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Extracellular ATP activates MAP kinase cascades through a P2Y purinergic receptor in the human intestinal Caco-2 cell line

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ABSTRACT

Background: ATP exerts diverse effects on various cell types via specific purinergic P2Y receptors. Intracellular signaling cascades are the main routes of communication between P2Y receptors and regulatory targets in the cell.

Methods and results: We examined the role of ATP in the modulation of ERK1/2, JNK1/2, and p38 MAP kinases (MAPKs) in human colon cancer Caco-2 cells. Immunoblot analysis showed that ATP induces the phosphorylation of MAPKs in a time- and dose-dependent manner, peaking at 5 min at 10 μM ATP. Moreover, ATPγS, UTP, and UDP but not ADP or ADPβS increased phosphorylation of MAPKs, indicating the involvement of, at least, $P2Y_2$/$P2Y_4$ and $P2Y_6$ receptor subtypes. RT–PCR studies and PCR product sequencing supported the expression of $P2Y_2$ and $P2Y_4$ receptors in this cell line. Spectrofluorimetric measurements showed that cell stimulation with ATP induced transient elevations in intracellular calcium concentration. In addition, ATP-induced phosphorylation of MAPKs in Caco-2 cells was dependent on Src family tyrosine kinases, calcium influx, and intracellular Ca^{2+} release and was partially dependent on the cAMP/PKA and PKC pathways and the EGFR.

General significance: These findings provide new molecular basis for further understanding the mechanisms involved in ATP functions, as a signal transducer and activator of MAP kinase cascades, in colon adenocarcinoma Caco-2 cells.

1. Introduction

Extracellular nucleotides exert diverse effects on a variety of tissues and cell types via specific purinergic receptors consisting of P2X and P2Y families [1]. P2X ionotropic receptors form nonselective channels that allow, in most cases, anion (Cl^-), monovalent cation or Ca^{2+} influx from the extracellular space. Seven members of the P2X family have been cloned in mammals ($P2X_{1-7}$) [2]. The P2Y receptors are coupled to heterotrimeric G proteins and are divided in eight subtypes in mammals ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) [3–5]. Intracellular signaling cascades are the main routes of communication between P2Y receptors and regulatory targets in the cell. Pharmacologically, the human $P2Y_2$ is activated equipotently by ATP and UTP while the human $P2Y_4$ receptor responds preferentially to UTP [2].

Adenosine triphosphate (ATP), being a natural ligand at both P2X and P2Y receptors may participate in the regulation of cell growth in non-neoplastic, as well as neoplastic tissues [6–8]. It has been proposed that purinergic signaling may play a role in regulating intestinal physiology [9], and it was shown that the $P2Y_6$ receptors mediate NaCl secretion in colonic epithelial cells [10]. Recently, activation of the $P2Y_6$ receptor by UDP has been shown to regulate cytokine expression in colonic Caco-2/15 cells by an ERK1/2 dependent mechanism [11]. Moreover, in the colonic cell line Caco-2, the expression of functional protein for at least the $P2X_7$, $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors was recently reported [7].

The source of extracellular ATP, apparently is the large pool of cytosolic ATP. Under most conditions, endogenous nucleotides are made available to the intestinal mucosa via hepatic supply and salvage pathway [12]. In the immediate vicinity of the cell surface, released ATP has been shown to reach a concentration high enough to stimulate P2-purinergic receptors in a human epithelial cell line, Intestine 407 [13]. No evidence for the existence of specialized ATP-containing vesicles in most of non-excitatory cells, such as endothelial and epithelial cells, has been demonstrated, and therefore, alternative mechanisms for the release of nucleotides have been postulated: (1) stress/hypoxia/mechanical stimulation, (2) vesicular trafficking, and (3) agonist-promoted stimulation [14].

Mitogen-activated protein kinases (MAPKs) are a large family of enzymes, expressed in all eukaryotic cells. Mammalian MAPKs are commonly divided into subfamilies that include the Extracellular-regulated protein kinases (ERKs), the c-Jun N-terminal kinases (JNKs), the p38, and the big MAP kinases (BMKs), but more subfamilies may exist [15]. They are activated via a unique dual phosphorylation mechanism, on a Thr-X-Tyr motif, located in the phosphorylation loop. Dual-specificity kinases termed MAP kinase kinases (MAPKKs,

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MEKs, or MKKs) catalyze this dual phosphorylation. MAPKKs are not highly specific, and may phosphorylate all members of a subfamily or even of two families [15]. The p38 MAPKs are strongly activated under stress conditions and therefore, together with the JNKs, are also known as stress-activated protein kinases (SAPKs). The substrate selectivity of MAPKs is conferred by specific interaction motifs located on the kinases and substrates [16,17]. In addition, MAPK cascade specificity is also mediated by scaffolding proteins that organize pathways in specific modules through simultaneous binding of several components [18]. To date, however, there is little information on whether the multiple mechanisms are simultaneously or individually used by a given stimulus or regarding the contribution of the different pathways. Activated MAPKs further propagate the signal by phosphorylating downstream targets such as transcription factors and other kinases.

Although the MAPK cascades leading to ERK1/2, JNK1/2, and p38 MAPK activation have been investigated in many cell types using different stimuli, it is not known whether ATP stimulates MAPKs in the colonic cell line Caco-2 and how nucleotide-mediated signaling is coupled to their activation. Caco-2 cells are derived from colorectal adenocarcinoma and when fully differentiated give rise to enterocytes-like properties [19]. As such, these cells represent a useful and well-accepted tool for metabolism and transport studies in the intestinal mucosa. In the present work, we used this cell line as an in vitro model for investigating potential effects of ATP on MAPKs.

