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Purinergic (ATP) signaling stimulates JNK1 but not JNK2 MAPK in osteoblast-like cells: Contribution of intracellular Ca^{2+} release, stress activated and L-voltage-dependent calcium influx, PKC and Src kinases

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ABSTRACT

This work shows that ATP activates JNK1, but not JNK2, in rat osteoblasts and ROS-A 17/2.8 osteoblast-like cells. In ROS-A 17/2.8 cells ATP induced JNK1 phosphorylation in a dose- and time-dependent manner. JNK1 phosphorylation also increased after osteoblast stimulation with ATPγS and UTP, but not with ADPβS. RT-PCR studies supported the expression of $P2Y_2$ receptor subtype. ATP-induced JNK1 activation was reduced by PI-PLC, IP_3 receptor, PKC and Src inhibitors and by gadolinium, nifedipine and verapamil or a Ca^{2+}-free medium. ERK 1/2 or p38 MAPK inhibitors diminished JNK1 activation by ATP, suggesting a cross-talk between these pathways. ATP stimulated osteoblast-like cell proliferation consistent with the participation of $P2Y_2$ receptors. These results show that $P2Y_2$ receptor stimulation by ATP induces JNK1 phosphorylation in ROS-A 17/2.8 cells in a way dependent on PI-PLC/IP_3/intracellular Ca^{2+} release and Ca^{2+} influx through stress activated and L-type voltage-dependent calcium channels and involves PKC and Src kinases.

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It is well established that extracellular nucleotides elicit a wide range of responses in many cell types. These include roles in exocrine and endocrine secretion, platelet aggregation, vascular endothelial cell-mediated vasodilatation, nociceptive mechanosensory transduction, cotransmission and neuromodulation in most nerve types in the peripheral and central nervous systems. In addition, they have been shown to modulate cell proliferation, differentiation and apoptosis [1].

Growing evidence suggests that extracellular nucleotides, signaling through P2 receptors, may play important roles in the regulation of bone metabolism [2,3]. There are two main families of P2 receptors: the P2X receptor family of ligand-gated nonselective ion channels and the P2Y receptor family of G-protein-coupled receptors [1,4]. Currently, seven P2X receptor subunits have been cloned ($P2X_{1-7}$) and eight P2Y receptor subtypes ($P2Y_{1, 2, 4, 6, 11, 12, 13 \text{ and } 14}$) activated by purine and/or pyrimidine nucleotides have been reported in vertebrates [1,4,5]. Most P2Y receptors activate phosphatidylinositol-specific phospholipase C (PI-PLC)[1] leading to increases in inositol trisphosphate (IP_3), diacylglycerol (DAG) and mobilization of intracellular calcium [4,6,7].

ATP acting through P2Y receptors has been shown to modulate mitogen-activated protein kinases (MAPKs) signaling pathways in several cell types including osteoblasts [8–14]. Family members of the MAPKs mediate a wide variety of cellular behaviours such as proliferation, differentiation and apoptosis in response to extracellular stimuli [15]. Four different subgroups within the MAPK family have been described: extracellular signal-regulated kinases (ERKs), c-jun N-terminal or stress-activated protein kinases (JNK/SAPK), ERK 5/big MAP kinase 1 (BMK1), and the p38 group of protein kinases [15]. MAPK family members have been shown to be important signaling components linking mechanical stimuli to cellular responses, including cell growth, differentiation, and metabolic regulation in various cell types. In osteoblast-like cells, we have recently reported that ATP stimulation of P2Y receptors, presumably the $P2Y_2$ subtype, sensitizes them to mechanical stress leading to calcium influx through gadolinium sensitive channels (SAC influx) and activation of ERK1/2 and p38 MAPKs [13]. JNK is activated by numerous extracellular stimuli and represents a major physiological mechanism of regulation of c-Jun, a component of the AP1 transcription factor [16]. However, the involvement of P2Y receptors in the ATP signaling through JNK in osteoblasts has not been studied.

In the present work, we have investigated signal transduction pathways involved in the regulation of JNK by ATP and the expression of the main P2Y receptor subtypes, which might mediate the effects of the purinergic agonist on MAPKs in the rat osteoblast-like cell line ROS-A 17/2.8. The data obtained expand the results of previous studies on ERK1/2 and p38 MAPK [13], demonstrating that

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[1] *Abbreviations used:* PI-PLC, phosphatidylinositol-specific phospholipase C; IP_3, inositol trisphosphate; DAG, diacylglycerol; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK/SAPK, c-jun N-terminal or stress-activated protein kinases.

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ATP stimulates phosphorylation of JNK1 but unexpectedly not of JNK2, through activation of the $P2Y_2$ receptor/PI-PLC/IP_3/intracellular calcium release signaling pathway. JNK1 modulation by ATP also involves PKC and Src activation and the participation of L-VDCC and ATP-dependent SAC influx.

Materials and methods

Materials

ATP, ATPγS, UTP, ADPβS, nifedipine, verapamil, neomycin sulfate, 2-Aminoethyl diphenyl borate (2-APB), Ham's F-12 medium, RPMI-1640, DMEM, α-MEM, bovine serum albumin (BSA), collagenase and $GdCl_3$ were from Sigma Chemical Co. (St. Louis, MO, USA). Inhibitors Ro 318220 and SB203580 were from Calbiochem (San Diego, CA, USA). PD98059 and U0126 were from TOCRIS (Ellisville, MO, USA). PP1 was from BIOMOL (Plymouth Meeting, PA, USA). Fetal bovine serum (FBS) was from Natocord (Córdoba, Argentina). TRIzol Reagent was from Invitrogen (Carlsbad, CA, USA). Reverse Transcription System was from Promega (Madison, WI, USA). Primers listed in Table 1 were synthesized in Ruralex (Buenos Aires, Argentina). CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit was from Promega (Madison, WI, USA). Cell Signaling Technology (Beverly, MA, USA) supplied several antibodies as follows: polyclonal phospho-SAPK/JNK (Thr183/Tyr185) (1:1000) which detects both p46 (pJNK1) and p54 (pJNK2) SAPK/JNK dually phosphorylated at threonine 183 and tyrosine 185; polyclonal SAPK/JNK (1:500) which recognizes both total JNK1 (46 kDa) and JNK2 (54 kDa) proteins; mouse monoclonal phospho-p44/42 MAP kinase (Thr202/Tyr204) (1:1500); polyclonal p44/42 MAP Kinase (1:1000), and polyclonal caspase-3 (1:1000) which detects full-length caspase-3 (35 kDa) and its large fragment resulting from cleavage (17 kDa). Anti-actin (20-33) antibody (1:15000) was from Sigma Chemical Co. (St. Louis, MO, USA). Polyclonal antibody against p38α (1:1000), polyclonal phospho-p38 (1:1000), goat anti-mouse (1:5000) and goat anti-rabbit (1:10000) peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein size markers were from Amersham Biosciences (Piscataway, NJ, USA), Immobilon P (polyvinylidene difluoride) membranes and ECL chemiluminescence detection kit were from Amersham (Little Chalfont, Buckinghamshire, England). All other reagents used were of analytical grade.

