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Modulation of ERK 1/2 and p38 MAPK signaling pathways by ATP in osteoblasts: Involvement of mechanical stress-activated calcium influx, PKC and Src activation

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Abstract

There is evidence that extracellular nucleotides, acting through multiple P2 receptors, may play an important role in the regulation of bone metabolism by activating intracellular signaling cascades. We have studied the modulation of mitogen-activated protein kinase (MAPK) signaling pathways and its relationship to changes in intracellular calcium concentration ($[Ca^{2+}]_i$) induced by ATP in ROS-A 17/2.8 osteoblastic cells. ATP and UTP (10 μM) increased $[Ca^{2+}]_i$ by cation release from intracellular stores. We have found that when the cells are subsequently subjected to mechanical stress (medium perturbation), a transient calcium influx occurs. This mechanical stress-activated calcium influx (MSACI) was not observed after ADP stimulation, indicating that $P2Y_2$ receptor activation is required for MSACI. In addition, ERK 1/2 and p38 MAPK were activated by ATP in a dose- and time-dependent manner. This activation was almost completely blocked using neomycin (2.5 mM), an inhibitor of phosphoinositide-phospholipase C (PI-PLC), Ro 318220 (1 μM), a protein kinase C (PKC) inhibitor, and PP1 (50 μM), a potent and selective inhibitor of the Src-family tyrosine kinases. Ca^{2+}-free extracellular medium (containing 0.5 mM EGTA) and the use of gadolinium (5 μM), which suppressed MSACI, prevented ERK 1/2 and p38 phosphorylation by ATP. Altogether, these results represent the first evidence to date suggesting that $P2Y_2$ receptor stimulation by ATP in osteoblasts sensitizes mechanical stress activated calcium channels leading to calcium influx and a fast activation of the ERK 1/2 and p38 MAPK pathways. This effect also involves upstream mediators such as PI-PLC, PKC and Src family kinases.

Keywords: ATP; MAPK; Ca^{2+}; Osteoblasts; P2Y receptors

1. Introduction

In the last few years, the biological actions of extracellular nucleotides have been studied in many cell and tissue types. There is increasing evidence that adenosine triphosphate (ATP) may function as an important local messenger in bone acting through P2 receptors. P2 receptors are divided into the P2X receptor family, which are ligand-gated nonselective ion channels, and the P2Y receptor family, which are G-protein-coupled receptors. At least seven P2X subtypes have been cloned and characterized, and evidence for about 11 P2Y subtypes has been found in vertebrates (Dalziel & Westfall, 1994; Ralevic & Burnstock, 1998). The main signal transduction pathway of P2Y activation involves phospholipase C, which leads to generation of IP_3 and a transient rise of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Bowler et al., 1999). It has been reported that P2Y receptors

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are expressed in osteoblasts of rat and human origin (Hoebertz, Townsend-Nicholson, Glass, Burnstock, & Arnett, 2000; Maier, Glatz, Mosbacher, & Bilbe, 1997). Several studies have demonstrated that extracellular nucleotides increase cell proliferation and differentiation through specific P2 purinoceptors. In various cell types, including osteoblasts, modulation of mitogen-activated protein kinase (MAPK) signaling pathways has been implicated in the regulation of cell proliferation and differentiation as well as in other cellular responses triggered by extracellular stimuli (Zarubin & Han, 2005). 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 (Zarubin & Han, 2005). Particularly, ERKs are involved in cell proliferation/transformation and survival whereas p38 MAPKs participate in many cellular processes, such as inflammatory responses, cell cycle control, apoptosis, and differentiation of several cell types including osteoblasts (Hu, Chan, Wang, & Li, 2003).

Several studies on the modulation of MAPK signal transduction pathways have been reported for osteoblasts; however, the action of ATP on MAPKs in these cells has been poorly studied.