2. Materials and methods

2.1. Materials

ATP, ATPγS, UTP, UDP, ADP, ADPβS, Fura-2/pentaacetoxymethyl ester (Fura-2/AM), 2-aminoethyl diphenyl borate (2-APB), tyrphostin AG1478, high-glucose Dubelcco's modified Eagle's medium (DMEM), and anti-actin antibody were from Sigma-Aldrich (Saint Louis, MO, USA). Ro 31-8220, PP2, and Rp-cAMP (adenosine 3′, 5′-cyclic monophosphorothiodate, Rp-isomer, triethylammonium salt) were from Calbiochem (San Diego, CA, USA). Fetal bovine serum (FBS) was from Natocord (Córdoba, Argentina). Mouse monoclonal anti-phospho p44/42 MAP kinase (ERK 1/2) (Thr202/Tyr204), rabbit polyclonal anti-phospho SAPK/JNK (Thr183/Tyr185), rabbit monoclonal anti-phospho MKK3/6 (Ser189/207), rabbit polyclonal anti-phospho SEK1/MKK4 (Ser257/Thr261), rabbit polyclonal anti-ERK1/2, and polyclonal anti-JNK1/2 antibodies were from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal anti-phospho p38 (Thr180/Tyr182), rabbit polyclonal anti-p38α, and goat anti-rabbit and anti-mouse peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein size markers, Immobilon P (polyvinylidene difluoride; PVDF) membranes, and ECL chemiluminescence detection kit were from Amersham Biosciences (Piscataway, NJ, USA). All other reagents used were of analytical grade.

2.2. Cell culture

The human colon cancer cell line Caco-2 was supplied by the American Tissue Culture Bank (Bethesda, MD, USA). The cells were cultured at 37 °C in DMEM containing 10% FBS, 1% nonessential amino acids, penicillin 100 IU/ml, streptomycin 100 mg/ml, and gentamicin 50 mg /ml under a humid atmosphere of 5% CO_2 in air. Cultures were passaged every 3 days with fresh medium. The treatments were performed employing 70% confluent cultures in DMEM with 0.1% FBS. For intracellular calcium measurements, cells were plated onto glass coverslips and allowed to grow until confluence (2–3 days after plating) before use.

2.3. SDS–PAGE and immunoblotting

Caco-2 cells were treated with ATP, ATPγS, UTP, UDP, ADP, and ADPβS in the presence or absence of inhibitors. When the treatment was performed in a Ca^{2+}-free buffer, the medium was replaced by buffer C (in mM: 138 NaCl, 5 KCl, 1 $MgCl_2$, 5 glucose, 10 HEPES (pH 7.4) plus 0.5 EGTA) 30 min before cell stimulation. Treatment of the cells was performed in the cell-metabolic incubator to minimize cellular stress. Then the cells were washed with PBS buffer plus 25 mM NaF and 1 mM Na_3VO_4, and lysed in buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 3 mM KCl, 1 mM EDTA, 1% Tween-20, 1% Nonidet P-40, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 mM NaF, and 1 mM Na_3VO_4. The lysates were incubated on ice for 10 min, vortexed for 45 sec and maintained on ice for another 10 min. After centrifugation at 14,000×*g* and 4 °C during 15 min the supernatant was collected and proteins were quantified by the Bradford procedure [20]. Lysate proteins dissolved in 6× Laemmli sample buffer were separated (25 μg/lane) on SDS–polyacrylamide (10%) gels [21] and electro-transferred to PVDF membranes. After blocking for 1 h at room temperature with 5% nonfat milk in TBST buffer (50 mM Tris pH 7.2–7.4, 200 mM NaCl, 0.1% Tween-20), the membranes were incubated overnight with the appropriate dilution of primary antibody in TBST plus 1% nonfat milk or 5% BSA. After washing, membranes were incubated with the appropriate dilution of horseradish peroxidase-conjugated secondary antibody in TBST plus 1% nonfat milk. Finally, the blots were developed by ECL with the use of Kodak BioMax Light film and digitalized with a GS-700 Imaging Densitomer (Bio-Rad, Hercules, CA, USA).

2.3.1. Stripping and reprobing membranes

The complete removal of primary and secondary antibodies from the membranes was achieved by incubating them in stripping buffer (62. 5 mM Tris–HCl pH 6.8, 2% SDS, and 50 mM β-mercaptoethanol) at 55 °C for 30 min with agitation. Then, the membranes were washed for 10 min in TBST (1% Tween-20) and blocked, as indicated above, for 1 h at room temperature. After that, membranes were ready to reprobe with the corresponding antibodies.

2.4. Total RNA isolation and reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA from Caco-2 cells grown in monolayer was isolated using Invitrogen Trizol reagent according to the manufacturer's instructions. The RNA pellet was dissolved in RNase-free water and then reverse transcription reaction was performed employing oligo $(DT)_{12-18}$ to obtain cDNA using the commercial kit "Superscript II Reverse Transcriptase" (Invitrogen, Carlsbad, CA, USA) by following the instructions detailed in the data sheet. Next, first-strand cDNA was diluted in a 50-μl PCR amplification reaction mix by combining the reagents as indicated in the kit. All samples were first denatured at 94 °C (3 min) and the PCR reaction then allowed to proceed for 35 amplification cycles: denaturation (45 sec, 94 °C), annealing (45 sec at 67 °C ($P2Y_2$) or 56.5 °C ($P2Y_4$)), and extension (90 sec at 72 °C ($P2Y_2$) or 1 min at 72° C ($P2Y_4$)). Then, a final extention step of 10 min at 72 °C was performed. Transcripts for $P2Y_2$ and $P2Y_4$ receptors [22] were assayed by PCR using the JumpStart REDTaq ReadyMix PCR Reaction Mix from Sigma-Aldrich and specific primers. The primers used were previously checked using the BLASTn program of the GenBank; their sequence ($P2Y_2$: forward: 5′-CTCTACTTTGTCACCAC-CAGCGCG-3′, reverse: 5′-TTCTGCTCCTACAGCCGAATGTCC-3′; $P2Y_4$: forward: 5′-CCACCTGGCATTGTCAGACACC-3′, reverse: 5′-GAGTGAC-CAGGCAGGGCACGC-3′) was based on the known sequences in the coding region of the human receptors. The predicted product size for $P2Y_2$ is of 637 bp and of 425 bp for $P2Y_4$. The resulting PCR products

were analyzed by 2% agarose gel electrophoresis and visualized by staining with ethidium bromide.

2.5. Purification of PCR products and sequencing

The PCR products seeded in a 2% agarose gel electrophoresis were visualized with UV light and the resulting bands were cut from the gel and transferred into appropriate sterile tubes. Purification was performed employing the "S.N.A.P. Gel Purification Kit" (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Purified PCR products were sequenced through the DNA Sequencing Facility Service of Ruralex (Buenos Aires, Argentina). The sequences obtained were analyzed running a BLASTn program at the GenBank database.