Cell culture

ROS 17/2.8 osteoblastic cells (rat osteosarcoma-derived) were provided by Dr. Irina Mathow (Facultad de Farmacia y Bioquímica—IDEHU, Universidad de Buenos Aires, 1113 Buenos Aires, Argentina). The cell line was previously renamed ROS-A 17/2.8 alluding to its pharmacological profile characteristic of $P2Y_2$ receptor expression. The mouse C2C12 myoblast cell line was provided by Dr. Enrique Jaimovich (Universidad de Chile, Santiago de Chile, Chile) and the breast cancer epithelial cell line MCF-7 was from American Type Culture Collection (Manassas, VA, USA). Cells were seeded at a density of 10,000 cells per cm^2 and cultured at 37 °C in appropriate medium (Ham's F-12 for ROS-A 17/2.8 cells, DMEM for C2C12 myoblasts or RPMI-1640 for MCF-7 cells) containing 10% FBS under humidified air (5.5% CO_2). After 48 h, the medium was replaced by FBS-free medium and the cells were starved for 18–21 h before Western blot analysis.

Osteoblast isolation

Calvarial osteoblasts were obtained from 5-day-old neonatal rats. Briefly, calvaria were incubated in PBS containing 4 mM EDTA at 37 °C for two 10-min periods and the supernatants were discarded. Subsequently, calvaria were rinsed in PBS and subjected to digestion with 200 U/ml collagenase in PBS for four 15-min periods. Cells released during the first digestion were discarded and those released during the subsequent digestions were spun down, collected and combined after centrifugation during 10 min at 1500 rpm. Then, cells were cultured at 37 °C in α-MEM supplemented with 10% FBS, 1% penicillin and streptomycin under humidified air (5.5% CO_2).

Western blot analysis

Cells were treated with ATP, ATPγS, UTP or ADPβS in the presence or the absence of the calcium channel blocker Gd^{3+} or various inhibitors (nifedipine, verapamil, neomycin, compounds 2-APB, Ro318220 and PP1) when indicated. 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) 15 min before cell stimulation. Treatment of the cells was performed in the cell-metabolic incubator to minimize cellular stress. Then 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 s and maintained on ice for another 10 min. After centrifugation at 14,000g and 4 °C during 15 min the supernatant was collected and proteins were quantified by the Bradford method [17]. Lysate proteins dissolved in 6× Laemmli sample buffer were separated (30 μg/lane) on SDS–polyacrylamide gels (10% or 12% acrylamide for analysis of capase-3 cleavage) 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 5% BSA. 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 reprobing 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. 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, the membranes were ready to reprobe with the corresponding antibodies.

Reverse transcription-polymerase chain reaction (RT-PCR)

Transcripts for $P2Y_1$, $P2Y_2$ and $P2Y_4$ were assayed by RT-PCR using the Reverse Transcription System from Promega. Specific primers and the products expected to yield are listed in Table 1. Total RNA was isolated from cell monolayers employing TRIzol Reagent from Invitrogen. Next, total RNA was transcribed to cDNA by a reverse transcription reaction employing $Oligo(dT)_{15}$ and AMV Reverse Transcriptase. First-strand cDNA was diluted in a 100-μl PCR amplification reaction mix by combining the reagents indicated in the kit. The PCR mixture was incubated as indicated in Table 1. The resulting PCR products were analyzed by 2% agarose gel electrophoresis and visualized by staining with ethidium bromide.

Table 1
Sequences of primers used for RT-PCR, cycles employed and predicted product size

Primer	Sequence	Product size	Cycles	Source
$P2Y_1$	Forward 5′-CATCTCCCCCATTCTCTT-3′ Reverse 5′-GTTGCTTCTTCTTGACCTGT-3′	663 bp	• Initial denaturation: 5 min at 95 °C • 30 amplification cycles: - denaturation: 1 min at 95 °C - annealing: 1 min at 53 °C - extension: 30 s at 72 °C • Final extension: 7 min at 72 °C	Ref. [18]
$P2Y_2$	Forward 5′-GCTTCAACGAGGACTTCAAGTA(C/T)GTGC-3′ Reverse 5′-AGGTGAGGAAGAGGATGCTGCAGTAG-3′	301 bp	• Initial denaturation: 5 min at 98 °C • 40 amplification cycles: - denaturation: 45 s at 95 °C - annealing: 45 s at 58 °C - extension: 45 s at 72 °C • Final extension: 7 min at 72 °C	Ref. [19]
$P2Y_4$	Forward 5′-CCAGAGGAGTTTGACCACTA-3′ Reverse 5′-CACCAAGGCCAGGGAGGA-3′	447 bp		

[^{3}H]-thymidine incorporation assay

ROS-A 17/2.8 cells were cultured in 24-multiwell plates at a density of 14,000 cells per well. After 48 h, the medium was replaced by Ham's F-12 without FBS and the cells were starved for 18–21 h. Cell treatment was performed in quadruplicate and started by adding the indicated agonists or vehicle. Before the treatment was finished, 0.5 μCi [^{3}H] -thymidine/well was added and the cells were further incubated for 1 h at 37 °C. The treatment was stopped by aspirating the medium and washing three times with PBS. Cells were incubated 30 min at 4 °C with 12% trichloroacetic acid (TCA) per well and then washed three times with cold 12% TCA. The samples were solubilized with 1 N NaOH and the radioactivity was counted in a liquid scintillation counter. Proteins were quantified by the Bradford method [17]. [^{3}H]-thymidine incorporation was quantified as cpm per milligram of protein and the results expressed as stimulation % with respect to the control.

Cell proliferation assay

The number of viable cells in proliferation was determined employing the commercial CellTiter 96® AQueous One Solution Cell Proliferation Assay kit from Promega. ROS-A 17/2.8 cells were plated into 96-multiwell plates (350 cells/well) and treated in a similar manner than in the [^{3}H]-thymidine incorporation assay. Before the treatment was finished, the medium containing the indicated agonists was replaced by medium containing MTS (20 μl MTS/100 μl Ham's F-12). Cells were incubated during 60–90 min at 37 °C (5.5% CO_2) and subsequently the absorbance was measured at 490 nm. Control wells (without cells) containing the same volumes of culture medium and MTS solution as in the experimental wells were used to substract background absorbance from absorbance values of the samples. The results were obtained as absorbance per well and expressed as % stimulation with respect to the control.

