific Laboratories Ltd, Naperville, IL) and snap-frozen in isopentane in a liquid nitrogen bath. Samples were then stored at −80 °C until processing, and cut into 5 μm sections in a cryostat maintained at −20 °C. Tissue sections were air-dried and fixed for 10 min in acetone. $P2X_7$ expression was evaluated using the indirect immunoperoxidase technique. Immunohistochemical staining was performed using the rabbit anti-$P2X_7$ antibody (1:300 dilution; Alomone, Jerusalem, Israel). Briefly, frozen sections were incubated at room temperature with normal horse serum for 30 min and then with the anti-$P2X_7$ antibody for 1 h. Negative control experiments were performed by pre-absorbing the $P2X_7$ receptor antibodies with homologous peptide antigens and/or by omission of the primary $P2X_7$ antibodies. After rinsing in PBS for 10 min, tissue sections were incubated for 30 min with a goat anti-rabbit peroxidase conjugate IgG (Zymed Laboratories Inc.). Additional rinsing was followed by development with a solution containing hydrogen peroxide and diaminobenzidine, dehydrated, lightly counterstained in Harris's hematoxylin, dehydrated again, and mounted in histological mounting medium. Sections were examined by two independent observers using light microscopy at ×400 magnification.

2.8. Intracellular calcium measurements

HeLa cells treated with 0, 1, or 2 ng/ml IFNγ were grown on glass coverslips for 48 h to subconfluence. The cells were then loaded with 2.5 μM Fura-2-AM (Molecular Probes) for 40 min at room temperature in culture medium. The coverslips were washed in PBS and mounted in a three-compartment superfusion chamber attached to the stage of an inverted microscope (NIKON DIAPHOT 300 TMD) whose base was formed by a coverslip containing the cells. The central chamber containing the cells had a volume of 200 μl, and was perfused with Ca^{2+}-containing saline (PBS supplemented with 1 mM $CaCl_2$) or Ca^{2+}-free saline (PBS supplemented with 1 mM EGTA) at room temperature at a rate of 1 ml min^{-1}. The intracellular calcium concentration of groups of 15–30 cells was monitored continuously with the use of a fluorescence photometer (Photon Technology; Princeton, NJ). Fura-2 was excited alternately at 340 and 380 nm, and the emission at 510 nm was measured. The ratio measurement, which is proportional to the intracellular calcium concentration, was determined every 100 ms. The nucleotides were injected in bolus, allowing the drugs to persist in the presence of the cells for 1 min, or the ATP was perfused continuously while the temperature was kept constant at 37 °C.

2.9. Electrophysiology

Cells were grown in 35 mm plastic culture dishes (Falcon) and incubated in the presence or absence of 1 or 2 ng/ml IFNγ for 24–48 h. The cells were then transferred to an inverted microscope (Zeiss Axiovert 100; Carl Zeiss; Jena, Germany). The culture medium was exchanged for an extracellular solution containing, in mM: 135 NaCl, 5 KCl, 1 $MgCl_2$, and 10 Na-HEPES, pH 7.4. Unless specified in the text, the experiments were performed at 23–25 °C. ATP and UTP were applied locally to the cell of interest using a microperfusion device. Recordings were carried out using the conventional whole cell patch-clamp technique [40]. Ionic currents were recorded in whole cell configuration, using an EPC-7 amplifier (List Electronic, Darmstadt, Germany) according to standard patch-clamping techniques [40]. Gigaohm seals were formed after offset potential compensation, using heat-polished micropipettes of 5–10 MΩ filled with an intrapipette solution (in mM: 135 KCl, 5 NaCl, 2 $MgCl_2$, 0.1 K-EGTA, and 10 K-HEPES, pH 7.4). ATP and UTP were applied to the cell surface by pneumatic injection, using a second micropipette filled with a different concentration of nucleotides dissolved in extracellular solution and connected to a PPM-2 pneumatic pump (NeuroPhore BH-2 system, List). Data were collected using pClamp and Fetchex software, version 6.0, and a Digidata 1200 interface (Axon Instruments) and plotted using Origin software version 4.0 (Microcal Inc.).

3. Results

3.1. Expression of P2X and P2Y mRNA on HeLa cells

In order to determine which P2X and P2Y isoforms are expressed by cervical epithelial cells, mRNA was isolated from HeLa cells and was analyzed by RT-PCR. Amplified PCR products of the expected sizes were obtained for $P2X_3$ (324 bp), $P2X_4$ (393 bp), $P2X_5$ (142 bp), $P2X_6$ (176 bp) and $P2X_7$ (396 bp) (Fig. 1A). The expression was faint for

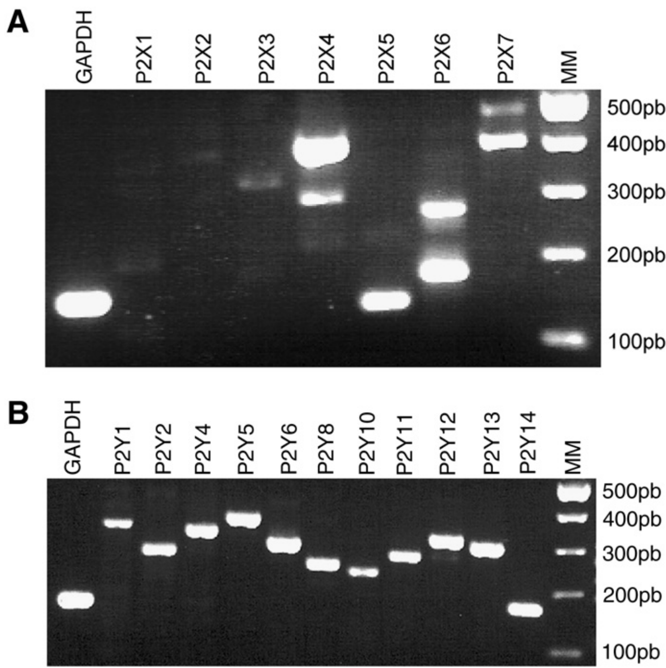

Fig. 1. P2X and P2Y mRNA is expressed in human cervical epithelial cells. (A) P2X RT-PCR products in HeLa cells. (B) P2Y RT-PCR products in HeLa cells. Peripheral blood lymphocytes were used as positive controls for expression of the P2X receptors. All the PCR amplifications were performed at least 3 times.