2.6. Intracellular calcium measurements

Intracellular Ca^{2+} changes in Caco-2 cells were monitored by using the Ca^{2+}-sensitive fluorescent dye Fura-2. Cells grown onto glass coverslips were loaded with 1 μM of Fura-2/AM in buffer A (in mM: 138 NaCl, 5 KCl, 1 $MgCl_2$, 5 glucose, 10 HEPES (pH 7.4), 1.5 $CaCl_2$, and 0.1% bovine serum albumin (BSA)), in the dark during 30 min at 37 °C. Unloaded dye was washed out and cells were stored in buffer B (buffer A without BSA and Fura-2/AM) in the dark at room temperature for 30 min prior to use to allow complete intracellular dye deesterification. For fluorescence measurements, the coverslips containing dye-loaded cells were then mounted on a chamber and placed on inverted microscope (Nikon Diaphot 200) and maintained at 25–30 °C. The excitation wavelength was switched between 340 and 380 nm employing a dual-excitation monochromator from an SLM-Aminco 8100 spectrofluorimeter connected to the epifluorescence port of the microscope through an optic fiber. Emitted cellular fluorescence was collected at 510 nm and ratios from 340/380 nm signals were obtained, thus making the measurement independent of variations in cellular dye content, dye leakage, or photobleaching.

2.7. Statistical evaluation

Statistical significance of data was evaluated using Student's *t*-test and probability values below 0.050 ($p<0.050$) were considered significant. Quantitave data are expressed as the means ± SD from the indicated set of experiments.

3. Results

In the present study, we examined whether ATP modulates ERK1/2, JNK1/2, and p38 MAPK phosphorylation in Caco-2 cells. For this purpose, we first performed time– and dose–response studies. Serum-starved cells were exposed for 1 to 15 min to 10 μM ATP. As shown in Fig. 1A (left), the three members of the MAPK family were activated in a time-dependent manner, reaching the maximum within 5 min of ATP exposure. Fig. 1A (right) shows no changes in protein expression of MAPKs after cell exposure to ATP. MAPKs were also phosphorylated in a dose-dependent manner up to 100 μM ATP (Fig. 1B). Furthermore, ATP also activates MKK3/6 and MKK4, upstream kinases of p38 and JNK1/2 MAPK signaling pathways, respectively (Fig. 2).

It has been reported that, in Caco-2 cells, a variety of P2Y purinergic receptor types are expressed [7,23]. Thus, to obtain

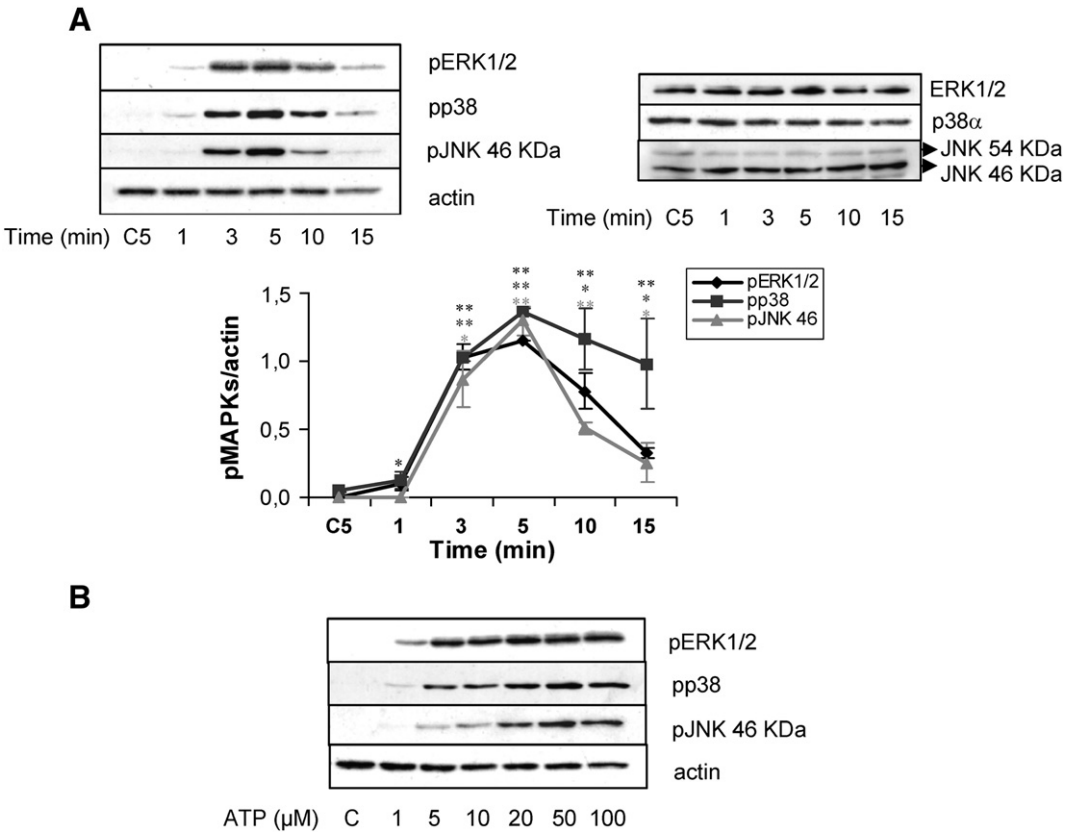

Fig. 1. Time– and dose–response profiles of ATP-dependent ERK 1/2, JNK1/2, and p38 MAPK phosphorylation in Caco-2 cells. Caco-2 cells were treated with (A) 10 μM ATP 1–15 min or (B) 1–100 μM ATP for 5 min. After cell lysis, comparable aliquots of lysate proteins were separated by SDS–PAGE followed by Western blotting with anti-pERK1/2 antibody and then the membranes were stripped and reprobed with anti-pp38 and -pJNK1/2 phosphospecific antibodies and with anti-actin as loading control. Total MAPK antibodies were used in A (right panel) as control of protein expression. Representative immunoblots and quantification by scanning volumetric densitometry of blots from 3 independent experiments are shown. Means ± SD are given. $^{*}p<0.05$, $^{**}p<0.01$, where *p* values refer to differences in phosphorylation of MAPKs between cells in the presence or absence of ATP.