Statistical analysis

Statistical significance of data was evaluated using Student's *t*-test [20] and probability values below 0.05 ($P < 0.05$) or 0.01 ($P < 0.01$) were considered significant or highly significant, respectively. Quantitative data are expressed as mean ± standard deviation (SD) from the indicated set of experiments.

Results

In this work, we first characterized the effects of ATP on the phosphorylation (activation) of c-Jun NH2-terminal kinases (JNKs) in the ROS-A 17/2.8 osteoblast-like cell line. To achieve this objective, we treated serum-starved ROS-A 17/2.8 cells with 1–100 μM ATP for 5 min and then JNK phosphorylation was evaluated by Western blot analysis using a phospho-specific antibody (Fig. 1 A). The results show highest phosphorylation levels of JNK1 (46 kDa) at 10 μM ATP that were sustained up to 100 μM ATP. In addition, JNK1 was activated in a time-dependent manner, reaching the maximum within 5 min of treatment with 10 μM ATP and returning to basal levels after 15 min (Fig. 1B). On the other hand, the JNK2 isoform (54 kDa) was not activated by ATP in ROS-A 17/2.8 cells at any of the doses and times indicated or at longer times (up to 120 min; not shown). In order to demonstrate that the phospho-JNK 1/2 antibody also recognizes the phosphorylated form of JNK2, we used MCF-7 cells as a positive control, in which JNK1/2 phosphorylation by ATP has been recently reported [14]. Moreover, we have observed that total JNK2 expression was greater than JNK1 in ROS-A 17/2.8 cells (Fig. 1A and B).

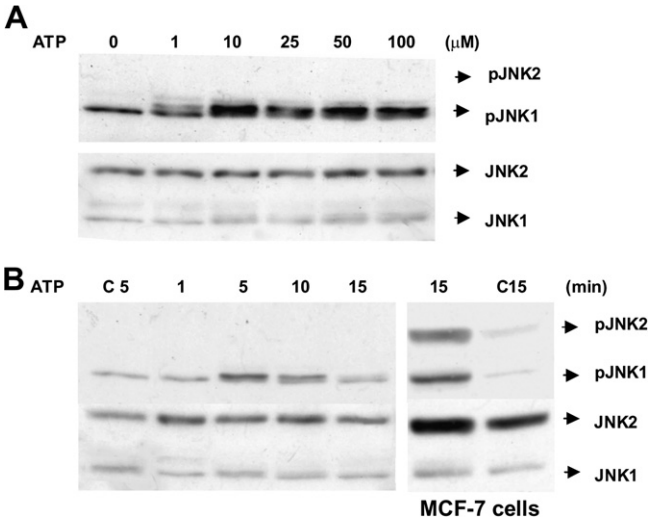

Fig. 1. Dose– and time–response patterns of ATP effects on JNK1 phosphorylation in osteoblast-like cells. Serum-starved (21 h) ROS-A 17/2.8 osteoblast-like cells were treated with 1–100 μM ATP during 5 min (A) or with 10 μM ATP during 1–15 min. MCF-7 cells (used as a positive control to show that phospho-JNK1/2 antibody also recognizes phospho-JNK2) were treated with 5 μM ATP during 15 min (B). Subsequently, Western blot analysis was performed as described in Methods employing an antibody that recognizes the phosphorylated (active) state of both JNK1 and JNK2 isoforms. Only the 46 kDa isoform indicated as pJNK1, is activated by ATP in osteoblast-like cells. Controls using vehicle instead of ATP are indicated as 0 in the dose–response and C5 or C15 (control during 5 or 15 min, respectively) in the time–response studies. An antibody directed against the total form (active plus inactive) of JNK1 (46 kDa) and JNK2 (54 kDa) was used as loading and expression control. Note that JNK2 is expressed to a greater extent than JNK1. The results are representative of at least three experiments performed independently.

Significant phosphorylation of JNK1 was observed after 5 min treatment of osteoblast-like cells with ATPγS (0.5–50 μM), a non-hydrolysable analogue of ATP or with 10 μM UTP, both known agonists acting on $P2Y_2/P2Y_4$ receptors (Fig. 2A and B). ADPβS (10–100 μM), a potent agonist of the $P2Y_1$ receptor, was not able to stimulate JNK1 phosphorylation (Fig. 2C). The biological activity of ADPβS was controlled by using the mouse C2C12 myoblast cell line, known to express the $P2Y_1$ receptor in culture [21]. As Fig. 2C shows, ADPβS stimulated JNK1 and JNK2 phosphorylation in C2C12 cells after 5 min treatment at a concentration of 50 μM. These results suggest then a role for $P2Y_2$ or $P2Y_4$ receptors as the main P2Y receptor subtypes involved in JNK1 stimulation by ATP and rules out the participation of the $P2Y_1$ receptor in this response in osteoblast-like cells.

Studies were performed to confirm the physiological validity of the above results using rat primary osteoblast cells. Treatment of calvarial osteoblasts with 10 μM ATP and UTP during 5 min induced JNK1 but not JNK2 phosphorylation (Fig. 2 D). Moreover, ADPβS treatment was without effects on either JNK isoform. As observed in Fig. 2D, again, JNK2 expression was greater than JNK1 in osteoblasts like in the osteoblastic cell line ROS-A 17/2.8. This key experiment validates our data and supports that ATP plays a physiological role in stimulating the JNK1 signaling pathway through $P2Y_2$ or $P2Y_4$ receptors in osteoblasts.

In order to evaluate the expression of $P2Y_1$, $P2Y_2$ and $P2Y_4$ purinergic receptor genes in ROS-A 17/2.8 cells, reverse transcription-polymerase chain reaction (RT-PCR) studies were performed using the primers indicated in Table 1. As Fig. 3 shows, a DNA fragment of the expected size (301 bp) for the $P2Y_2$ receptor subtype was obtained. On the other hand, we could not amplify $P2Y_4$ or $P2Y_1$ DNA fragments. These results support the expression of the $P2Y_2$ receptor subtype in ROS-A 17/2.8 cells and exclude the participation of the $P2Y_4$ receptor in mediating JNK1 activation by ATP.

