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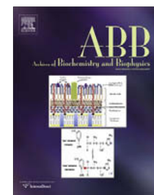

ATP modulates transcription factors through $P2Y_2$ and $P2Y_4$ receptors via PKC/MAPKs and PKC/Src pathways in MCF-7 cells

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ABSTRACT

In this work, we studied the involvement of PKC and Src in the phosphorylation of ERK1/2, p38 and JNK1 MAPKs and in the modulation of ATF-1, c-Fos, c-Jun and Jun D transcription factors by ATP in MCF-7 breast cancer cells. RT-PCR studies and nucleotide sequence analysis confirmed first the expression of $P2Y_2$- and $P2Y_4$-receptor subtypes. The use of specific inhibitors and Src antisense oligonucleotides showed that PKC, but not Src, plays a role in the phosphorylation of MAPKs by ATP. ATP stimulated the expression of c-Fos and the phosphorylation c-Jun, Jun D and ATF-1. PKC and Src only participated in c-Fos induction and in ATF-1 phosphorylation. Pharmacological inhibition of MAPKs demonstrated that c-Fos induction and phosphorylation of c-Jun and Jun D, but not of ATF-1, depend on MAPK activation. These results suggest that stimulation of $P2Y_2$ and $P2Y_4$ receptors by ATP modulates transcription factors through PKC/MAPKs and PKC/Src pathways in MCF-7 cells.

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Introduction

Extracellular nucleotides are important signaling molecules in both intracellular and extracellular microenvironments, and possible effectors of neoplastic transformation [1]. They can modulate cellular function by activation of membrane-bound P2 receptors. There are two principal families of P2 receptors: P2X, which are ligand-gated ion channels; and P2Y, which belong to the group of G-protein-coupled receptors (GPCRs)[1]. Seven mammalian P2X-receptor subtypes ($P2X_{1-7}$) and eight mammalian P2Y-receptor subtypes ($P2Y_{1,2,4,6,11-14}$), have been cloned [2]. Distinct P2-receptor subtypes have been identified in primary samples of a variety of human cancer tissue and cell lines. Recent evidence suggests that they may be involved in the regulation of proliferation, differentiation and apoptosis [3]. Thus, identification of P2Y receptors and the characterization of signaling pathways associated with them might contribute to the development of novel therapeutic tools in the treatment of cancer.

It has been observed in several kinds of cells that extracellular nucleotides through P2 receptors modulate multiple signaling pathways including phosphorylation of the mitogen-activated protein kinases (MAPKs) and activation of transcription factors [4–7]. The MAPKs are a family of serine/threonine kinases that play an essential role in signal transduction by modulating gene transcription in the nucleus in response to changes in the cellular environment [8]. In MCF-7 breast cancer cells, we previously showed that ATP increases the $[Ca^{2+}]_i$ and the phosphorylation of the MAPKs ERK1/2, p38 and JNK1/2. In addition, our pharmacological studies suggested that this effect was mediated by $P2Y_{2/4}$-receptor activation [9].

The protein kinase C has been involved in the transduction of signals for cell proliferation and differentiation [10]. Interestingly, increased levels of PKC have been associated with malignant transformation in a number of cell lines including breast [11].

Src family tyrosine kinases are non-receptor proteins (Src, Yes, Fyn, Frk, Brk, and others) that share a similar structure and can modulate a variety of cellular functions including proliferation, survival, adhesion and migration. When phosphorylated at tyrosine 527, Src is inactive. Activation is accomplished by dephosphorylation of this tyrosine residue, and the resulting conformational change facilitates autophosphorylation at tyrosine 416. After autophosphorylation Src is in an active state [12]. Src has been found to be overexpressed or highly activated in a number of human neoplasms, including breast [13]. Both PKC and Src kinases have been implicated in the phosphorylation of MAPKs by ATP in osteoblasts [5,14].

The AP-1 (activating protein-1) transcription factor is a dimeric complex whose major components are the products of the c-Jun and c-Fos protooncogenes, the c-Jun and c-Fos family proteins [15]. This complex consists of homodimers of Jun proteins (c-Jun, Jun B, Jun D) or heterodimers of Jun and Fos proteins (c-Fos, Fos

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[1] *Abbreviations used:* GPCRs, G-protein-coupled receptors; MAPKs, mitogen-activated protein kinases; AP-1, activating protein-1; TRE, TPA response element; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcription-polymerase chain reaction.

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B, Fra-1, Fra-2) that bind to a regulatory DNA sequence, the TPA response element (TRE) [16]. AP-1 transcription factor plays a critical role in multiple signal transduction pathways in many cells. In breast cancer cells, previous studies have suggested that it is an important regulator of cell growth and invasion [17].

In this work, we studied the participation of PKC and Src family kinases in ATP-induced MAPK phosphorylation, in the induction of c-Fos and in the phosphorylation of c-Jun, Jun D and ATF-1 transcription factors in MCF-7 breast cancer cells. The expression of $P2Y_2$- and $P2Y_4$-receptor subtypes was also investigated. Our data suggest that Src family kinases modulate the purinergic nucleotide-dependent phosphorylation of ATF-1 and c-Fos transcription factors, while PKC participates both in the phosphorylation of MAPKs and in the induction/phosphorylation of c-Fos and ATF-1 transcription factors by ATP, through $P2Y_{2/4}$-receptor subtypes, in MCF-7 cells.