$P2X_3$ mRNA, while $P2X_1$ and $P2X_2$ were completely absent. RT-PCR for the housekeeping gene, GAPDH, was performed to control for the quantity of cDNA loaded on the gel. Peripheral blood lymphocytes, used as a positive control for expression of all the P2X receptors, showed expression of $P2X_1$ and $P2X_2$ (data not shown), confirming the quality of the $P2X_1$ and $P2X_2$ PCR probes used for the epithelial cells. Amplification of $P2X_4$ and $P2X_6$ mRNA resulted in two fragments, one of the expected size and a second band. We had previously observed additional fragments for $P2X_2$ and $P2X_3$ in macrophages, which were ascribed to non-specific products of PCR amplification and alternative splicing [46]. Similarly, amplified PCR products of the expected sizes were obtained in HeLa cells for $P2Y_1$ (327 bp), $P2Y_2$ (259 bp), $P2Y_4$ (311 bp), $P2Y_6$ (283 bp), $P2Y_{11}$ (260 bp), $P2Y_{12}$ (307 bp), $P2Y_{13}$ (293 bp) and $P2Y_{14}$ (168 bp), and also the orphans genes $P2Y_5$ (351 bp), $P2Y_8$ (237 bp) and $P2Y_{10}$ (227 bp) (Fig. 1B).

3.2. IFNγ modulates $P2X_7$ receptor expression in HeLa cells

The synthesis of $P2X_7$ receptor protein was then assessed with and without pre-treatment with IFNγ, and IFNγ plus TNFα, using the Western blot assay. As shown in Fig. 2 the 70 kD band characteristic of the full-length $P2X_7$ receptor [41] was present on control untreated HeLa cells (Fig. 2A lane 1), besides an additional 40 kDa band, likely corresponding to truncated $P2X_7$ protein. We observed a dose-dependent increase in levels of the full-length $P2X_7$ receptor band following IFNγ treatment, with the concomitant disappearance of the 40 kDa band (Fig. 2A lanes 2–5). It has been shown that the pro-inflammatory cytokine TNFα has a synergistic effect on $P2X_7$ receptor modulation in macrophages [42,43]. The HeLa cells were thus co-treated with different concentrations of IFNγ plus TNFα, as described in the Materials and methods. However, the presence of TNFα did not have an additional effect over IFNγ alone (data not shown).

3.3. Effect of IFNγ treatment on transcription of the $P2X_7$ gene

Since IFNγ treatment had a positive effect on $P2X_7$ receptor protein expression, we evaluated whether IFNγ could affect $P2X_7$ at the transcriptional level. We thus incubated the cells with 0, 0.5, 1 or 2 ng/ml of IFNγ for 72 h and measured $P2X_7$ transcription by real-time PCR. There was a large dose-dependent increase in the amount of $P2X_7$ mRNA after IFNγ treatment (Fig. 2B). A noticeable effect was observed with 0.5 ng/ml IFNγ, but became much larger after treatment with 2 ng/ml IFNγ.

3.4. HeLa cells become sensitive to ATP-induced lysis after IFNγ treatment

HeLa cells are known to be more resistant to ATP-induced lysis than primary cervical cells [26]. But after treatment with IFNγ, the HeLa cells became sensitive to ATP as a function of the IFNγ dose (Fig. 3A). The mode of cell death was next analyzed by double-staining with Annexin V and PI. We observed that untreated cells are completely resistant to apoptosis induced by ATP, but the IFNγ-treated cells became sensitive to ATP-mediated cell death, with characteristics of both apoptosis and necrosis (Fig. 3B). We next investigated the effect of the irreversible $P2X_7$ antagonist, oATP, on apoptosis in the presence of ATP. We pre-treated HeLa cells that were incubated with IFNγ with oATP before incubation with ATP. Pre-treatment with oATP inhibited ATP-induced apoptosis, further confirming the IFNγ-mediated modulation of $P2X_7$ receptor expression (Fig. 3C).

A similar effect was observed with another human epithelial cell line, the intestinal epithelial HCT8 cells, after treatment with IFNγ. We observed that HCT8 cells were 50% more sensitive to ATP-mediated apoptosis than HeLa cells in the absence of IFNγ treatment, but became very sensitive to ATP-induced apoptosis after pre-treatment with increasing concentrations of IFNγ (Fig 3D).

To determine whether IFNγ could also modulate $P2X_7$ receptor expression in primary human epithelial cells, we incubated explants of human intestinal epithelial tissue with IFNγ for 24 h and then

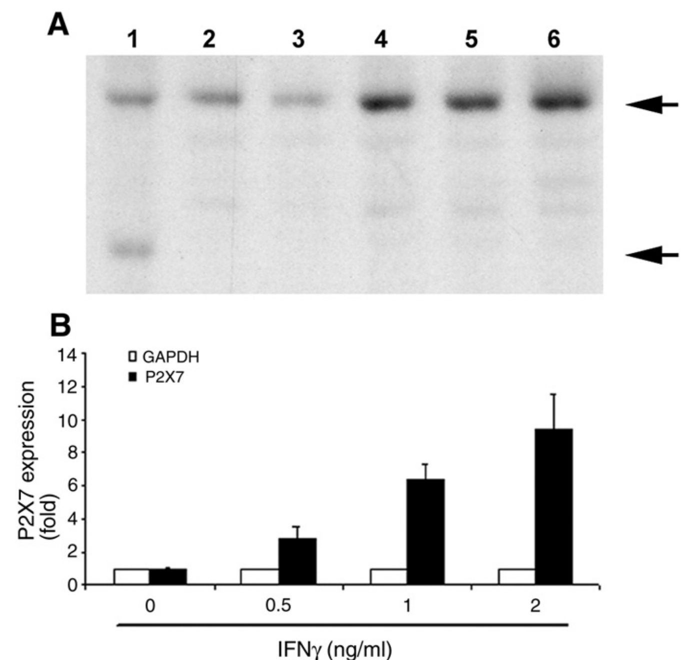

Fig. 2. The pro-inflammatory cytokine IFNγ positively modulates protein and mRNA expression of the $P2X_7$ receptor. (A) Western blot analysis of total protein extracts from untreated HeLa cells and HeLa cells treated with IFNγ (0.2, 0.5, 2 and 5 ng/ml) for 2 days. Lane 1: untreated HeLa cells. Lanes 2–5: cells treated with IFNγ at 0.2, 0.5, 2 and 5 ng/ml, respectively. Lane 6: the J774 macrophage cell line used as a positive control. Upper lower arrows indicate the 70 kDa and 40 kDa bands. The results shown are representative of two experiments performed on different days. (B) Cells were treated with control buffer or increasing concentrations of IFNγ for 3 days. Total mRNA was measured by real-time PCR, as described in the Materials and methods. The experiment was performed on two separate days, with similar results.