We have previously shown by spectrofluorimetric measurements in Fura-2 loaded osteoblastic cells that treatment with ATP sensitizes them to mechanical stress leading to calcium influx (stress-activated calcium (SAC) influx), which is blocked by the trivalent cation gadolinium (Gd^{3+}) and participates in the stimulation of ERK1/2 and p38 MAPKs [13]. Therefore, the involvement of extracellular calcium in JNK1 activation by ATP was evaluated. Incubation of ROS-A 17/2.8 cells during 15 min with a Ca^{2+}-free buffer containing 0.5 mM EGTA partially (59.7 ± 6.1%) inhibited JNK1 activation by ATP (Fig. 4A). This result suggests that extracellular calcium participates in JNK1 phosphorylation by ATP. As demonstrated before, cell viability was not affected by incubation in a Ca^{2+}-free medium during 15 min, since more than 90% of the cells excluded the Trypan Blue dye (not shown). To rule out any apoptotic effect due to EGTA or even ATP on ROS-A 17/2.8 cells, the expression of cleaved caspase-3 in the same experiment was examined. The blot of Fig. 4A, right, shows that caspase-3 was not cleaved at any condition studied, indicating that these agents did not induce apoptosis.

Then, we investigated if gadolinium-sensitive ATP-dependent SAC influx contributes to the phosphorylation of JNK1 in osteoblast-like cells. Accordingly, cell pretreatment with 2.5 μM Gd^{3+} for 2 min inhibited ATP activation of JNK1 by 55 ± 15%; also, JNK1 phosphorylation was diminished by 61.3 ± 3.2% upon preincubation for 15 min with 5 μM nifedipine and verapamil, two L-type voltage-dependent calcium channel (L-VDCC) blockers (Fig. 4B). These results suggest that Ca^{2+} influx through Gd^{3+}- and L-VDCC channels participates in the activation of JNK1 by ATP in ROS-A 17/2.8 cells.

Owing to the fact that a partial inhibition of JNK1 was achieved in calcium-free buffer and in the presence of SAC and L-VDCC blockers, it may be assumed that JNK1 modulation by ATP also involves an intracellular calcium mobilization-dependent mechanism. To investigate if JNK1 stimulation by ATP is dependent on intracellular Ca^{2+} release, the effect of the purinergic agonist on

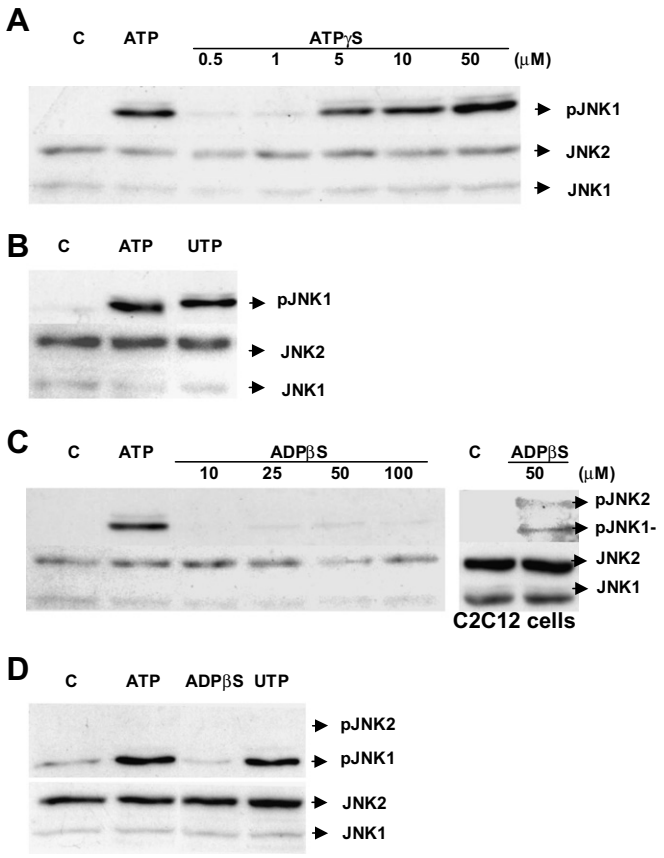

Fig. 2. Pharmacological profile of the P2Y receptors involved in the activation of JNK1 by ATP in osteoblasts. ROS-A 17/2.8 osteoblastic cells were treated for 5 min with 10 μM ATP, 0.5–50 μM ATPγS (the non-hydrolysable analogue of ATP) (A) or with 10 μM UTP (B), both known agonists of the $P2Y_2/P2Y_4$receptors or with 10–100 μM ADPβS (C), a potent agonist of the $P2Y_1$ receptor. The mouse C2C12 myoblast cell line, known to express the $P2Y_1$ receptor in culture [21], was treated with 50 μM ADPβS during 5 min and used as a positive control for the agonist. Primary osteoblasts (neonatal rat calvaria-derived) were treated for 5 min with 10 μM ATP, ADPβS or UTP (D). Controls using vehicle instead of purinergic agonists are indicated as (C). Levels of phospho-JNK1 and JNK2 were then measured with the phospho-specific antibody as in Fig. 1. Total JNK1/2 antibody was used as loading control. The results are representative of at least three experiments performed independently.

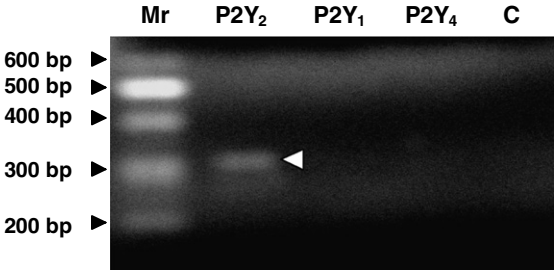

Fig. 3. Presence of $P2Y_2$ purinergic receptor subtype mRNA in ROS-A 17/2.8 cells. After total RNA extraction, a RT-PCR amplification with specific primers against $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptor subtypes was performed as indicated in Methods. cDNA products were analyzed thereafter by agarose gel (2%) electrophoresis. The arrow indicates the amplification product of $P2Y_2$ receptor subtype at the expected size of 301 bp. $P2Y_1$ (663 bp) and $P2Y_4$ (447 bp) were not detectable. (Mr) 100 bp ladder; (C) control without RNA. A representative gel with ethidium bromide-stained RT-PCR products is shown.