Materials and methods

Materials

ATP, RPMI-1640 medium and Calphostin C were from Sigma–Aldrich Co. (St. Louis, MO, USA). Ro318220 was from EMD Chemicals, Inc. (San Diego, CA, USA). PD98059, SB203580, SP600125 and PP2 were from Tocris Bioscience (Ellisville, MO, USA). Src antisense (5′-CTC TTG TTG CTA CCC ATG GTCC-3′) and mismatch (5′-AGC AAC GAC AAG TCA AGC TG-3′) oligonucleotides, and RT-PCR primers were from Ruralex (Buenos Aires, Argentina). Fetal bovine serum (FBS) was from Natocord (Córdoba, Argentina). Monoclonal antibodies recognizing dually phosphorylated ERK1/2 (Thr202, Tyr204), dually phosphorylated JNK1/2 (Thr183/Tyr185), phosphorylated CREB (Ser 133)/ATF-1 (Ser 63), phosphorylated c-Jun (Ser 73)/Jun D (Ser 255), and phosphorylated Src (Tyr 416) were from Cell Signaling Technology (Beverly, MA, USA). Polyclonal antibodies recognizing dually phosphorylated p38 (Thr180/Tyr182) and p38α, monoclonal anti c-Src antibody, polyclonal anti c-Fos, polyclonal 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) membranes and ECL chemiluminescence detection kit were from GE Healthcare (Little Chalfont, Buckinghamshire, England). All other reagents used were analytical grade.

Cell culture

The human breast cancer epithelial cell line MCF-7 (American Type Culture Collection, Manassas, VA, USA) was seeded at a density of 20,000 cells per cm^2 and cultured at 37 °C in RPMI-1640 medium containing 10% FBS under humidified air (5.5% CO_2). After 48 h, the medium was replaced by RPMI-1640 without FBS and cells were starved for 24 h before agonist stimulation. Controls with vehicle (water) were used.

Western blot analysis

MCF-7 cells were treated with ATP in the presence or absence of the inhibitors Ro318220, Calphostin C, PD98059, SB203580, SP600125 and PP2. Cells were then 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 s 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 method [18]. Lysate proteins dissolved in 6× Laemmli sample buffer were separated (30 μg/lane) using SDS–polyacrylamide gels (10% acrylamide) and electrotransferred to PVDF membranes. After blocking with 5% non-fat milk in TBST buffer (50 mM Tris, pH 7.2–7.4, 200 mM NaCl, 0.1% Tween 20), the membranes were incubated 90 min with the appropriate dilution of primary antibody in TBST plus 1% non-fat milk. After washing, the membranes were incubated with the appropriate dilution of horse radish peroxidase-conjugated secondary antibody in TBST plus 1% non-fat 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).

Stripping and re-probing of membranes

The complete removal of primary and secondary antibodies from the membranes was achieved by incubating the membranes 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. Membranes were then washed for 10 min in TBST (1% Tween 20) and blocked, as indicated above, for 1 h at room temperature. Thereafter, membranes were ready to re-probe with the corresponding antibodies.

Total RNA isolation

Total RNA from MCF-7 cells grown in monolayer was isolated using Invitrogen Trizol reagent. First, MCF-7 cells were lysed directly in the culture dish by the addition of 1 mL per 10 cm^2 of Trizol reagent, passing the cell lysate several times through a pipette. The homogenized sample was incubated for 5 min at 15 °C to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 mL of chloroform per 1 mL of Trizol Reagent was added and the mix was shaken vigorously. The sample was centrifuged at 12,000*g* for 15 min at 4 °C. The aqueous phase was transferred to a fresh tube, and RNA was precipitated by mixing it with isopropyl alcohol. Incubation for 10 min at 15 °C followed and then an additional centrifugation at 12,000*g* for 15 min at 4 °C was performed. The precipitated RNA (pellet) was washed once with 75% ethanol and centrifuged at no more than 7500*g* for 5 min at 4 °C. To end the procedure, the RNA pellet was dried briefly and dissolved in RNase-free water.

Reverse transcription-polymerase chain reaction (RT-PCR)

Transcripts for $P2Y_2$ and $P2Y_4$ in MCF-7 cells were studied by RT-PCR using the Superscript II Reverse Transcriptase from Invitrogen, the JumpStart REDTaq ReadyMix PCR Reaction Mix from Sigma–Aldrich Co. and specific primers. The sequence of the primers ($P2Y_2$: forward: 5′-CTCTACTTTGTCACCACCAGCGCG-3′, reverse: 5′-TTCTGCTCCTACAGCCGAATGTCC-3′; $P2Y_4$: forward: 5′-CCACCT GGCATTGTCAGACACC-3′, reverse: 5′-GAGTGACCAGGCAGGGCAC GC-3′) was based on the known sequences in the coding region of the human receptors [19]; and was previously checked using the BLASTn program of the GenBank. The predicted PCR product sizes for $P2Y_2$ and $P2Y_4$ receptors were of 637 bp and 425 bp, respectively. After total RNA isolation, the reverse transcription reaction was carried out employing Oligo $(DT)_{12-18}$ and Superscript II Reverse Transcriptase according to manufacturer's instructions. First-strand cDNA was diluted in a 50 μl PCR amplification reaction mix by combining the reagents as described in the kit. The PCR samples were first denatured at 94 °C (3 min) and then the reaction was allowed to proceed for 35 amplification cycles: denaturation (45 s, 94 °C), annealing (45 s at 67 °C ($P2Y_2$); 56.5 °C ($P2Y_4$)) and extension (90 s at 72 °C ($P2Y_2$); 1 min at 72 °C ($P2Y_4$)). Next, a final extension step of 10 min at 72 °C was performed. The resulting PCR

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

PCR products purification and sequencing

PCR products 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), according to manufacturer's instructions. Purified PCR products were sequenced through the DNA Sequencing Facility Service of Ruralex. The sequences obtained were analyzed running a BLASTn program at the GenBank database.

Cell treatment with c-Src antisense and mismatch oligonucleotides

Cells were pre-treated with c-Src antisense oligonucleotides as follows. MCF-7 cells were seeded in plates at a density of 20,000 cells per cm^2 and cultured at 37 °C in RPMI-1640 medium containing 10% FBS under humidified air (5.5% CO_2). When cells reached 50–60% confluence, they were washed twice with PBS solution. Then 4 mL of serum-free RPMI-1640 medium were added to each plate. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Antisense or mismatch oligonucleotides (antisense: 5′-CTC TTG TTG CTA CCC ATG GTCC-3′; mismatch: 5′-AGC AAC GAC AAG TCA AGC TG-3′) [20] were used at a final concentration of 20 μM. Cells were incubated with the oligonucleotides/liposome mixture at a final volume of 5 mL for 24 h at 37 °C in a CO_2 incubator. After 24 h, medium was changed to serum-free RPMI for 1 h and then followed by Western blot analysis of cell extracts.