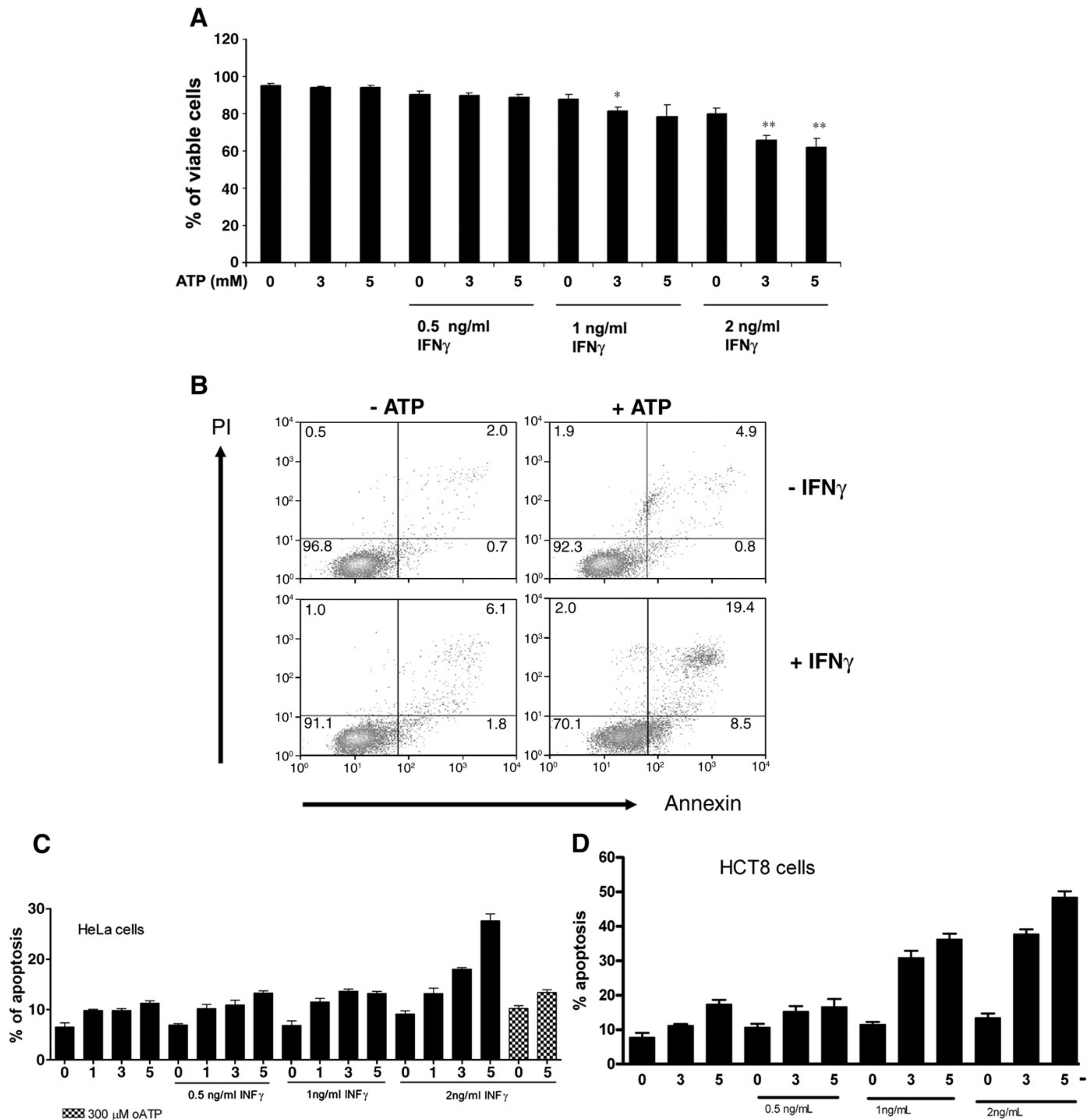

Fig. 3. IFNγ increases the sensitivity to ATP-mediated cell death. HeLa cells were incubated in the presence or absence of IFNγ (0.5, 1, or 2 ng/ml) for 3 days. The cells were then treated with increasing concentrations of ATP for 2 h, and maintained in cell culture for an additional 24 h. (A) The loss of viability induced by ATP after IFNγ incubation, measured by cytofluorimetry (viable cells = cells negative for both Annexin V and PI). B shows the increase in the number of apoptotic cells after incubation with IFNγ (2 ng/ml). Phosphatidylserine exposure on dying cells was measured by labeling with Annexin V-FITC, and loss of plasma membrane integrity was measured by PI staining. C and D show the formation of hypo-diploid nuclei after ATP treatment in HeLa cells (C) and HCT8 cells (D). Note that the pre-treatment with 300 μM oATP inhibited ATP-induced apoptosis. The data shown in A and B are from a representative experiment performed in triplicate repeated on two separate occasions. The data shown in C and D are mean ± SEM of three and four independent experiments performed in triplicate (*$P<0.05$ for 3 mM ATP compared with 0 mM ATP, cells treated with 1 ng/ml IFNγ; **$P<0.001$ for 3 and 5 mM ATP compared with 0 mM ATP, cells treated with 2 ng/ml IFNγ).

examined the samples by immunohistochemistry for the presence of the $P2X_7$ receptor. We observed an increase in $P2X_7$-specific immunostaining on epithelial cells from explants incubated with IFNγ (Fig. 4A–B) compared with untreated ones (Fig. 4C–D), demonstrating that the effects of IFNγ on $P2X_7$ expression are not limited to epithelial cell lines. Quantification of the effect of IFNγ on $P2X_7$ expression is shown in Fig. 4E.