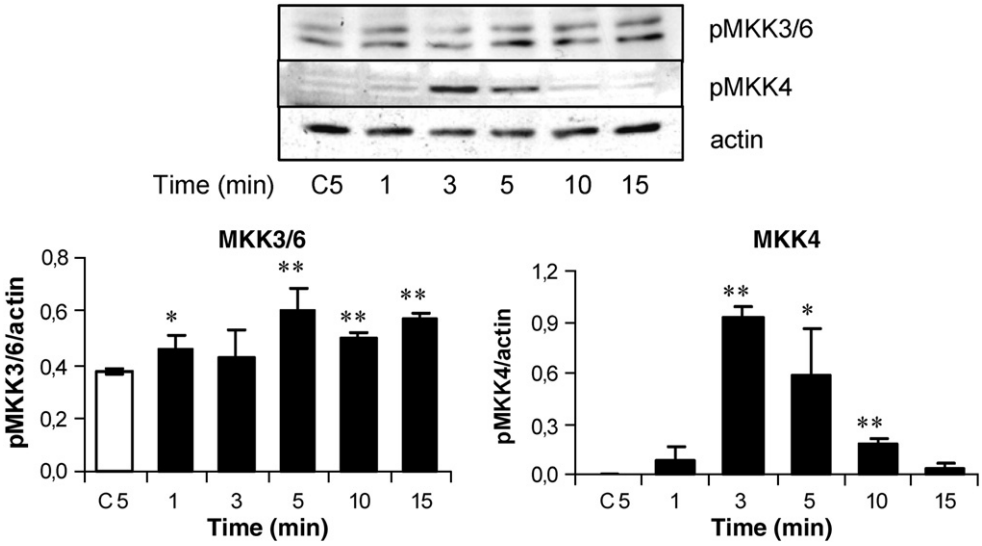

Fig. 2. Time profile of MKK3/6 and MKK4 phosphorylation. Caco-2 cells were stimulated with 10 μM ATP 1–15 min as described and immunoblotted using phospho-MKK3/6 and MKK4 antibodies. Representative immunoblots and quantification by scanning volumetric densitometry of blots from 3 independent experiments are shown. Means ± SD are given. $^{*}p<0.05$, $^{**}p<0.01$, where p values refer to differences in phosphorylation of MAPKKs between cells in the presence or absence of ATP.

information on purinergic receptor subtype/s involved in the activation of ERK1/2, JNK, and p38 MAPK in Caco-2 cells, we evaluated the action of different purinergic and pirimidinergic agonists (Fig. 3A). We found that stimulation of p46 JNK and p38 MAPK phosphorylation by UTP (10 and 50 μM) was more potent than the effect of ATP, while the phosphorylation of ERK1/2 was similar (10 μM) or lower (50 μM). Similar results were observed when Caco-2 cells were exposed to UDP, an agonist of the $P2Y_6$ receptor. As shown in Fig. 3A (right panel), p46 JNK and p38 phosphorylation by 50 μM UDP was comparable to the ATP response at the same dose. The use of ATPγS (10 and 50 μM), a nonhydrolyzable analog of ATP, induced a weaker phosphorylation of the three MAPKs when compared to the response elicited by ATP. ADPβS (10 and 50 μM), a potent agonist of the $P2Y_1$ receptor and a nonhydrolyzable analog of ADP, was not able to stimulate the phosphorylation of none of the MAPKs, whereas ADP (10 and 50 μM) triggered a weak response. As shown in Fig. 3B, the time profile of ATPγS-induced phosphorylation of MAPKs (5–120 min) revealed a lower activity than ATP.

Considering this pharmacological evidence, RT–PCR studies were performed to determine the expression of $P2Y_2$ and $P2Y_4$ receptors in Caco-2 cells. The polymerase chain reaction was carried out using specific pairs of forward and reverse primers [22] which amplified two DNA fragments of 425 bp and 637 bp, matching the expected size for the $P2Y_4$ and $P2Y_2$ receptor subtypes, respectively (Fig. 3C). To identify the fragments obtained, PCR products were extracted from the agarose gel, purified, and sequenced as indicated in Materials and methods. The data were analyzed running a BlastN Program of the GenBank database and the sequences obtained for the $P2Y_2$ and $P2Y_4$ DNA fragments were given GenBank accession numbers FJ599700 and FJ599702, respectively. Next, we studied the modulation of intracellular calcium concentration ($[Ca^{2+}]_i$) by ATP, performing spectrofluorimetric measurements in Fura-2-loaded cells. As shown in Fig. 4, stimulation of Caco-2 cells with ATP (10 μM) elicited a rapid and transient increment in $[Ca^{2+}]_i$ (+ 70%, $n=6$) that returned to the baseline after 90 sec. To further determine whether activation of MAPKs correlates with changes in $[Ca^{2+}]_i$ levels induced by ATP, Caco-2 cells were preincubated during 30 min in a calcium-free buffer (plus 0. 5 mM EGTA) or with 2-APB, an IP_3 receptor (IP_3R) blocker. As shown in Fig. 5, the absence of Ca^{2+} and the presence of 2-APB in the medium decreased ATP effects on phosphorylation of MAPKs. In addition, similar results were obtained when Caco-2 cells were incubated with the intracellular Ca^{2+} chelator BAPTA-AM (not shown). These results suggest that both extracellular calcium influx and intracellular calcium release participate in ATP-dependent activation of ERK1/2, p46 JNK, and p38 MAPK pathways. Cell viability was not affected by incubation in a Ca^{2+}-free medium during 30 min, since more than 95% of the cells excluded the trypan blue dye (not shown).