Statistical analysis

Statistical significance of data was evaluated using Student's *t*-test [21], and probability values below 0.05 ($P < 0.05$) were considered significant. Quantitative data are expressed as means ± standard deviation (SD) from the indicated set of experiments.

Results

We previously reported pharmacological evidence involving $P2Y_{2/4}$ receptors in ATP-induced increases in $[Ca^{2+}]_i$ and in the phosphorylation of the MAPKs ERK1/2, p38 and JNK1 in MCF-7 human breast cancer cells [9]. To confirm the identity of the receptor subtypes which mediate the response to ATP, we studied their expression in MCF-7 cells by RT-PCR employing specific pairs of forward and reverse primers (see Materials and methods). We amplified two DNA fragments of 425 bp and 637 bp. These fragments match with the predicted amplification product sizes for the $P2Y_4$- and $P2Y_2$-receptor subtypes, respectively; as indicated by a positive control of RNA from Caco-2 cells [19,22] (see Fig. 1). Sequence analysis by running a BLASTn Program of the GenBank database revealed that the RT-PCR products obtained exhibited ~99% sequence identity with human $P2Y_4$ and $P2Y_2$ receptors (GenBank Accession Nos. FJ599701 and FJ599699, respectively). These results support, then, the expression of the $P2Y_2$- and $P2Y_4$-receptor subtypes in MCF-7 cells.

P2Y receptors are members of the GPCR superfamily [23]. Signaling from P2Y receptors to the MAPK cascades can proceed by several distinct pathways, some of which involve PKC and/or Src [5,24–28]. To determine the participation of PKC in ATP-induced phosphorylation of ERK1/2, p38 and JNK1 MAPKs in MCF-7 cells, Ro318220 and Calphostin C, two well known PKC inhibitors, were used. As revealed by Western blot analysis, 5 μM Ro318220 (Fig. 2A) and 0.1 μM Calphostin C (Fig. 2B) reduced the phosphorylation of ERK1/2, p38 and JNK1 MAPKs induced by ATP, suggesting the involvement of PKC.

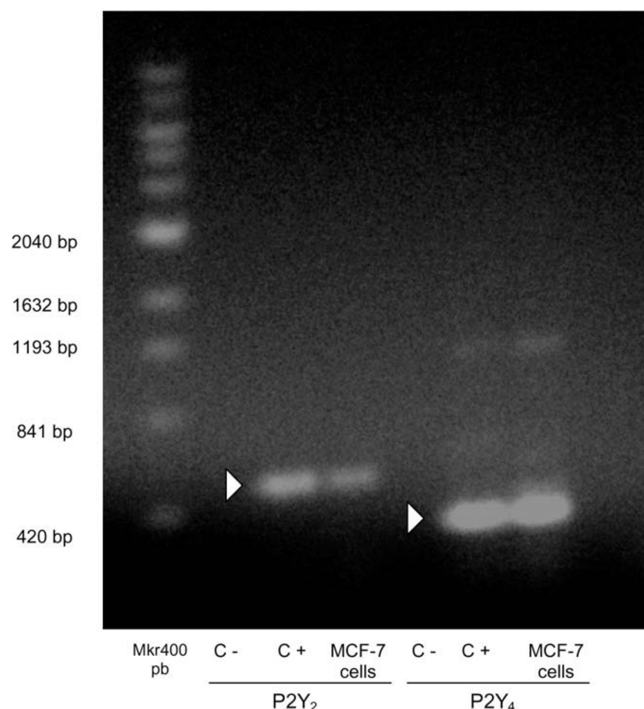

Fig. 1. RT-PCR analysis of $P2Y_2$ and $P2Y_4$ mRNA receptor subtypes in MCF-7 cells. RT-PCR amplification with specific primers against $P2Y_2$- and $P2Y_4$-receptor subtypes was performed after total RNA isolation, as described in Materials and methods. Then, PCR products were analyzed by agarose gel 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 the human colonic adenocarcinoma cells Caco-2, known to express $P2Y_2$ and $P2Y_4$ receptors [20]. A representative gel containing ethidium bromide-stained RT-PCR products is shown.

As shown in Fig. 3, 5 μM ATP stimulated the phosphorylation of Src tyrosine kinase at its Tyr 416. Src phosphorylation was almost suppressed by the PKC inhibitor Ro318220 (5 μM), thus indicating that ATP leads to Src phosphorylation through a mechanism involving PKC. In addition, we studied the participation of Src in the phosphorylation of MAPKs by ATP by testing the effects of different concentrations of PP2, an inhibitor of Src family kinases. As seen in Fig. 4, although PP2 completely blocked Src phosphorylation (Tyr 416) by ATP, it did not affect the phosphorylation of ERK1/2, and it increased the phosphorylation of p38 and JNK1 MAPKs. Transfection with specific antisense and mismatch oligonucleotides against human c-Src [20] was performed to confirm the results obtained using PP2. Fig. 5A and B reveal significant c-Src knock-down after cell transfection with antisense oligonucleotides, as the total amount of c-Src protein was markedly reduced when compared with control and mismatch oligonucleotide conditions. Fig. 5C shows no significant changes in the phosphorylation of ERK1/2, p38 and JNK1 MAPKs after cell transfection with c-Src antisense oligonucleotides. Altogether, these findings suggest that although Src is phosphorylated at its Tyr 416 by ATP, it does not participate in the phosphorylation of the MAPKs studied.