3.5. ATP and UTP elicit calcium fluxes in HeLa cells

To identify the P2-receptor subtypes that may be functional in HeLa cells, we measured changes in $[Ca^{2+}]_i$ induced by nucleotides. In Ca^{2+}-containing PBS solution, successive application of ATP and UTP (100 μM) induced a rapid transient increase of $[Ca^{2+}]_i$, regardless of which nucleotide was added first (Fig. 5A, B).

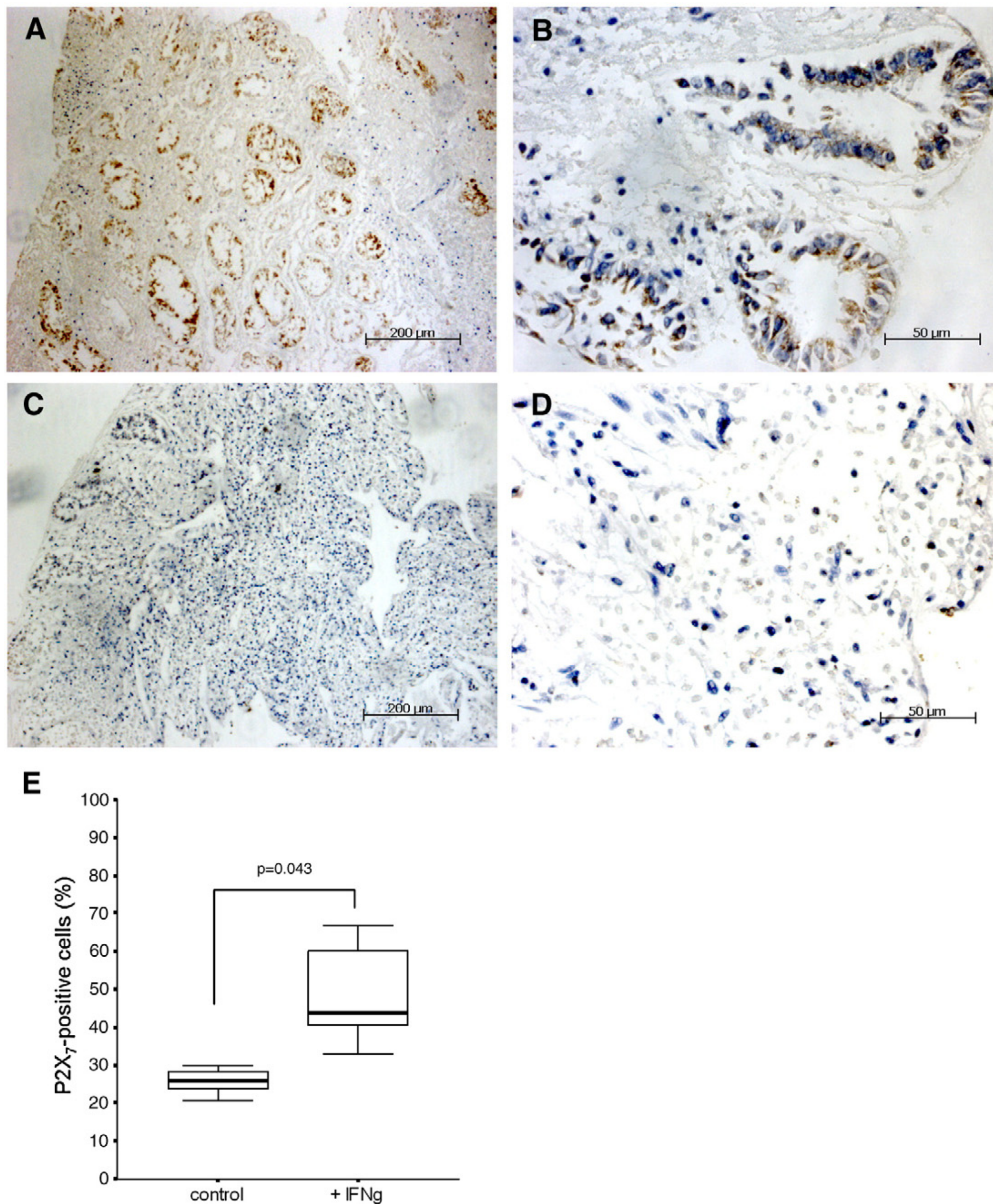

Fig. 4. Effect of IFNγ on $P2X_7$ expression in the normal colonic epithelium. Human colonic samples from endoscopic biopsies were treated in organ cultures with or without the addition of 2 ng/ml IFNγ for 24 h. Frozen sections were stained by immunoperoxidase using an antibody against the $P2X_7$ receptor. A–D shows immunostaining for $P2X_7$ receptors on human epithelial tissues from untreated (C and D) and IFNγ-treated (A and B) samples. Sections were examined using light microscopy at ×100 (A and C) and ×400 magnification (B and D). E, The density of $P2X_7$-positive cells is given as the percentage of immunoreactive cells among epithelial cells in the crypts and in the surface epithelium. Numbers are representative of five different experiments. Horizontal bars represent medians, boxes represent the 25th and 75th percentiles, and vertical bars represent ranges. Differences were analyzed using the Wilcoxon signed rank test.

Removing Ca^{2+} from the extracellular bath barely reduced the ATP responses, suggesting that the main cytosolic $[Ca^{2+}]_i$ increase was due to release from intracellular stores (Fig. 5C). In addition, the ATP response was only partially blocked by pre-treatment with the $P2X_7$ antagonist, oATP (Fig 5D), and the UTP response was completely blocked by suramin treatment (data not shown). To investigate possible cross-desensitization between ATP and UTP in nucleotide-induced $[Ca^{2+}]_i$ changes, HeLa cells were perfused with 100 μM ATP or UTP, until the $[Ca^{2+}]_i$ response returned to basal levels, and the cells were then exposed to 100 μM of a second agonist (ATP or UTP) in the continued presence of the first agonist. As shown in Fig. 5E, UTP pre-treatment markedly, but not completely, reduced the $[Ca^{2+}]_i$ response to ATP, whereas ATP pre-treatment completely abolished the UTP-induced $[Ca^{2+}]_i$ changes (Fig. 5F). UTP and ATP promoted dose-dependent $[Ca^{2+}]_i$ fluxes transiently, with responses starting at 100 nM for both nucleotides, and an EC50 of 17 ± 4 and 27 ± 5 μM for UTP and ATP, respectively (Fig 5G). The nucleotides UDP, ADP and αβ-meATP also elicited changes in $[Ca^{2+}]_i$, beginning at 5 μM for ADP and UDP and 25–100 μM for αβ-meATP (Fig. 5H).