We then investigated if the kinases Src, PKA, and PKC play a role in the activation of MAPK cascades by ATP. To that end, Caco-2 cells were preincubated in the presence or absence of PP2, a potent and selective inhibitor of the Src family tyrosine kinases [24], Rp-cAMP, a highly specific competitive antagonist for all activators of the cAMP signal pathway [25], or PKC inhibitor, Ro 318220 [26]. Then, cells were stimulated with 10 μM ATP for 5 min, followed by Western blot analysis of cell lysates with the corresponding anti-phospho MAPK antibodies. As shown in Fig. 6A, PP2 (10–75 μM) inhibited by 90% ERK1/2 phosphorylation and completely suppressed the phosphorylation of p38 and p46 JNK MAPKs by ATP. Furthermore, in Caco-2 cells, ATP activated c-Src, increasing its phosphorylation at Tyr 416 (+2-fold) which was also suppressed by its PP2 inhibitor (Fig. 6B). The cAMP antagonist and the PKC inhibitor diminished ATP effects on the three kinases (Fig. 6C). These results suggest the participation of Src kinases and, to a lesser extent, of PKA and PKC in the phosphorylation of the MAPKs by ATP in Caco-2 cells. As activation of the epidermal growth factor receptor (EGFR) triggers mitogenic signaling in gastrointestinal mucosa, and its expression is also upregulated in colonic cancers and most neoplasms [27–29], in this study, we examined whether ATP-mediated stimulation of MAPKs in Caco-2 cells involves cross-communication between P2YR and EGFR signaling systems. To ascertain the role of ATP-induced EGFR activation in triggering the mitogenic pathways, we inhibited EGFR kinase activity with the specific inhibitor AG1478, a tyrphostin that selectively blocks the tyrosine kinase of the EGF receptor by interaction with its ATP-binding site and by inducing the formation of inactive EGF receptor dimmers [30]. Then, Western blot analyses were performed to study the phosphorylation of MAPKs by ATP. As shown in Fig. 7, the selective antagonist AG1478 dose-dependently diminished ATP-induced p46 JNK and p38 MAPK phosphorylation, while its effects on ERK1/2 were less pronounced. These data suggest the participation of EGFR in activation of MAPKs induced by ATP in Caco-2 cells.

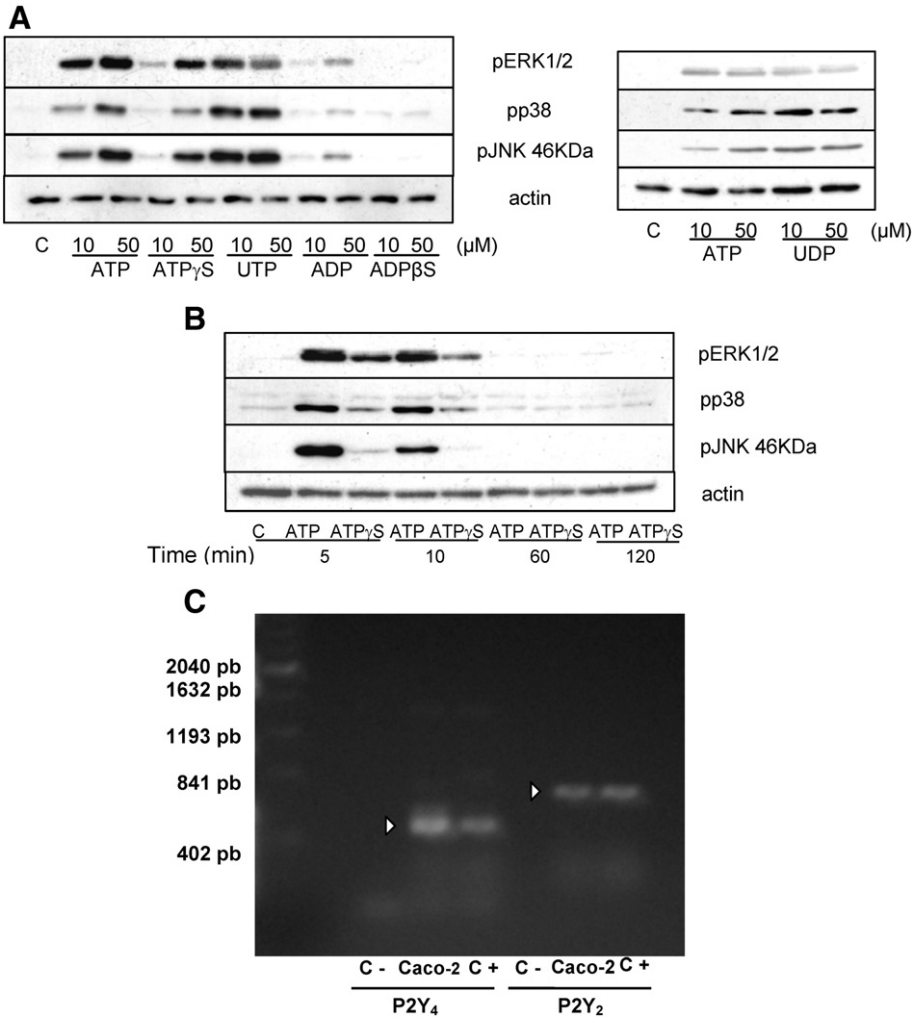

Fig. 3. Phosphorylation of MAPKs by extracellular nucleotides in Caco-2 cells. (A) Caco-2 cells were stimulated by the addition of 10 and 50 μM ATP or the same doses of ATPγS, UTP, ADP, ADPβS, and UDP for 5 min. (B) Caco-2 cells were exposed for 5–120 min to 10 μM ATP or ATPγS. Cell lysate proteins were immunoblotted as described in the legend to Fig. 1. The immunoblots shown are representative of at least three independent experiments. (C) Analysis of P2Y receptor subtypes present in Caco-2 cells. RT–PCR studies with specific primers against $P2Y_2$ and $P2Y_4$ receptor subtypes was performed after total RNA isolation. Then, PCR products were analyzed by agarose gel electrophoresis (2%). The amplification products of $P2Y_2$ (637 bp) and $P2Y_4$ (425 bp) receptor subtypes are indicated by the arrows. The figure shows the 400 bp PCR marker (Mkr), the negative controls (C−) made using water instead of cDNA and the positive controls (C+) performed with RNA isolated from human breast cancer cells. A representative gel with ethidium bromide-stained RT–PCR products is shown.