MAPKs phosphorylate many transcription factors and therefore enhance their transcriptional activity [8]. In view that ATP activates MAPKs in MCF-7 cells, we studied the phosphorylation of CREB/ATF-1 and c-Jun, Jun D transcription factors as well as the expression of c-Fos in response to ATP. Fig. 6A shows maximum phosphorylation of ATF-1 after a 15 min treatment with ATP while

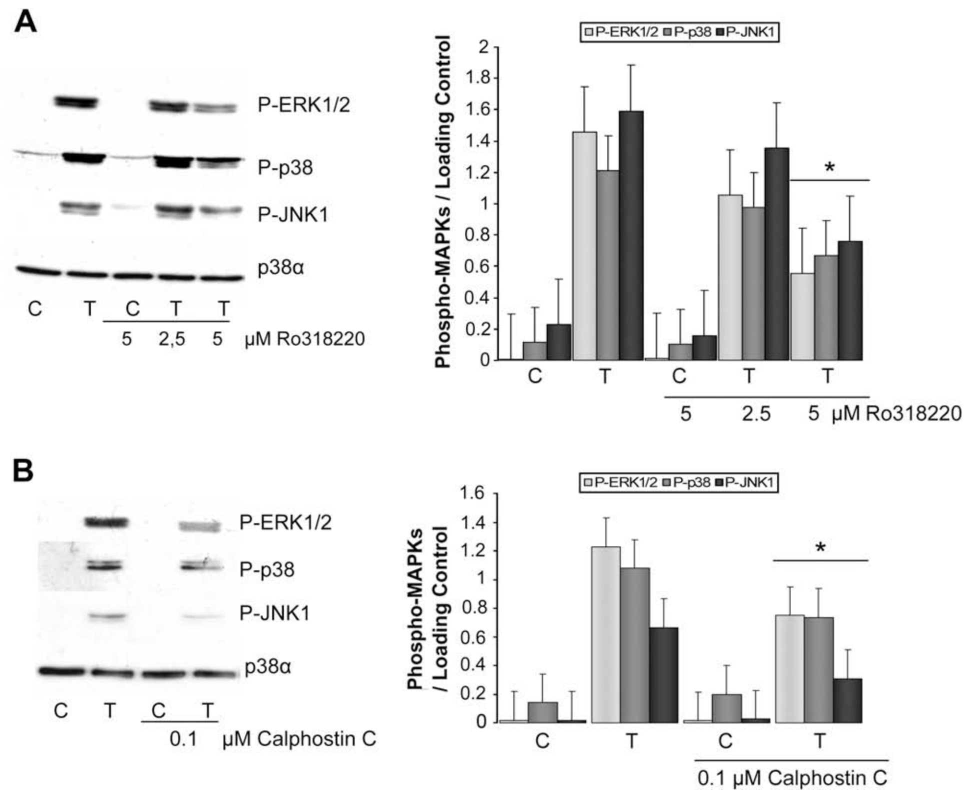

Fig. 2. Inhibitors of PKC reduce the phosphorylation of ERK1/2, p38 and JNK1 MAPKs by ATP. Cells were pre-incubated for 20 min with 2.5 and 5 μM Ro318220 (A) or 0.1 μM Calphostin C (B), both PKC inhibitors. Then, cells were treated with 5 μM ATP (T) or vehicle (C) for 5 min and the phosphorylation of MAPKs was evaluated by Western blot analysis as described in Materials and methods, employing antibodies that recognize the active (phosphorylated) forms of ERK1/2 (pERK1/2), JNK1/2 (pJNK1/2) and p38 (pp38). An antibody directed against the total form (active plus inactive) of p38α isoform was used as loading control. The membrane was stripped and re-probed three times. The vertical bar chart shows the ratio of optical density quantification of each phospho-MAPK versus loading control by imaging densitometry. The results are shown as means ± SD. $^{*}P < 0.05$.

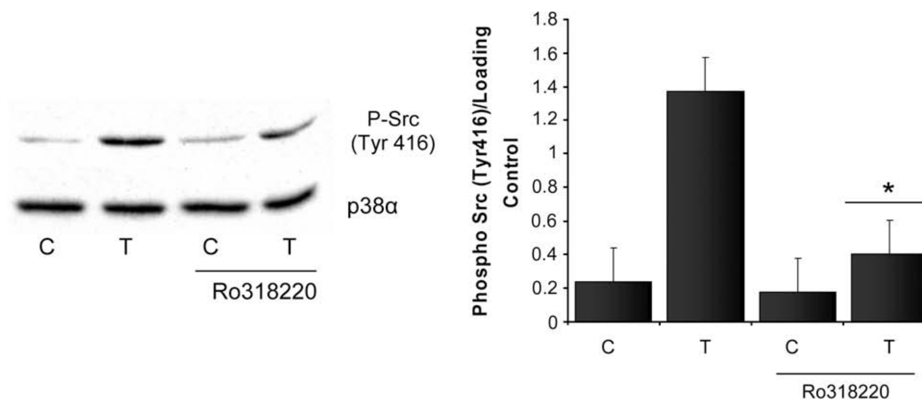

Fig. 3. ATP stimulates Src activation through PKC. Cells were pre-incubated for 20 min with 5 μM Ro318220. Then, cells were treated with 5 μM ATP (T) or vehicle (C) for 5 min and phosphorylation of Src at its Tyr 416 was evaluated by Western blot analysis as described in Materials and methods. An antibody directed against the total form of p38α isoform was used as loading control. The figure shows a representative immunoblot and the quantification of the blots, expressed as arbitrary densitometry units, of three independent experiments. The results are shown as means ± SD. $^{*}P < 0.05$.

phosphorylation of CREB was not detected. In addition, highest levels of c-Jun and Jun D phosphorylation and of c-Fos induction were observed at 30 min. As 17β-estradiol induces the phosphorylation of ATF-1 and CREB in C2C12 cells [29], this muscle cell line was used as a positive control to check that the anti-phospho-CREB antibody also recognizes phospho-ATF-1 (Fig. 6B).