3.6. IFN-γ treatment modifies the pattern of ATP and UTP-induced $[Ca^{2+}]_i$ responses

HeLa cells were perfused with buffer saline containing 1 mM ATP at 37 °C. Under these conditions, we observed a transient rise

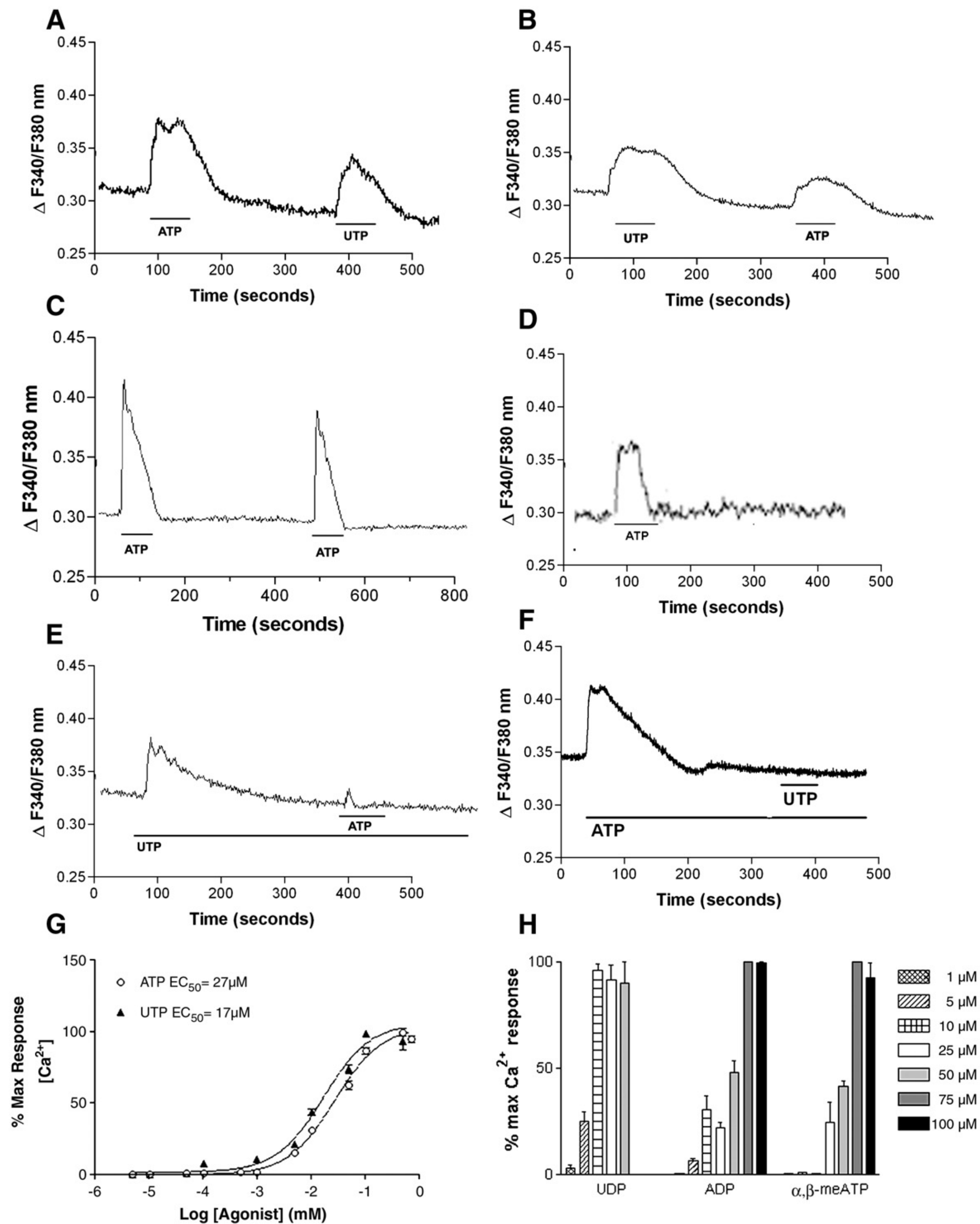

Fig. 5. Effect of different nucleotides on intracellular Ca^{2+} fluxes. HeLa cells were loaded with Fura-2, and nucleotides were added in a bolus of 1-min duration in a continuous perfusion chamber. (A) Effects of 100 μM ATP on $[Ca^{2+}]_i$ followed by stimulation with 100 μM UTP. (B) Same as in A, but UTP was applied first. (C) 100 μM ATP was added in Ca^{2+}-containing saline (first bar) and in calcium-free saline (second bar). (D) Effect of 100 μM ATP on $[Ca^{2+}]_i$ in cells pre-treated with oATP. (E and F) The effect of ATP/UTP on cross-desensitization of Ca^{2+} responses. (E) Addition of 100 μM ATP during prolonged stimulation with 100 μM UTP, showing a small remaining response to ATP. (F) Addition of 100 μM UTP during prolonged stimulation with 100 μM ATP, showing that pre-stimulation with ATP blocks UTP-induced calcium responses. (G) Dose response curves for ATP- and UTP-induced $[Ca^{2+}]_i$ responses. Cells were exposed to the indicated concentration of ATP (open circles) or UTP (filled triangles). (H) Changes in $[Ca^{2+}]_i$ in response to 1, 5, 10, 25, 50, 75 and 100 μM of the indicated nucleotides. The $[Ca^{2+}]_i$ is given as the fluorescence ratio (F340/F380 nm), where fluorescence is proportional to the intracellular calcium concentration. In A–F, representative data of a single experiment with 20 cells per incubation are shown. The experiments were repeated at least three times with similar results. Values in G and H represent mean ± SEM of five (G) and six (H) independent experiments.