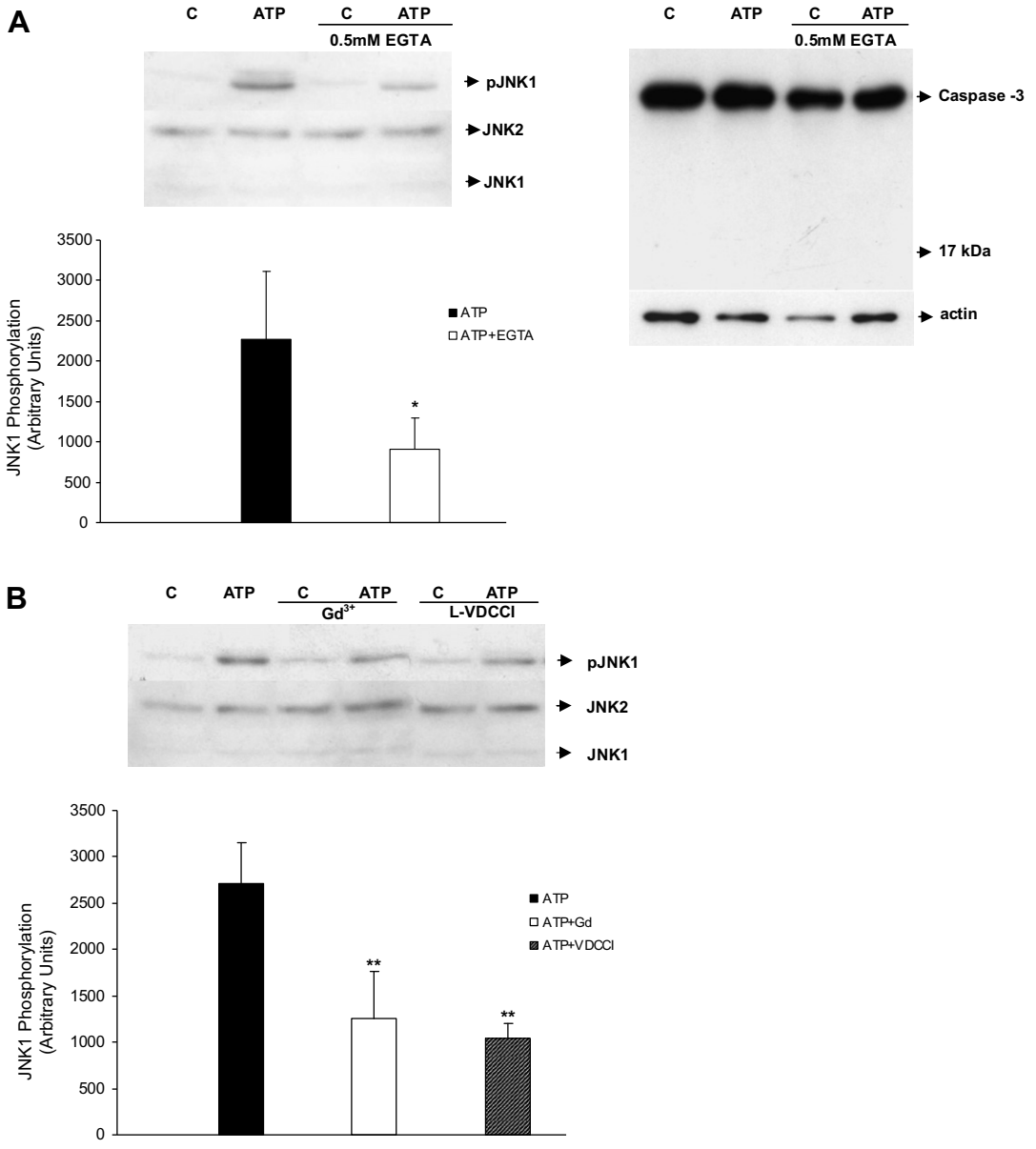

Fig. 4. Participation of extracellular Ca^{2+}, stress activated (SAC) and L-type voltage-dependent (L-VDCC) calcium influx in ATP activation of JNK1 in osteoblast-like cells. ROS-A 17/2.8 cells were incubated in control medium or calcium-free medium containing 0.5 mM EGTA (A) or in 1.5 mM calcium medium in the presence or absence of 2.5 μM Gd^{3+}, SAC inhibitor, or 5 μM nifedipine and verapamil, L-VDCC blockers (L-VDCCI) (B). Then, the cells were stimulated by the addition of either vehicle (C) or 10 μM ATP (ATP) for 5 min. Under these conditions SAC influx is activated by ATP [13]. Cell lysate proteins were immunoblotted with pJNK1/2 phospho-specific antibody as in Fig. 1. Only the 46-kDa isoform is activated by ATP and indicated as pJNK1. Total JNK1/2 antibody was used as loading control. Anti caspase-3 antibody was used to evaluate any apoptotic effects due to EGTA or even ATP, (A) (right blot). The fragment of 17 kDa generated by proteolysis of caspase-3 in apoptotic cells, was not observed. Total anti-actin was used as loading control. Representative immunoblots are shown. Bars show the quantification of the blots expressed as arbitrary densitometry units of JNK1 phosphorylation levels with respect to controls. The results are shown as mean ± SD ($n = 3$). $^{*}p < 0.05$; $^{**}p < 0.01$.

this MAPK was analyzed by Western blot after incubation of osteoblast-like cells with 2.5 mM neomycin, a PI-PLC inhibitor, or with 150 μM 2-APB, an IP_3 receptor (IP_3R) blocker. ATP-dependent JNK1 phosphorylation was completely abolished by cell treatment with neomycin and 2-APB (Fig. 5A and B, respectively). The results support P2Y receptor type/PI-PLC activation followed by IP_3 generation and calcium release from intracellular stores as an essential event of the signal transduction pathway involved in JNK1 phosphorylation by ATP.

The participation of PKC and Src-family of tyrosine kinases as upstream mediators in the ATP-activated JNK1 signaling pathway was studied. JNK1 phosphorylation by ATP was strongly suppressed (83.5 ± 5%) by Ro318220 (1 μM), a known inhibitor of all PKC isoforms (Fig. 5C), and inhibited (50 ± 25%) by PP1 (50 μM), a potent and selective inhibitor of the Src-family of tyrosine kinases (Fig. 5D). These data involve the PKC and Src family members in purinergic activation of JNK1 in ROS-A 17/2.8 cells.

In addition, the inhibitor of p38 MAPK SB203580, completely suppressed p38 activation and reduced JNK1 phosphorylation by ATP almost totally. Although this inhibitor elevated the basal levels of ERK1/2 phosphorylation, it had no effect on ERK1/2 activation by ATP (Fig. 6A). The effect of ERK1/2 inhibition on ATP-induced JNK1 activation was also examined by using two inhibitors of MEK1/2 (the kinases upstream of ERK1/2). As shown in Fig. 6B, PD98059 dose-dependently diminished JNK1 phosphorylation induced by ATP whereas U0126 abolished it. These results may reflect the operation of a cross talk mechanism between the p38/ERK and JNK signaling pathways.