4. Discussion

MAP kinases regulate diverse cellular programs including embryogenesis, proliferation, differentiation, and apoptosis based on clues derived from the cell surface and the metabolic state and environment of the cell [31]. Our study on ERK1/2, JNK1/2, and p38 MAPK modulation by ATP demonstrated that purinergic activation stimulates the phosphorylation of the three MAPK family members in Caco-2 cells and in a dose- and time-dependent manner. As in other cell types [32–34], we observed that both JNK 46 kDa and 54 kDa are expressed in this cell line, but only p46 is activated by ATP implying that this isoform is the major contributor to JNK activity in stimulated Caco-2 cells. The magnitude of MAPKs phosphorylation by ATPγS, the nonhydrolyzable analog of ATP, was lower than the activation by ATP at the same doses. Marked MAPK activation was observed after cell treatment with ATP, UTP, and UDP, but no significant MAPK changes in phosphorylation were detected in the case of ADP, probable product of ATP hydrolysis, suggesting that the higher effect on activation of MAPKs after ATP treatment of Caco-2 cells is not caused by its hydrolysis product under the experimental conditions. Furthermore, ADPβS, a potent agonist of the $P2Y_1$ receptor [35,36], did not stimulate the phosphorylation of ERK1/2, JNK1/2, and p38 MAPK. The different response exerted by ATP and its nonhydrolyzable analog on the phosphorylation of MAPKs could be due to the receptor subtypes expressed or agonist specificity in Caco-2 cells. In other cell types such as neuroblastoma and astrocytoma cells, it was reported that ATPγS is an agonist at recombinant $P2Y_2$ receptors, but it was less potent than UTP and ATP [37,38]. Moreover, in human hepatocytes, ATPγS through $P2Y_2$ and $P2Y_4$ interaction caused an increase in $[Ca^{2+}]_i$, although the effect was lower than the induced by ATP or UTP [39]. In addition, the nonhydrolyzable analog seems to be more specific than ATP since it does not act as a $P2Y_1$ agonist as ATP [2]. The different patterns of MAPK phosphorylation induced by ATP and UTP may be related to the stimulation of a different pathway and/or the affinity of the agonist for P2Y receptors. It is known that the $P2Y_2$ receptor is activated by ATP and UTP, and the human $P2Y_4$ receptor has a strong preference for UTP as agonist [36,40], but recent reports have postulated that both agonists can activate the $P2Y_{11}$ receptor stimulating different signaling pathways [41]. Our results suggest then that ATP stimulates phosphorylation of MAPKs in Caco-2 cells through $P2Y_2$, $P2Y_4$, and/or $P2Y_{11}$ receptors, in accord with reports on the expression of these P2Y receptor subtypes in other human intestinal carcinoma cells [7,8,42]. We obtained further evidence on the participation of $P2Y_2$ and $P2Y_4$ receptors in ATP actions in Caco-2

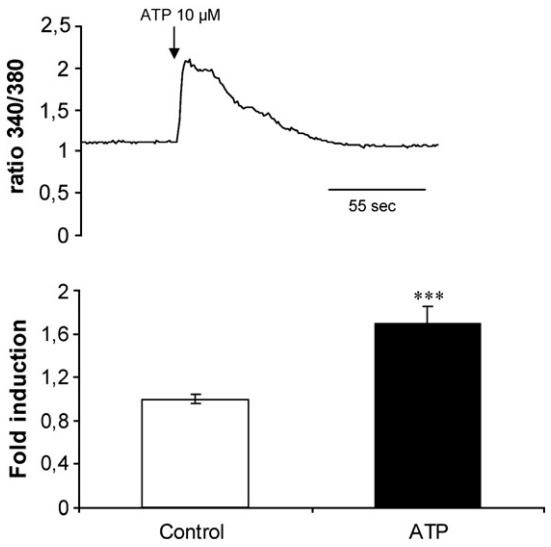

Fig. 4. Effects of ATP on intracellular calcium levels ($[Ca^{2+}]_i$) in Caco-2 cells. Changes in $[Ca^{2+}]_i$ were measured in Fura-2-loaded Caco-2 cells incubated in 1.5 mM Ca^{2+} as described in Materials and methods. Cells were stimulated with 10 μM ATP as indicated by the arrow. Time traces of ATP-treated cells representative from at least six independent experiments each performed onto no less than 40–50 cells are shown. Means ± SD are given. $^{***}p<0.001$ where p value refers to differences in $[Ca^{2+}]_i$ between cells in presence or absence of ATP.

cells by RT–PCR and sequence analysis of two amplified DNA fragments corresponding to the expected size for these purinergic receptor subtypes. It will be of interest to evaluate the expression of these receptors as a function of Caco-2 cells differentiation state.

It has been shown that purinergic agonists increase $[Ca^{2+}]_i$ in subconfluent and confluent Caco-2 cells [7,23]. In agreement with these results, here we demonstrated that ATP modulates $[Ca^{2+}]_i$ in subconfluent Caco-2 cells, the cell $Ca^{2+}{}_i$ response to the nucleotide being rapid and transient. Increases in $[Ca^{2+}]_i$ induced by ATP may result in activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which has also been shown to phosphorylate ERK1/2 [43]. However, it is not known whether there is a relationship between the rise in intracellular Ca^{2+} and stimulation of MAPKs by ATP in Caco-2 cells. Calcium influx from the extracellular environment is usually associated to P2X-R activation and as a secondary signal trigger by the opening of voltage-dependent Ca-channel following P2Y-R [1,2]. Nevertheless, the experiments using a Ca^{2+}-free medium plus EGTA and the IP_3R inhibitor 2-APB indicated that the Ca^{2+} signal mediates the activation of ERK1/2, p46 JNK, and p38 MAPK by the nucleotide, consistent with the role of Ca^{2+} as an upstream activator of MAPKs in Caco-2 cells.

P2Y receptors have been shown to couple to phosphorylation of ERK1/2, JNK1/2, and p38 MAPKs via activation of protein kinases such as phosphoinositide-3 kinase (PI3K) or protein kinase C (PKC) [33,44–46]. We also found that pharmacological inhibition of PKC diminished ATP-dependent phosphorylation of MAPKs, supporting the involvement of PKC as an upstream mediator in the activation of MAPK cascades in Caco-2 cells. The fact that 2-APB diminished ERK1/2, p46 JNK, and p38 MAPK phosphorylation suggests that calcium-dependent PKC isoforms may mediate such response.