in $[Ca^{2+}]_i$, which returned to basal levels after ~2 min (125 ± 16 s, $n = 4$) (Fig. 6A, C). The response was longer but comparable to the response observed with a small bolus application at room temperature. However, cells pre-incubated with 1–2 ng/ml IFNγ for 24 h responded to ATP perfusion with a rapid biphasic increase of $[Ca^{2+}]_i$, followed by a sustained plateau that slowly (4–5 min) (267 ± 28 s, $n = 5$) returned to $[Ca^{2+}]_i$ basal levels (Fig. 6B, C), compatible with the opening of a non-selective pore on the plasma membrane, as demonstrated for $P2X_7$ in other cell types [6,8,41,44]. The pre-treatment with the $P2X_7$ receptor

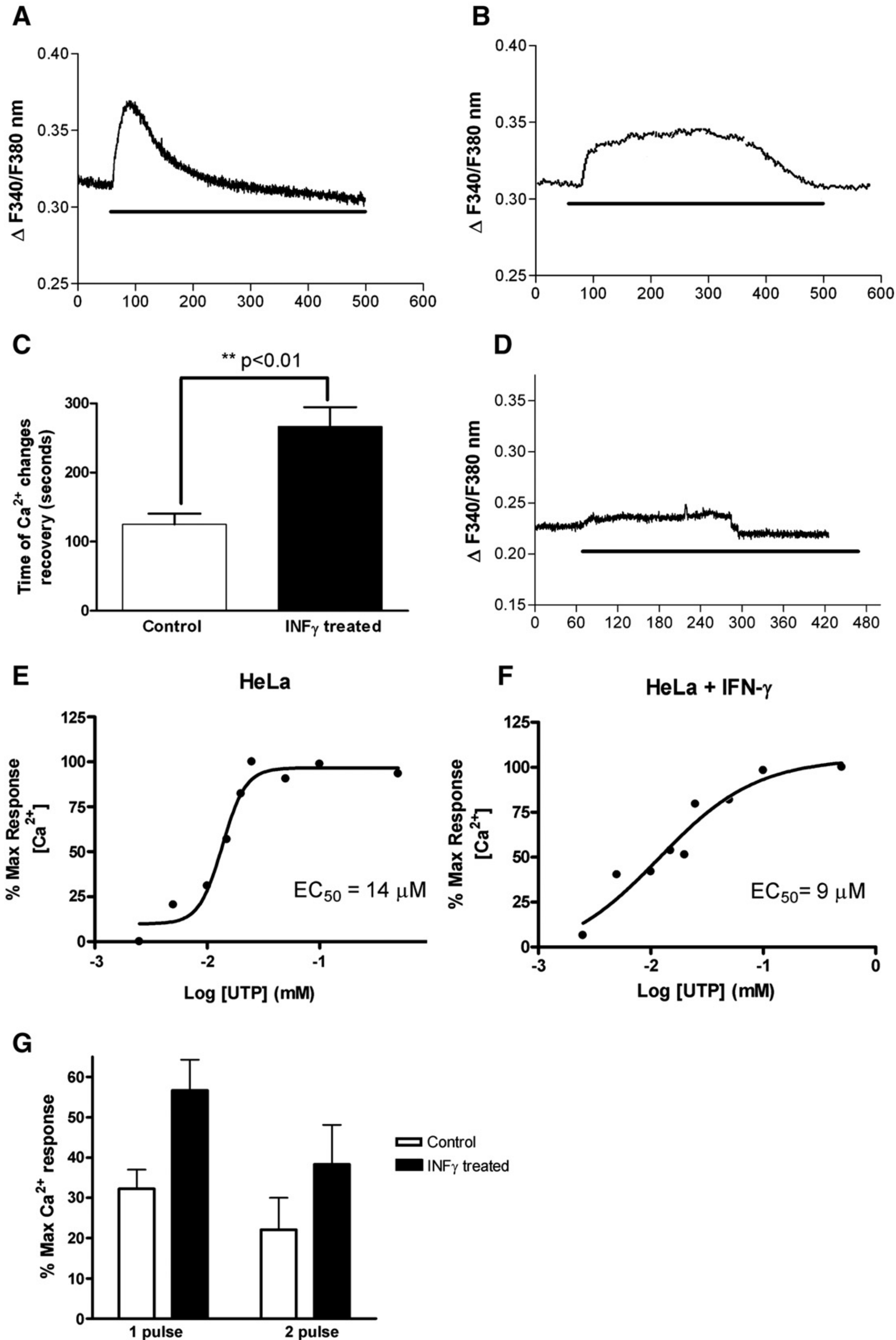

Fig. 6. Effect of IFNγ on ATP- and UTP-induced Ca^{2+} responses. (A) Untreated cells under perfusion with 1 mM ATP at 37 °C, showing a fast peak and gradual return to basal levels. (B) Cells pre-treated with 2 ng/ml IFNγ for 24 h, under perfusion with 1 mM ATP, showing a fast peak that remained at high levels for several minutes. (C) Graph showing the time needed for $[Ca^{2+}]_i$ changes shown in A and B to return to basal levels. (D) same in B, with cells pre-treated for 2 h with oATP. The $[Ca^{2+}]_i$ is given as the fluorescence ratio (F340/F380 nm), where fluorescence is proportional to the intracellular calcium concentration. (E and F) Dose response curves for UTP-induced $[Ca^{2+}]_i$ responses in untreated and IFNγ-treated cells, respectively. (G) UTP was added in a bolus of 1-min duration, followed by a second 1-min bolus 5 min later, in calcium-free saline and a continuous perfusion chamber at room temperature. Values in C represent mean ± SEM of four (A) and five (B) independent experiments. $n = 3$ and $n = 5$ for curves shown in E and F, respectively. Values in G represent mean ± SEM of 3 independent experiments.