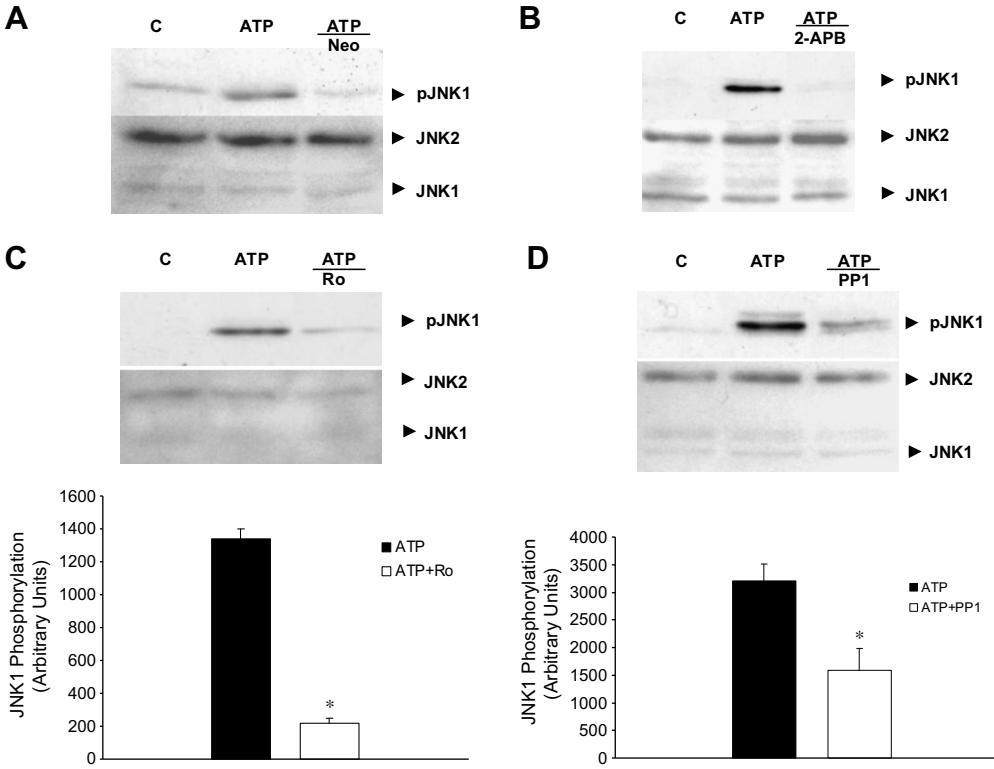

Fig. 5. Role of PLC, PKC and Src in JNK1 phosphorylation by ATP in osteoblast-like cells. ROS-A 17/2.8 cells were preincubated during 10 min in the presence or absence of 2.5 mM neomycin (A), 150 μM 2-APB (B), 1 μM Ro318220 (C) or 50 μM PP1 (D), followed by treatment with 10 μM ATP (ATP) or vehicle (C) during 5 min. Levels of pJNK1 were then measured with a phospho-specific antibody as in Fig. 1. Total JNK1/2 antibody was used as loading control. Representative immunoblots are shown. Bars show the quantification of the blots expressed as arbitrary densitometry units of JNK1 phosphorylation levels with respect to controls. The results are shown as mean ± SD ($n = 3$). $^{*}p < 0.05$.

Finally, the effects of ATP on ROS-A 17/2.8 cell proliferation were evaluated. Fig. 7A shows that cell treatment with 10 μM ATP for 24 and 48 h stimulated [^{3}H]-thymidine incorporation into DNA by 31 ± 8.4% and 39 ± 17.5%, respectively. These results suggest that the purinergic agonist induces proliferation in osteoblastic cells. In order to evaluate the subtype of purinergic receptor that may participate in the proliferative effects of ATP, cell proliferation after treatment of ROS-A 17/2.8 cells with ATPγS, UTP (both agonists at $P2Y_2$ receptor) or ADPβS (agonist at $P2Y_1$ receptor) was evaluated using the MTS assay as described in Methods. Fig. 7B shows that both ATPγS and UTP stimulated osteoblastic cell proliferation. The increments were 139.2 ± 5.3% and 171.7 ± 8.7% over the control for 10 and 50 μM ATPγS, respectively, and 66.7 ± 14.3% for 10 μM UTP. On the other hand, 10 μM ADPβS did not induce significant changes in ROS-A 17/2.8 cell proliferation. Only when a higher dose (five times) of ADPβS was used, a stimulation of [^{3}H]-thymidine incorporation similar to that of 10 μM ATPγS was achieved. These results suggest that $P2Y_2$ receptors participate in the proliferative effects of ATP on ROS-A 17/2.8 osteoblastic cells. The involvement of the JNK1 signaling pathway in such mitogenic action should be addressed in future investigations.

Discussion

The results presented here demonstrate that ATP modulates the c-Jun NH2-terminal kinases (JNKs) in ROS-A 17/2.8 rat osteosarcoma cells. Thus, we extended previous findings reporting the regulation of ERK 1/2 and p38 MAPK pathways by ATP in the same osteoblastic cell line [13]. As in the case of these MAPKs, ATP was able to phosphorylate JNK1 MAPK in a time- and dose-dependent manner in ROS-A 17/2.8 cells. The transient activation of MAPK signaling pathways by extracellular nucleotides through purinergic receptors has been reported in other cell systems. In particular, extracellular ATP was shown to activate JNK signaling and proliferation in hepatocytes of regenerating liver *in vivo* after $P2Y_2$ stimulation [22]. However, activation of the JNK signal transduction pathway by ATP in osteoblasts has not been reported until the present study.

We have observed that JNK2 isoform (54 kDa) expression was greater than JNK1 in all cell types studied (ROS-A 17/2.8, primary osteoblasts, C2C12 and MCF-7 cells). However, unlike in C2C12 and MCF-7 cells, JNK2 was not phosphorylated by ATP at any of the doses or times studied neither in osteoblasts nor in osteoblast-like cells. JNK1 and JNK2 have been considered redundant isoforms, both of which contribute to JNK activity [16]. However, recent studies have suggested that JNK1 and JNK2 also have different functions [23–25]. In particular, in osteoblasts, staurosporine or thapsigargin-induced apoptosis may occur via activation of JNK1, caspase-3-like proteases, and transcriptional factors including AP-1 and NF-κB [26,27]. The function of JNK in apoptosis depends on the cell type, nature of the apoptotic stimulus, duration of its activation, and activity of other signaling pathways [28]. Our results show that ATP exerts proliferative effects in osteoblastic cells and does not induce apoptosis as judged by the experiment showing that caspase-3 was not cleaved after ATP treatment. Accordingly, in tumor cell lines JNK1 activation was necessary to phosphorylate the transcription factor Sp1 and to shield Sp1 from the ubiquitin-dependent degradation pathway during mitosis, thus increasing cell growth rate [29]. Of interest, in mouse fibroblasts it has been shown that JNK1 contributes to c-Jun phosphorylation more than JNK2, especially after cellular stimulation [30].