To establish a relationship between the activation of MAPKs and transcription factors, specific pharmacological inhibitors of p38 (SB203580), ERK1/2 (PD98059) and JNK (SP600125) were used. These compounds were first assayed to test their effectiveness to inhibit the phosphorylation of MAPKs when used at the concentrations most frequently described in the literature. Fig. 7 shows that 20 μM SB203580, 20 μM PD98059 and 40 μM SP600125 inhibited ATP-dependent phosphorylation of p38, ERK1/2 and JNK1, respectively. Next, we evaluated the phosphorylation of c-Jun, Jun D and ATF-1, and the induction of c-Fos. As seen in Fig. 8A–D, pharmacological inhibition of MAPKs diminished c-Fos protein induction as well as the phosphorylation of c-Jun and Jun D by ATP, but not of ATF-1, thus suggesting their dependence on p38, ERK1/2 and JNK activation.

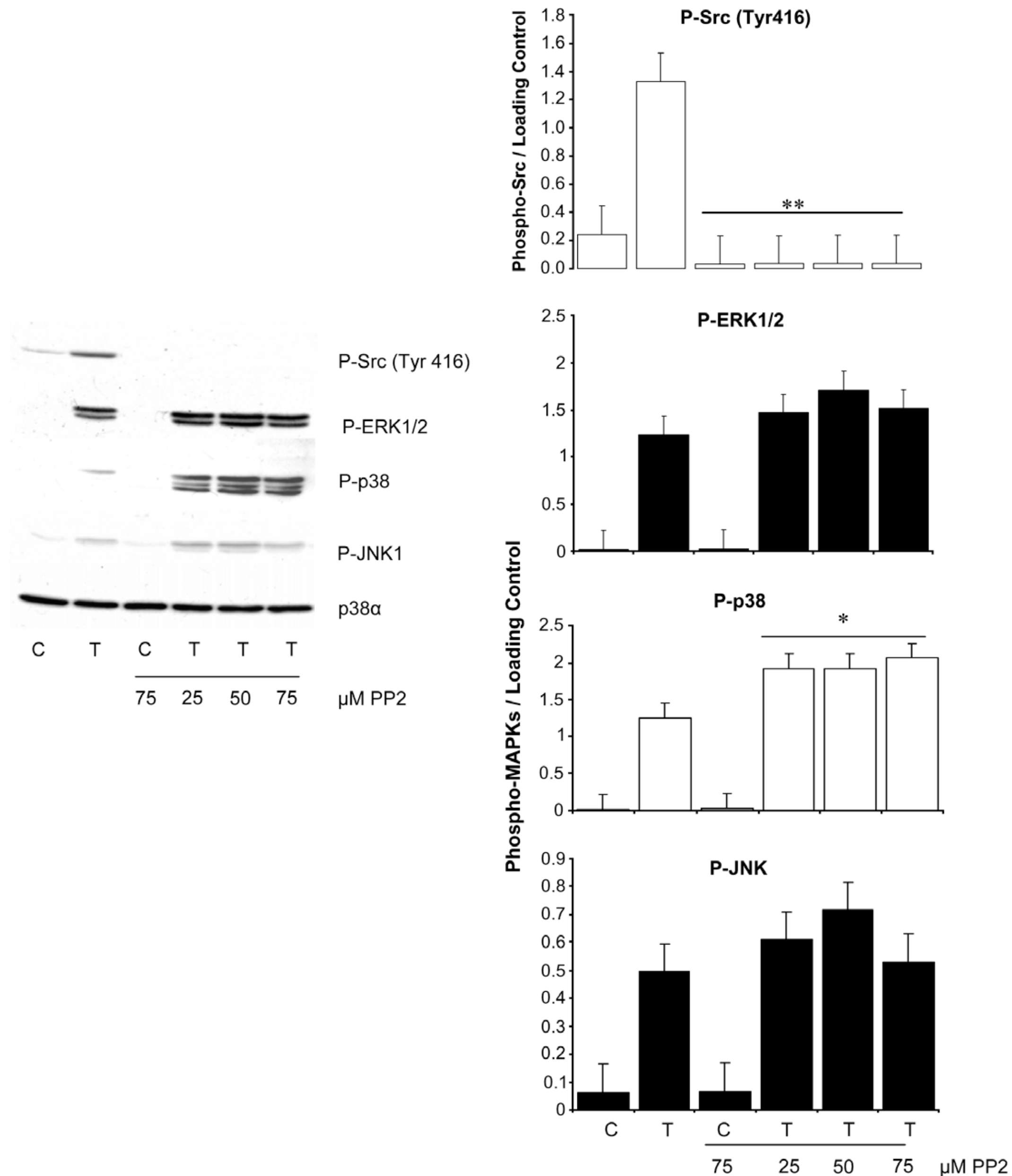

Fig. 4. PP2 suppresses the phosphorylation of Src (Tyr 416) but does not reduce the activation of MAPKs by ATP. MCF-7 cells were pre-incubated for 30 min with different PP2 concentrations (25–75 μM). Then, cells were treated with 5 μM ATP (T) or vehicle (C) for 5 min and Western blot analysis was performed. Antibodies that recognize the active form of Src (phosphorylated at its Tyr 416), the phosphorylated forms of p38, ERK1/2 and JNK1/2 MAPKs and the total form of p38α isoform were used. The membrane was stripped and re-probed four times. A representative immunoblot is shown. Bars show the quantification of three independent experiments, expressed as arbitrary densitometry units of Src and MAPKs phosphorylation levels with respect to controls. The results are shown as means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

Although Src was activated by ATP it could not be implied in the phosphorylation of MAPKs by the nucleotide. Therefore, we also explored its relationship with the phosphorylation/induction of transcription factors in MCF-7 cells. As seen in Fig. 9, PP2 strongly reduced ATF-1 phosphorylation and diminished c-Fos protein induction, but had no effect on c-Jun and Jun D phosphorylation by ATP. This result supports the participation of Src in the modulation of ATF-1 and c-Fos transcription factors by the purinergic nucleotide.