In addition, we evaluated the participation of cAMP/PKA in purinergic activation of MAPK pathways. We found that phosphorylation of MAPKs by ATP is partially suppressed by the cAMP antagonist Rp-cAMP. Among P2Y receptors, the human $P2Y_{11}$ is dually coupled to PLC and adenylyl cyclase stimulation in other cell types [14,47,48], therefore we cannot rule out the participation of $P2Y_{11}$ receptor in ATP actions in Caco-2 cells. However, studies in other cell lines where ATP elevates cAMP levels implicate the activation of P2Y receptors different from $P2Y_{11}$ [49] or involve an indirect mechanism increasing prostaglandin release and in this way regulating cAMP formation [50].

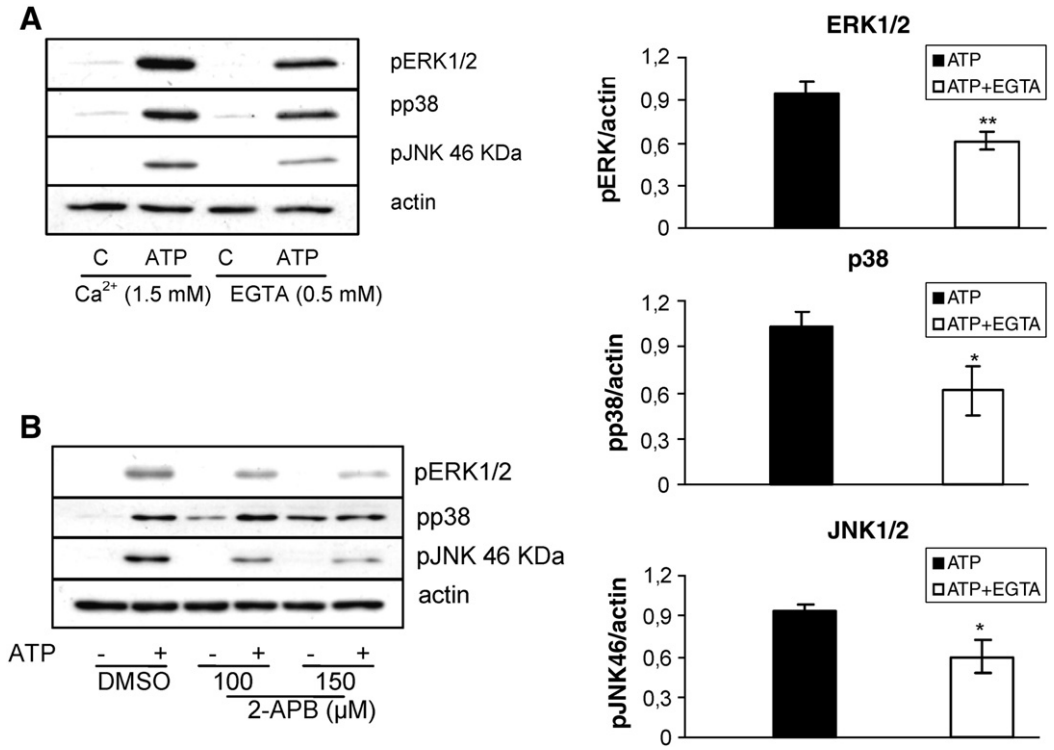

Fig. 5. EGTA and 2-APB affect ATP-dependent phosphorylation of MAPKs in Caco-2 cells. Cells were treated with 10 μM ATP during 5 min in the presence or absence of EGTA (0.5 mM) or 2-APB (100–150 μM). The cells were then lysed and comparable aliquots of lysate proteins were separated by SDS–PAGE followed by Western blotting as in Fig. 1. The immunoblots are representative of at least three independent experiments. Bars show the quantification of the blots expressed as arbitrary units of MAPK phosphorylation levels with respect to controls (mean ± SD; $^{*}p<0.05$, $^{**}p<0.01$).

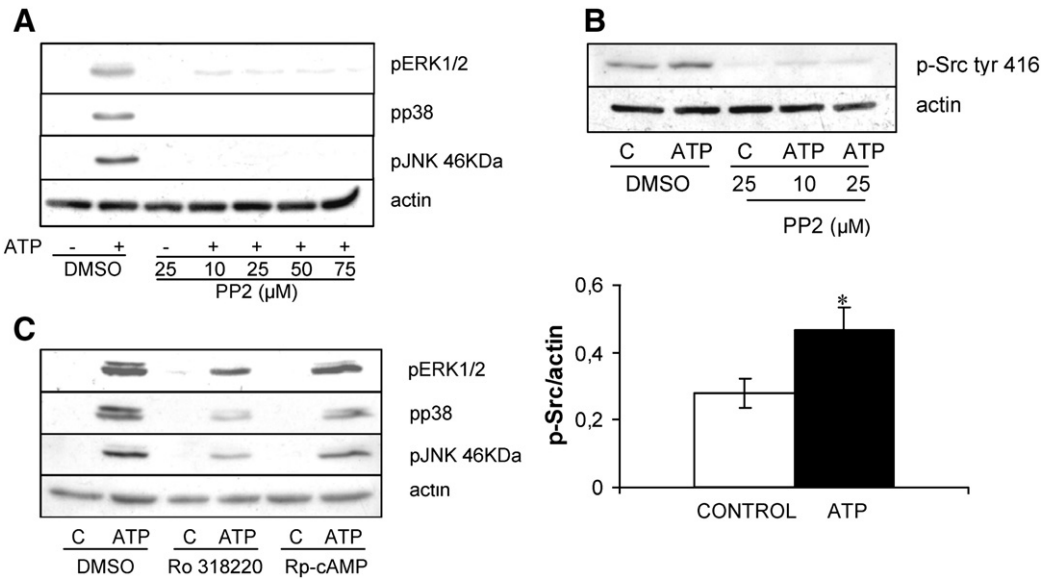

Fig. 6. Effects of Src inhibitor PP2, cAMP antagonist Rp-cAMP, and PKC inhibitor Ro 318220 on ATP-dependent phosphorylation of MAPKs. Caco-2 cells were treated with 10 μM ATP during 5 min in the absence or presence of (A and B) PP2 (10–75 μM) or (C) Rp-cAMP (50 μM) and Ro 318220 (0, 2 μM). The cells were then lysed, comparable protein aliquots were separated by SDS–PAGE followed by Western blotting as in Fig. 1. Representative immunoblots of at least three independent experiments are shown. Bars show the quantification of the blots expressed as arbitrary units of Src phosphorylation levels with respect to control (mean ± SD; $^{*}p<0.05$).