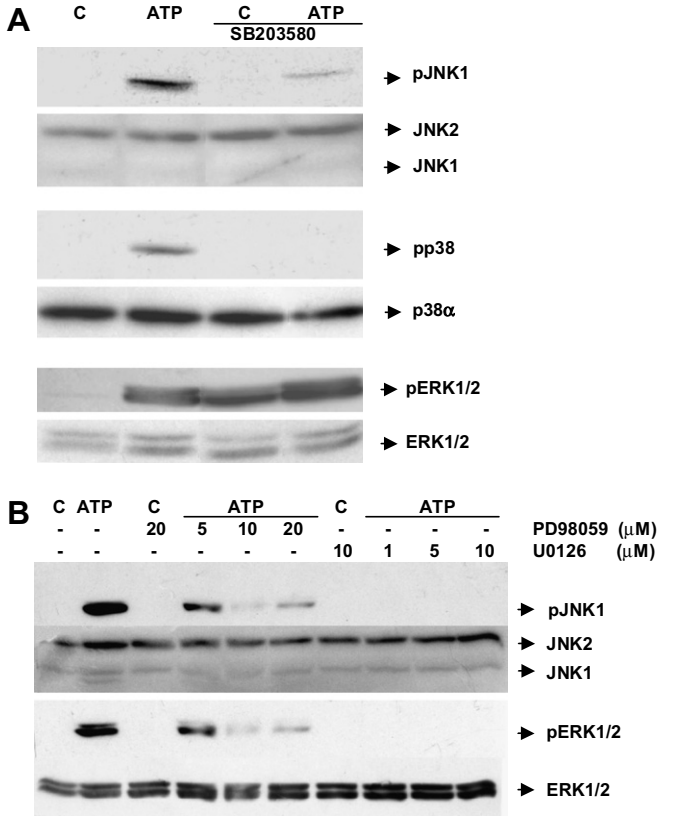

Fig. 6. Effect of p38 and ERK1/2 inhibition on JNK1 phosphorylation by ATP. ROS-A 17/2.8 cells were preincubated during 10 min in the presence or absence of 20 μM SB203580 (A) or during 30 min with PD98059 or U0126 at the concentrations indicated (B) followed by treatment with 10 μM ATP (ATP) or vehicle (C) for 5 min. Cell lysate proteins were immunoblotted with pJNK1/2 phospho-specific antibody as in Fig. 1. Total JNK1/2, p38α and ERK1/2 antibodies were used as loading control. The immunoblots shown are representative of three separate experiments.

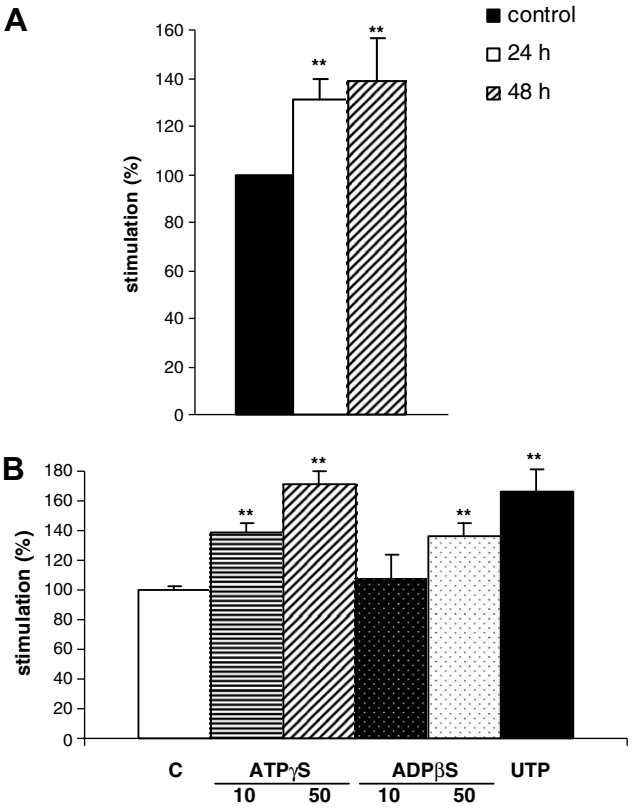

Fig. 7. Effect of ATP on osteoblast-like cell proliferation. ROS-A 17/2.8 cells were treated with 10 μM ATP or vehicle (C) during 24 or 48 h. Then, cellular proliferation was evaluated by measurement of [^{3}H]-thymidine incorporation into DNA as described in Methods (A). ROS-A 17/2.8 cells were treated with vehicle (C), the indicated concentrations of ATPγS and ADPβS or 10 μM UTP during 24 h. Then, cellular proliferation was evaluated by the MTS assay as described in Methods (B). The results are express as % stimulation with respect to the control (mean ± SD ($n = 4$) $^{**}p < 0.01$).

We could not rule out the participation of JNK2 in the physiological actions of ATP. For some MAPKs, the inactive form has been attributed a functional role. Such is the case of inactive JNK2 that in basal cellular conditions binds to the transcription factors ATF2, c-Jun and p53, promoting their proteasomal degradation [31,32]. Future investigations may elucidate the molecular mechanism underlying the different biological functions of JNK isoforms in osteoblast biology.