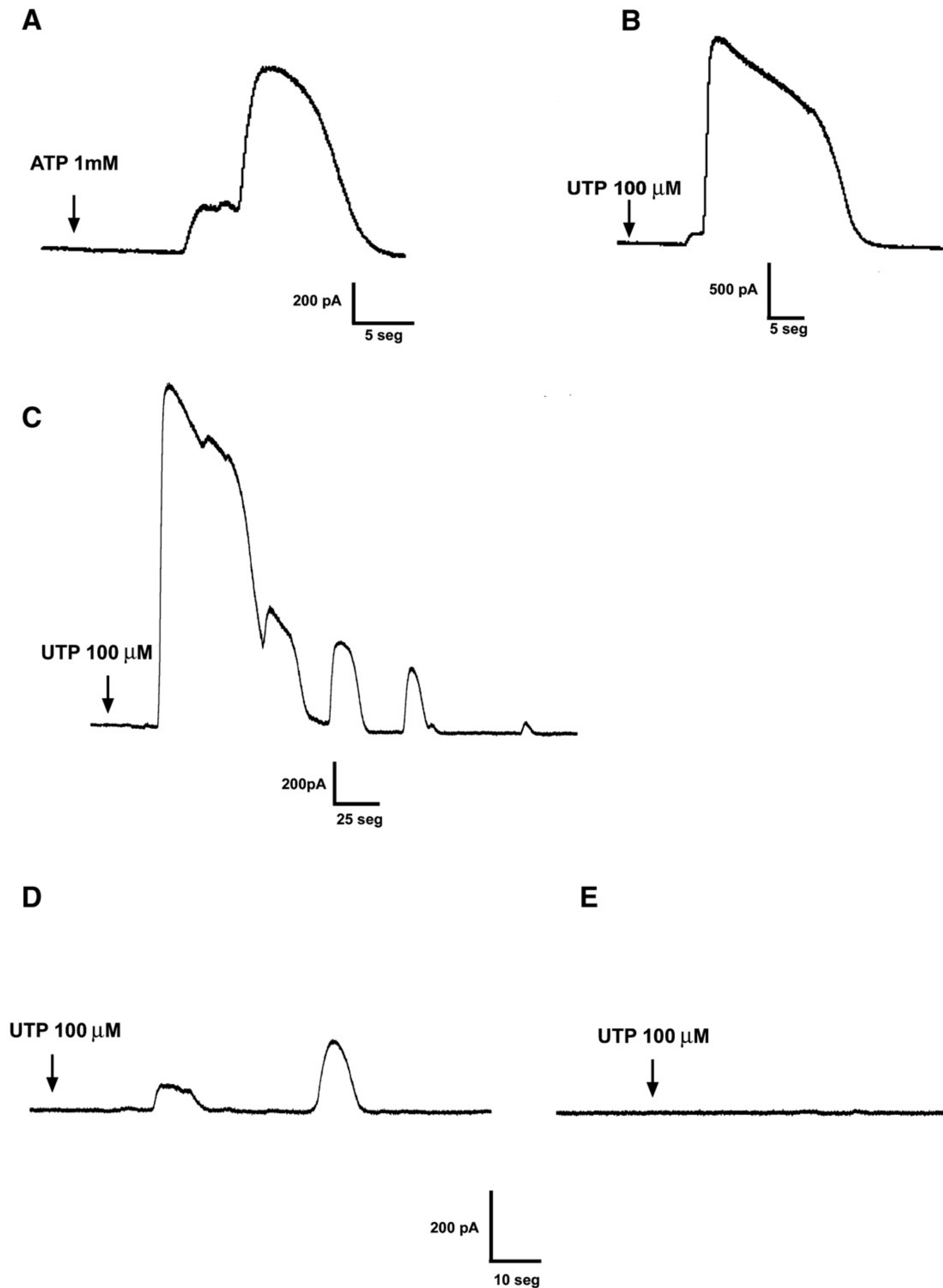

Fig. 7. Profile of ATP-activated currents in epithelial cells that were not treated with IFNγ. Whole cell currents were measured in HeLa cells kept at $V_H = -40$ mV. (A) Transient outward current after application of 1 mM ATP. (B and C) Transient outward currents after application of 100 μM UTP, showing a delay between stimulation with the nucleotide and initiation of the outward current. None of the cells tested responded with inward currents ($n = 11$). (D and E) Outward current desensitization after successive stimulation with 100 μM UTP. The first and second arrows indicate the fourth and fifth application of UTP.

antagonist, oATP, strongly inhibited the calcium response observed in IFNγ-incubated cells (Fig 6D), further suggesting a role for $P2X_7$ in the ATP-dependent effects.

In order to investigate the possibility that IFNγ could be affecting the activity of receptors other than $P2X_7$, we also characterized the effect of IFNγ on UTP-induced fluxes. In untreated cells, UTP promoted dose-dependent $[Ca^{2+}]_i$ fluxes transiently, with an EC50 of 14 μM (Fig. 6E). The EC50 decreased slightly (=9 μM) in IFNγ-incubated cells, but the UTP-induced response clearly did not fit a typical sigmoid curve (Fig. 6F), suggesting the presence of more than one functional P2Y receptor on IFNγ-incubated cells. Comparing the amplitude of the UTP-induced calcium responses in untreated and IFNγ-treated cells, we observed that two successive applications of 100 μM UTP induced a rapid transient increase of $[Ca^{2+}]_I$ in untreated cells, with similar amplitudes both times (Fig. 6G), However, in IFNγ-incubated cells,

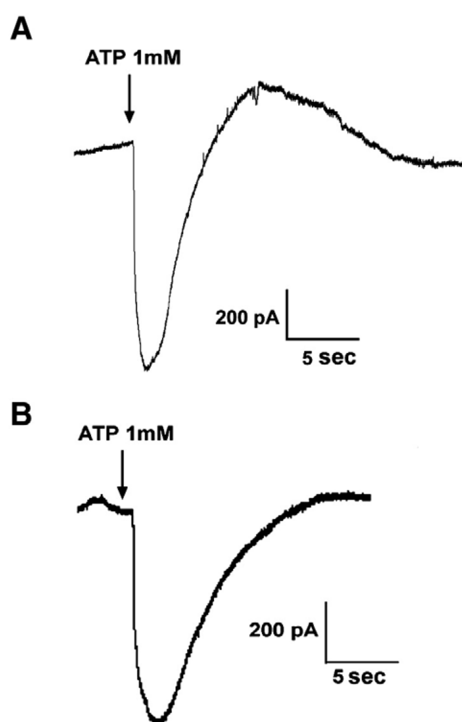

Fig. 8. IFNγ pre-treatment modifies ATP-mediated currents. Whole cell currents were measured in cells treated with 2 ng/ml IFNγ for 24 h and kept at a $V_H = -40$ mV. (A) A biphasic current induced immediately after ATP application. (B) Same cell as in A, showing that while the outward component of the response disappeared, the inward current does not desensitize. Note that the inward current starts immediately after ATP stimulation. Two of six cells responded as shown in A and B, while four other cells responded with outward currents, as shown for cells not treated with IFNγ.

the first response was bigger than in untreated cells, and the second response was smaller than the first one (Fig. 6G).