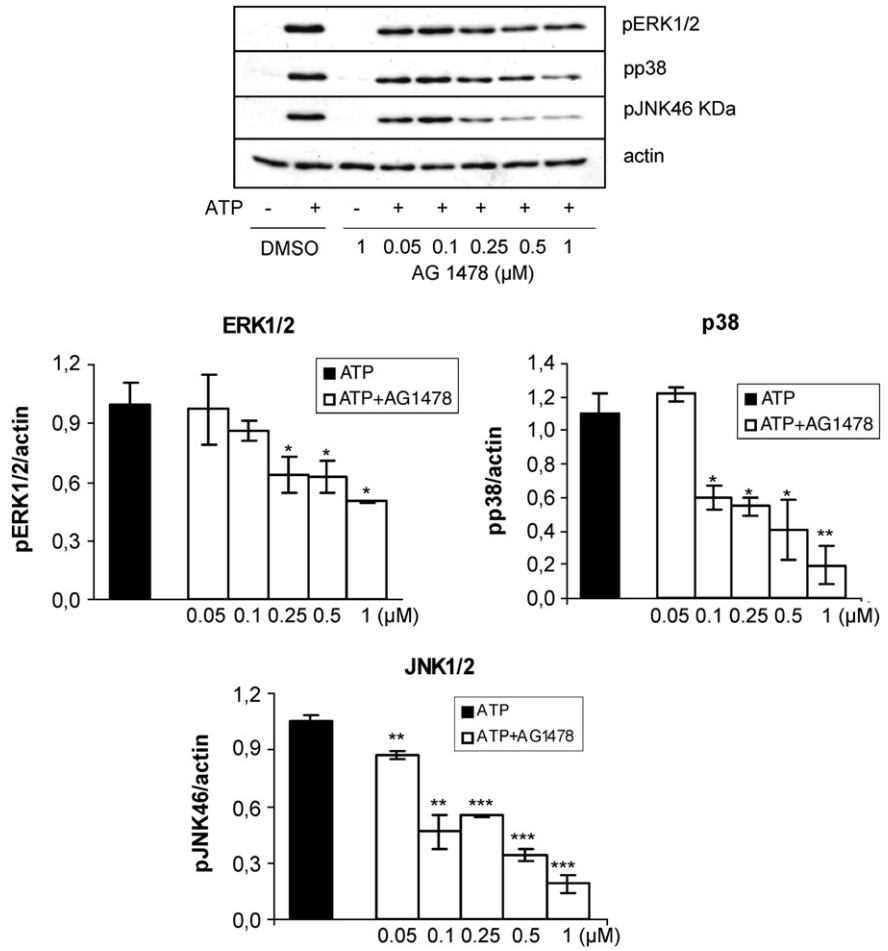

Fig. 7. EGFR contributes to ERK1/2, p46 JNK, and p38 MAPK phosphorylation induced by ATP. Caco-2 cells were preincubated with AG1478 (50–1000 nM) or DMSO (control) and stimulated for 5 min with 10 μM ATP. Cell lysates were immunoblotted against pERK1/2 and then the membranes stripped and reprobed with anti-pp38 and -pJNK1/2 phosphospecific antibodies and with anti-actin as loading control. Representative immunoblots and quantification by scanning volumetric densitometry of blots from 3 independent experiments are shown. Means ± SD are given. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, where p values refer to differences in MAPK phosphorylation between cells in presence or absence of AG1478.

P2Y receptor-mediated stimulation of ERK1/2 has also been shown to be dependent on the activation of tyrosine kinases such as Src or proline-rich tyrosine kinase (Pyk2) [45,51]. Recent studies have identified two SH3-binding domains in the C-terminal tail of the $P2Y_2$ receptor that are necessary for this G-protein-coupled receptor (GPCR) to bind directly and activate the nonreceptor tyrosine kinase Src and for Src-dependent transactivation of several receptor tyrosine kinases, including the EGFR and platelet-derived growth factor receptor (PDGFR) [52]. Our study indicates that in Caco-2 cells, the activation of the three MAPK cascades by ATP is also dependent on Src family tyrosine kinases and that EGFR participation is more important for ATP-mediated p46 JNK and p38 MAPK phosphorylation than for ERK1/2 activation in this intestinal cell line. GPCR may transactivate the EGFR via activation of tyrosine kinases such as Src, or via activation of metalloproteinases to generate EGFR ligands such as HB-EGF [53]. Previous studies of several different cell types have shown that growth factor receptors may activate similar signal transduction proteins and may use the same intracellular signaling cascades [45,52–54]. Therefore, it cannot be fully ruled out that stimulation of P2Y receptors in Caco-2 cells causes MAPK activation through multiple intracellular signaling cascades: one involving EGFR transactivation and the other involving an independent mechanism. Further studies are needed to identify the involvement of Src family members in EGFR transactivation by ATP in Caco-2 cells, as has been shown in other kind of cells [52,55].

In conclusion, our results provide evidence that expand our knowledge on the mechanism of action of ATP in Caco-2 cells. We show, for the first time, that ATP, UTP, and UDP acting through P2Y receptors, most likely $P2Y_2$, $P2Y_4$, $P2Y_6$ and probably $P2Y_{11}$, activate ERK1/2, JNK 46 kDa, and p38 MAPK. Modulation by ATP is Ca^{2+}-dependent and involves Src family kinases, to a lesser extent PKC, PKA, and the participation of EGFR. Further studies are in progress to determine the consequences of phosphorylation of MAPKs in response to ATP stimulation of Caco-2 cells on gene transcription and to identify the signal transduction pathways that mediate intestinal epithelial cell proliferation.

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