The stimulation of JNK1 achieved with 10 μM ATP or its non-hydrolysable analogue ATPγS was similar in magnitude, suggesting that not significant hydrolysis of ATP (used in most of the experiments) is taking place under the experimental conditions tested. In addition, UTP — an agonist at the $P2Y_2/P2Y_4$ receptors [4,33]— stimulated JNK1 in a similar manner than ATP. The fact that ADPβS, a potent agonist at the $P2Y_1$ receptor, did not activate JNK1 suggests that this receptor subtype does not mediate JNK1 activation by ATP in osteoblasts. Thus, the pharmacological response profile observed in ROS-A 17/2.8 cells involves $P2Y_2$ and/or $P2Y_4$ receptor subtypes in JNK1 modulation by ATP. The expression of multiple P2Y receptor subtypes in human bone and in osteoblastic cell lines has been reported [34,35]. However, in the case of the osteoblast-like ROS 17/2.8 cell line, it has been suggested that it does not normally express the $P2Y_2$ receptor subtype [36]. Moreover, in rat osteoblasts, $P2Y_4$ receptor mRNA was undetectable [37]. Because of these observations and based on the fact that our stock of cells responds to ATP and UTP with increments in $[Ca^{2+}]_i$ and MAPK phosphorylation, we renamed them as ROS-A 17/2.8 cells [13]. In the present work, we have amplified by RT-PCR a DNA fragment corresponding to the expected size of the $P2Y_2$ purinergic receptor subtype in osteoblast-like ROS-A 17/2.8 cells. It has been reported that ROS 17/2.8 cells undergo phenotypic maturation in culture [38]. Furthermore, it has been shown that primary osteoblasts become more responsive to ATP and express more $P2Y_2$ receptors as they differentiate into mature osteoblasts [39,40]. Then, and according to our results, it is expected that ROS 17/2.8 cells will express $P2Y_2$ receptors in culture. Under our experimental conditions, the $P2Y_1$ or $P2Y_4$ DNA fragments were not amplified by RT-PCR, thus suggesting that $P2Y_2$ is the main receptor subtype expressed in ROS-A 17/2.8 cells and that mediates JNK1 activation by ATP. Of relevance, data on *bona fide* primary osteoblasts described in this study, confirmed key results obtained with the osteoblast-like ROS-A 17/2.8 cell line and provided evidence on the physiological role of ATP-mediated JNK1 phosphorylation through $P2Y_2$ purinergic receptors in osteoblasts.

The participation of extracellular Ca^{2+} in ATP-induced JNK1 activation was evidenced by a significant inhibitory effect achieved after chelation of a nominally Ca^{2+}-free medium with EGTA. Moreover, we observed that gadolinium, an inhibitor of ATP-dependent SAC influx [13] reduced JNK1 phosphorylation by the nucleotide in osteoblastic cells, thus suggesting that ATP-dependent SAC influx participates in JNK1 activation, in a similar manner than we have previously reported for ERK1/2 and p38 MAPKs [13]. In addition, the L-type calcium channel blockers nifedipine and verapamil also reduced ATP-induced MAPK activation. In osteocytes, it has been reported that gadolinium and antisense oligonucleotides against

the α1c subunit of the L-type channel block calcium influx into the stretched cells [41]. Additionally, gadolinium was able to block voltage-dependent calcium channels in pituitary cells, leaving the possibility that the molecular identity of stretch channels may belong to the family of voltage-dependent calcium channels [42].

The participation of $P2Y_2$ type receptor/PI-PLC/IP_3 generation/intracellular Ca^{2+} release in JNK1 phosphorylation by ATP was evidenced by its suppression using neomycin and 2-APB. In accordance with these results, we have previously reported that PI-PLC inhibition by neomycin abolished ATP-dependent intracellular Ca^{2+} release and SAC influx [13]. The relative contribution of extracellular and intracellular Ca^{2+} to ATP stimulation of JNK1 under *in vivo* physiological conditions remains to be established.

In addition, the participation of PKC in the activation of JNK1 was shown in ROS-A 17/2.8 osteoblastic cells. The fact that 2-APB completely abolished ATP phosphorylation of JNK1 suggests that calcium-dependent PKC isoforms may mediate such response.

Moreover, the results presented in this study involve Src family members in purinergic activation of JNK1 in ROS-A 17/2.8 cells. Of relevance, two proline-rich, SH3 binding sites have been recently identified in the carboxyl-terminal tail of the human $P2Y_2$ nucleotide receptor that directly associates with the tyrosine kinase Src [43]. We have previously shown that PKC acts upstream on Src in ATP modulation of the ERK 1/2 and p38 MAPK signaling cascades in osteoblast-like cells [13]. Therefore, the action of PKC and Src inhibitors on JNK1 phosphorylation could be an indirect effect due to inhibition of ERK1/2 or p38 phosphorylation.

Finally, as an interesting finding, compounds SB203580 and PD98059 or U0126 which inhibit p38 and ERK1/2 MAPK, respectively, also inhibited ATP–JNK1 phosphorylation. As an explanation to this finding, several genetic and biochemical studies have evidenced cross talk between different MAPKs. In the case of p38 MAPK, the opposite effect was recently reported with SB220025, another inhibitor of this MAPK, which increased JNK activity in murine macrophages [44]. JNK and p38 are two closely related MAPK pathways having MKK and even scaffold proteins in common [45]. Our results are in line with those reported by Salh et al. [46] showing that H_2O_2-induced JNK activation in an intestinal cell line was inhibited by both PD98059 and UO126 at conventional doses used to inhibit MEK [46]. On the other hand, immunoblot and immunostaining analysis revealed that phosphorylation of ERK was increased by treatment with SB203580; whereas PD98059 increased the phosphorylation of p38, which implies a seesaw-like balance between ERK and p38 phosphorylation in osteoblastic SaOS-2 cells [47]. These experimental observations suggest a complex interaction between different MAPK signaling pathways that should be addressed in future investigations along with experiments employing additional methods to rule out any unspecific effects of the inhibitors.

It is well documented that activation of P2Y receptors in osteoblasts leads to induction of c-*fos*, an immediate early gene that plays a key role in the proliferation and differentiation of bone cells [6]. In addition, ATP activates the transcription factor Runx2, involved in osteoblast cell differentiation [48]. On the other hand, ATP stimulates osteoblast-like cell proliferation and synergistically potentiates different growth factors in inducing osteoblast DNA synthesis [49,50]. In agreement with these findings, we have observed that ATP stimulates ROS-A 17/2.8 osteoblast-like cell proliferation. Currently, we have not determined which MAPK participates in such effect. However, the activation of JNK1 by ATP may be a pro-survival signal that could contribute to well-documented effects of ATP on osteoblast proliferation and differentiation.

Altogether, the results of the present investigations show for the first time, that ATP acting through P2Y receptors, most likely of the $P2Y_2$ subtype, coupled to the PI-PLC/IP_3/intracellular Ca^{2+} release signaling pathway, activates JNK1 but not JNK2 in osteoblasts. In addition, JNK1 modulation by ATP is dependent on stimulation of PKC and Src family kinases and involves L-VDCC and SAC influx. Of potential physiological relevance, this signaling mechanism could play a role in bone remodelling by ATP as this purinergic agonist is able to stimulate osteoblastic cell proliferation [51].

Acknowledgments

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