Discussion

Eight P2Y-receptor subtypes have been cloned in mammals [2]. In this work, we document the expression of $P2Y_2$ and $P2Y_4$ purinoceptor subtypes in MCF-7 breast cancer cells, as revealed by RT-PCR analysis and nucleotide sequencing of the products obtained. This result is in general agreement with previous investigations by Dixon et al. [30] reporting molecular evidence on the presence of the P2U purinoceptor family in various human breast cancer cell lines, although $P2Y_2$ and $P2Y_4$ subclasses were not identified. The data are also in keeping with receptor agonist specificity studies using ATP, ATPγ-S, UTP and ADPβ-S, in which the PI–PLC pathway was involved in the response to ATP of MCF-7 cells [9]. Within P2Y-receptor subtypes only $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ act through the PI–PLC pathway [23]. The results shown here confirm the expression of $P2Y_2$- and $P2Y_4$-receptor subtypes strongly suggesting their involvement in the ATP effects on ERK1/2, p38 and JNK1 phosphorylation in MCF-7 cells.

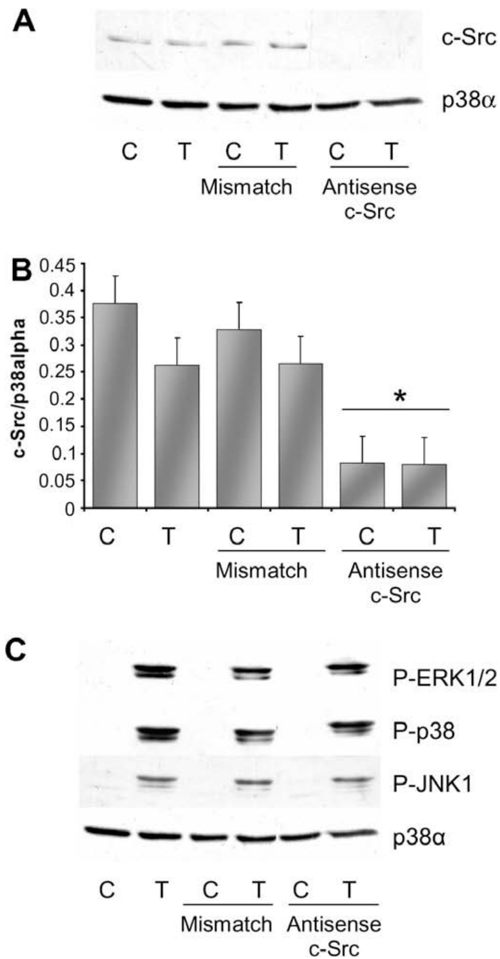

Fig. 5. The inhibition of c-Src expression does no affect the phosphorylation of ERK1/2, p38 and JNK1 by ATP in MCF-7 cells. Cells were transfected for 24 h with 20 μM mismatch or antisense oligonucleotides against human c-Src to reduce its expression, as described in Materials and methods. Then, cells were treated with 5 μM ATP (T) or vehicle (C) for 5 min. (A) Western blot analysis performed using antibodies that recognize total levels of c-Src and p38α (loading control). (B) The vertical bar chart shows the ratio of quantification of total Src versus loading control by imaging densitometry. The results are shown as means ± SD. $^{*}P < 0.05$, with respect to control conditions. (C) Phosphorylation of MAPKs was evaluated by Western blot analysis employing phosphospecific antibodies and a loading control as described in Fig. 2. A representative blot of three independent experiments is shown.

Activation of P2Y receptors by purinergic ligands can stimulate multiple intracellular signal transduction pathways [4]. In addition, activation of MAPKs through P2Y receptors can occur via upstream protein kinases such as Src and PKC [5]. Our studies indicate that ATP signaling through $P2Y_2$ and $P2Y_4$ receptors to p38, ERK1/2 and JNK1 MAP kinases depends on PKC as revealed by the use of Ro318220 and Calphostin C. In agreement with these results in C6 glioma cells [26] and astrocytes [25] $P2Y_2$-receptor activation also involves the participation of PKC in the phosphorylation of MAPKs.

Ro318220 is an inhibitor of all PKC isoenzymes that acts at the catalytic domain of the kinase; however, its apparent low specificity [12] could explain its failure to inhibit the ATP effect at the lowest concentration used (2.5 μM). The other PKC inhibitor, Calphostin C, acts through the regulatory domain of the calcium dependent- and calcium independent-PKCs. Therefore, our results suggest that these groups of kinases may be involved in the phosphorylation of MAPKs induced by ATP in MCF-7 cells. As signaling from $P2Y_{2/4}$ receptors to MAPK cascades in these cells depends on

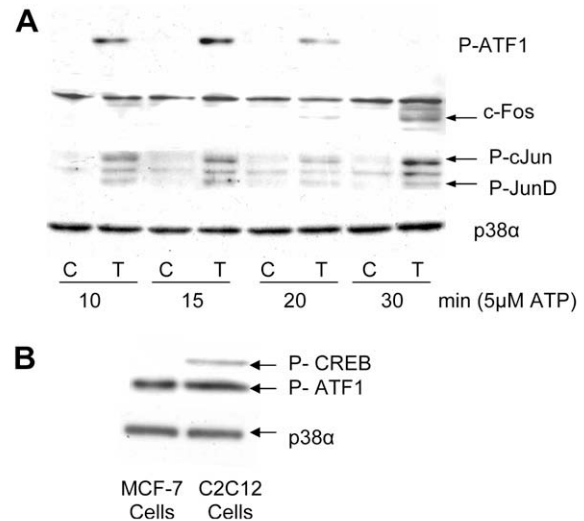

Fig. 6. Time-response profile of ATF-1, c-Jun and Jun D phosphorylation and c-Fos induction by ATP in MCF-7 cells. (A) Cells were treated with 5 μM ATP (T) or vehicle (C) for 10–30 min. Subsequently, Western blot analysis was performed employing antibodies that recognize the phosphorylated forms of ATF-1/CREB (P-ATF-1, P-CREB); c-Jun (P-c-Jun) and Jun D (P-Jun D); and the total forms of c-Fos and p38α (loading control). Blots are representative of at least three experiments performed independently. (B) C2C12 cells, treated with 17β-estradiol for 15 min, were used as a positive control to show that the anti-phospho-CREB antibody also recognizes phospho-ATF-1. In MCF-7 cells stimulated with ATP, only the phosphorylation of ATF-1 is detected.