3.7. ATP induces ion currents in HeLa cells

Application of 1 mM ATP induced an outward current in HeLa cells after 10–15 s (Fig. 7A). The same transient outward currents were observed in response to 100 μM UTP (Fig. 7B). Occasionally the outward currents were biphasic or took place in multiples steps, and displayed clear desensitization (Fig. 7C). We observed almost complete outward current desensitization after four or five successive applications of UTP (Fig. 7D). None of the cells tested responded with an inward current. The behavior of the responses was compatible with the activation of a Ca^{2+}-activated K^+ current, similarly to the $P2Y_2$-dependent response previously observed in other cell types [45,46].

A very different response was observed in HeLa cells pre-treated for 24 h with IFNγ. ATP application elicited biphasic responses composed of a fast inward current followed by an outward current (Fig. 8A). In some cells, we observed only the inward or the outward component of the current (data not shown). After continued ATP stimulation, the outward current disappeared, and only the inward component remained (Fig. 8B).

4. Discussion

Purinergic signalling is an important component of host defence against intracellular pathogens, mainly through activation of P2 receptors and particularly the $P2X_7$ receptor [47]. The $P2X_7$ receptor is involved in elimination of mycobacteria and chlamydiae in infected macrophages [9,11], and secretion of pro-inflammatory cytokines such as IL-1β and IL-18 [48]. Extracellular ATP is released from infected or stressed cells, and is now viewed as a “danger signal” that, acting through the $P2X_7$ receptor, can stimulate secretion of pro-inflammatory cytokines [49,50]. Ligation of the $P2X_7$ receptor has also been associated with homeostasis of T regulatory cells and regulation of autoimmune disease [51–54].

Our experiments suggest that, in the absence of IFNγ stimulation, HeLa cells express functional $P2X_7$ at a low level. Although $P2X_7$ mRNA and $P2X_7$ protein was detected in HeLa cells, the functional experiments demonstrate that ATP treatment does not elicit pore opening nor induce apoptosis, the two hallmarks of functional $P2X_7$ expression [1,55]. It has been proposed that a low level of expression of this receptor makes it unresponsive to ATP [56]. Similarly, it has recently been suggested that hetero-oligomerization of $P2X_7$ with a naturally occurring truncated form of the receptor could block the receptor's function in HeLa cells, as described by Feng et al. [35]. In support of this hypothesis, we observed the presence of a 40 kDa band on gels from HeLa cells that was not present in macrophage extracts, which could correspond to the truncated form of $P2X_7$. Another possibility is that our antibody cross-reacts with a different splice variant, since under our experimental conditions [56] we might not expect to observe the 40 kDa band described by Feng et al. Regardless, the new band disappears after IFNγ treatment, which is associated with a change in the functional state of the $P2X_7$ receptor.

Furthermore, IFNγ treatment clearly induces up-regulation of $P2X_7$ receptor and activity, as revealed by an increase in the levels of $P2X_7$ mRNA and protein, $P2X_7$-dependent calcium fluxes, and the presence of non-desensitizing inward currents. Interestingly, we could not detect the 40 kDa band in extracts of IFNγ-treated cells.

Most if not all cells express more than one subtype of P2 receptors [57]. But the observation that UTP-induced calcium signaling in HeLa cells did not completely eliminate subsequent ATP responses suggests that the cells express ATP receptors that are not sensitive to UTP. We could not detect calcium signaling using αβ-meATP at concentrations that activate $P2X_{1-3}$ receptors, implying that those P2X receptors are not functional in HeLa cells. On the other hand, the outward currents observed after ATP and UTP addition confirm the functional presence of P2Y, possibly $P2Y_2$ receptors, on the cells [58]. Our data on calcium fluxes suggest the presence of UDP-sensitive and ADP-sensitive P2Y receptors, possibly $P2Y_6$ and $P2Y_1$ subtypes, but this conclusion will require further studies. It has been proposed previously that $P2Y_2$ receptors on HeLa cells inhibit the activity of the Na^+/K^+-ATPase [33], but unlike the present study, the authors were able to detect mRNA for only the $P2Y_4$ and $P2Y_6$ subtypes on HeLa cells. Since $P2Y_4$ in humans is activated only by UTP [59], it should not be functional in HeLa cells, despite being present at the transcriptional level, as there were no UTP-induced calcium transients after perfusion with ATP. Our measurement of calcium fluxes in the presence of the $P2Y_2$ antagonist, suramin, confirms the functional expression of $P2Y_2$ receptors on HeLa cells. The UTP dose–response curve and sequential UTP pulses after IFNγ treatment suggest that this pro-inflammatory cytokine upregulates additional P2Y receptors on epithelial cells.

In conclusion, our study demonstrates that the most popular cell type in cell biology laboratories expresses a functional $P2X_7$ receptor, which can be upregulated under the inflammatory conditions found during microbial infection, thus amplifying inflammatory responses requiring stimulation of a cytosolic “inflammasome“ by extracellular danger signals [60,61]. Even though mRNA for a large number of purinergic receptors is present, HeLa cells appear to express a smaller subset of functional receptors, most likely for $P2Y_1$, $P2Y_2$ and $P2Y_6$. These receptors may also play a role in regulating essential functions of the epithelial cells, such as maintenance of the Na^+ and K^+ gradients across the plasma membrane and controlling infection by intracellular pathogens.

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