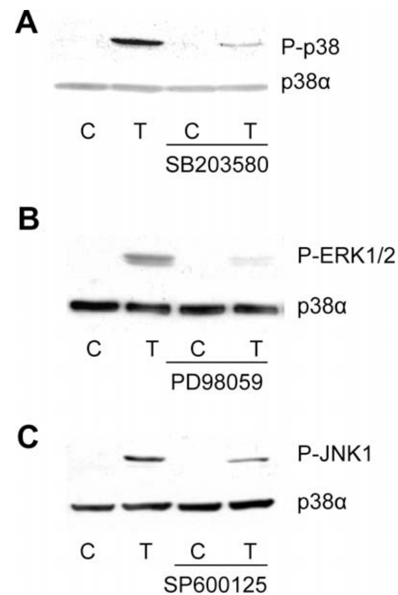

Fig. 7. Pharmacological inhibitors of MAPKs reduce their phosphorylation by ATP. MCF-7 cells were pre-incubated for 20 min with 20 μM SB203580 (A), 20 μM PD98059 (B) or 40 μM SP600125 (C). Then, cells were treated with vehicle (C) or 5 μM ATP (T) for 5 min, and Western blot analysis was performed employing antibodies that recognize the active (phosphorylated) forms of p38 (pp38), ERK 1/2 (pERK1/2) and JNK1/2 (pJNK1/2). An antibody directed against the total form (active plus inactive) of p38α isoform was used as loading control. These results are representative of at least three experiments performed independently.

PI/PLC and calcium [9], calcium dependent PKC isoforms are expected to participate.

Our results showed that Src family kinases were rapidly phosphorylated (within 5 min) at tyrosine 416 by ATP. Studies in human 1231N1 astrocytoma cells revealed the existence of proline-rich SH3 binding sites (PXXP) in the carboxyl-terminal tail of the

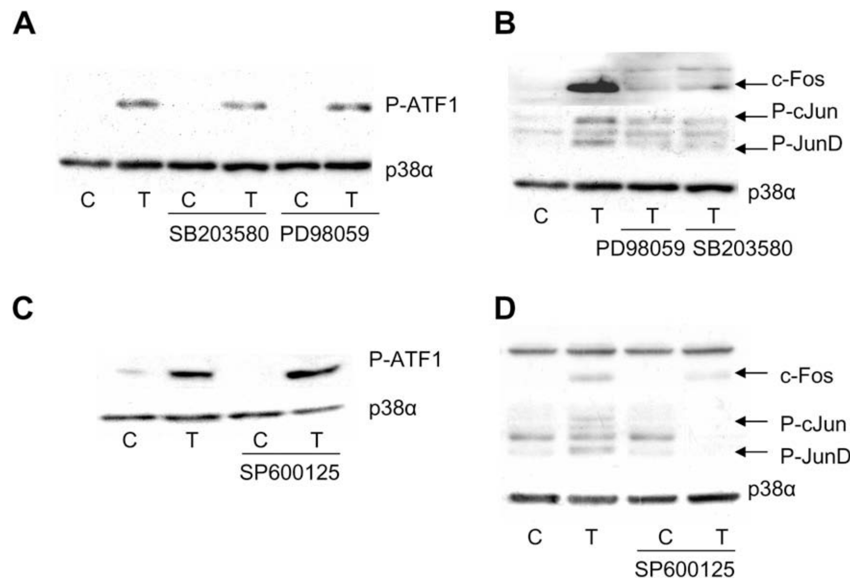

Fig. 8. Participation of MAPKs in the modulation of transcription factors by ATP. MCF-7 cells were pre-incubated for 20 min with 20 μM SB203580, 20 μM PD98059 or 40 μM SP600125. Then, cell treatment was performed by the addition of vehicle (C) or 5 μM ATP (T) for 15 (A and C) or 30 min (B and D). Cell lysate proteins were immunoblotted with P-CREB/P-ATF-1 phosphospecific antibody (A and C) or with anti-c-Fos and anti-P-c-Jun/P-Jun D antibodies (B and D). Anti-p38α was used as loading control. These results are representative of at least three experiments performed independently.

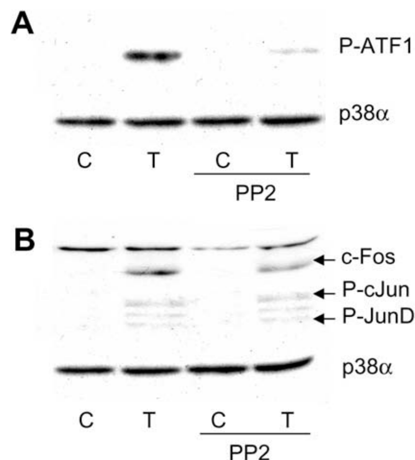

Fig. 9. Involvement of Src in the modulation of transcription factors by ATP. Cells were pre-incubated for 20 min with vehicle (DMSO) or 75 μM PP2, and were treated with 5 μM ATP (T) for 15 (A) or 30 min (B). Cell lysate proteins were immunoblotted with P-CREB/P-ATF-1 phosphospecific antibody (A) or with anti-c-Fos and anti-P-c-Jun/P-Jun D antibodies (B). Anti-p38α was used as loading control. These results are representative of at least three experiments performed independently.

$P2Y_2$ receptor that interact directly with Src after receptor stimulation [31]. Interestingly, Ro318220 inhibited Src phosphorylation at tyrosine 416 induced by ATP, suggesting that PKC is also involved in Src activation. This finding is consistent with previous results reported by others [5,32]. In addition, in NIH3T3 mouse fibroblasts and A7r5 rat aortic smooth muscle cell lines there is evidence of a protein tyrosine phosphatase, PTPα, which can directly activate Src kinase after its activation by PKC [32,33]. Thus, PTPα could participate in Src activation by ATP through a mechanism involving PKC in MCF-7 cells.

Although Src activity is thought to be necessary for the transactivation of growth factor receptors by G-protein coupled receptors (GPCRs), there are different interpretations about the role that these kinases play in the activation of downstream mitogenic signaling [31]. In rat-1 fibroblasts, a dominant-negative mutant of Src was used to demonstrate that it is important for linking GPCR activation with the activation of MAPKs [34,35]. On the other hand, experiments performed with embryonic fibroblasts derived from $Src^{-/-}$ mice indicated that Src is dispensable for GPCR-mediated activation of MAPKs [36]. Here, by the use of PP2, a potent and selective Src inhibitor; we showed that the phosphorylation of MAPKs by ATP is independent of Src in MCF-7 cells. The fact that this inhibitor increased p38 and JNK phosphorylation after cell treatment with ATP may be due to an off-target effect and/or to a stress response, as the MAPKs involved are stress sensitive. Cell transfection with c-Src antisense oligonucleotides confirmed the results obtained with PP2. Of relevance, we have recently obtained evidence demonstrating that ATP induces the phosphorylation of the serine/threonine kinase Akt through Src in MCF-7 cells [37]. In these experiments, both PP2 and Src antisense oligonucleotides, used under the same conditions as in this work, suppressed the phosphorylation of Akt by ATP suggesting that the reduction in Src expression is sufficient to attenuate Src activity. Our results are consistent with reports on the human lung cancer cell line A549, where the mitogenic effect upon activation of P2Y-receptor signaling by ATP and UTP does not involve Src kinases to regulate proliferation [38].

It is well known that purinergic signaling through $P2Y_2$ receptors leads to c-Fos expression in many cell types including breast [7,39–42]. Here, we confirmed the induction of c-Fos expression by ATP in MCF-7 cells and, in addition, provided information on the phosphorylation of c-Jun, Jun D and ATF-1. ATP induced the expression of c-Fos, and the phosphorylation of c-Jun and Jun D transcription factors with a similar temporal profile. Although it has been reported that ATP induces CREB phosphorylation in MCF-7 cells [7], we found that the purinergic nucleotide stimulates the phosphorylation of ATF-1 transcription factor and has no effect on CREB.

MAPKs play an essential role in signal transduction by modulating gene transcription in the nucleus in response to changes in the cellular environment [8]. Then, as expected, pharmacological inhibition of MAPKs reduced the induction/phosphorylation of c-Fos, c-Jun and Jun D by ATP; while PP2 had no effect on these transcription factors. On the other hand, the phosphorylation of ATF-1 by ATP was independent of MAPKs but dependent on Src. CREB/ATF-

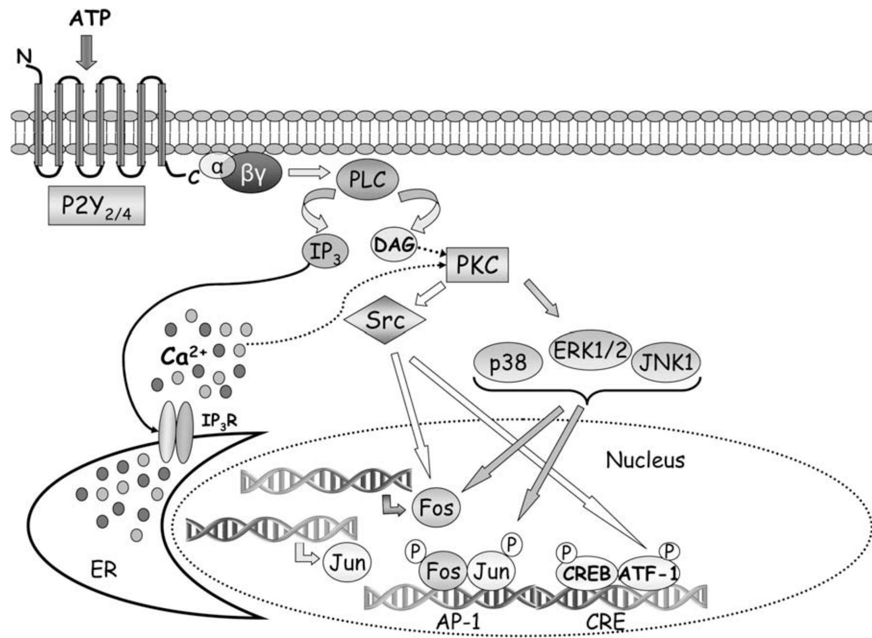

Fig. 10. Schematic model of ATP signaling pathway in MCF-7 cells. The scheme depicts, on the basis of the results obtained in previous and in this study, the ATP signaling pathway through $P2Y_{2/4}$ receptors in breast cancer cells. Dotted arrows stand for expected events.

1 can be phosphorylated by several kinases, including MAPKAP kinase-1β (also known as RSK-2), p70 S6 kinase (p70S6K) [43], and also Ca^{2+}–calmodulin-dependent kinases II and IV [44]. Therefore, the modulation of these kinases by Src cannot be excluded and should be further investigated in future studies in MCF-7 cells.

Altogether, these data support the expression of the $P2Y_2$- and $P2Y_4$-receptor subtypes in MCF-7 breast cancer cells. In addition, we suggest that their activation by ATP, leads to c-Fos induction and to the phosphorylation of c-Jun, Jun D and ATF-1 through PKC/MAPKs and PKC/Src pathways (schematically depicted in Fig. 10). These results are of potential relevance to understand the role of ATP signaling in breast tumor growth and progression.

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