Mechanical stimulation plays an important role in regulating bone metabolism. However, the mechanism by which bone cells sense and respond to mechanical stimulation is not well understood. Available evidence suggests that osteoblasts recognize mechanical or physical stress stimulation and transduce it into signals which, in turn, lead to cellular responses that involve changes in cell proliferation, differentiation, survival and gene expression (Koike, Shimokawa, Kanno, Ohya, & Soma, 2005; Yuge et al., 2003). It has been demonstrated that mechanical stimuli activate a variety of molecules known to function in signal transduction, including ion channel and G proteins, inositol trisphosphate, and many protein kinases, suggesting that multiple pathways are involved in mechanical stress signal transduction. A growing body of evidence shows that extracellular nucleotides released from the cells by mechanical stimulation are responsible for the conversion of mechanical forces into biochemical signals. Thus, it has been observed that mechanical stimulation in osteoclasts induces endogenous ATP release leading to activation of calcium influx through P2X receptors (Naemsch, Dixon, & Sims, 2001). Furthermore, studies with astrocytes have shown that stretch causes ATP release from the cells leading to activation of P2X and P2Y receptors and, in turn, of the ERK pathway (Neary, Kang, Willoughby, & Ellis, 2003). It has recently been reported, on the other hand, that by using oscillatory fluid flow, $P2Y_2$ receptors mediate mechanical stimulation-induced intracellular Ca^{2+} mobilization in osteoblasts (You, Jacobs, Steinberg, & Donahue, 2002).

In vitro experiments have shown that mechanical stimulation has a number of effects on bone cells, including an increase in $[Ca^{2+}]_i$, MAPK activation and modulation of gene expression (Simmons et al., 2003; Tanaka et al., 2005; Yuge et al., 2003). MAPK family members have shown to be important signaling components linking mechanical stimuli to cellular responses, including cell growth, differentiation, and metabolic regulation in various cell types. However, the sequential order of events mediating such responses remains unclear. Also, the initial sensor that recognizes mechanical stress has not been identified yet.

In the present work we have studied ATP modulation of intracellular Ca^{2+} and MAPK ERK 1/2 and p38 in the rat osteoblastic cell line ROS-A 17/2.8. Our data suggest that $P2Y_2$ receptors/PI-PLC stimulation by ATP in osteoblasts sensitizes mechanical stress activated channels which lead to calcium influx from the extracellular space and activation of p38 and ERK 1/2 signaling pathways. This effect also involves upstream mediators such as PKC and Src family kinases, and MKK3/6 in the case of p38 MAPK.

2. Materials and methods

2.1. Materials

Fura-2/pentaacetoxymethyl ester (Fura-2/AM), nifedipine, verapamil, neomycin sulfate, ATP, UTP, ADP, Ham's F-12 medium and $GdCl_3$ were from Sigma Chemical Co. (St. Louis, MO, USA). Ro 31-8220 was from Calbiochem (San Diego, CA, USA). PP1 was from BIOMOL (Plymouth Meeting, PA, USA). Fetal bovine serum (FBS) was from Natocord (Córdoba, Argentina).

Monoclonal antibodies recognizing dually phosphorylated ERK 1/2 (Thr202, Tyr204) (1:1500) and polyclonal anti phospho-Src (Tyr416) (1:300) antibody were from Cell Signaling Technology (Beverly, MA, USA). Polyclonal antibodies recognizing total ERK 1/ERK 2 (1:1000) were provided by New England Bio Labs (Beverly, MA, USA). Polyclonal antibodies recognizing dually phosphorylated p38 (Thr180, Tyr182) (1:1000) and MKK3/6 (Ser 189/207) (1:1000), polyclonal antibody against p38 α (1:1000), monoclonal anti-c-Src (1:500) antibody, and goat anti-rabbit and anti-mouse peroxidase-conjugated secondary antibodies, were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rhod (TRITC) AffiniPure Donkey Anti-Mouse IgG

(H + L) (1:200) was from Jackson ImmunoResearch (West Grove, PA, 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.

2.2. Cell culture

The rat osteoblastic cell line ROS 17/2.8 (rat osteosarcoma-derived) was provided by Dr. Irina Mathow (Facultad de Farmacia y Bioquímica-IDEHU, Universidad de Buenos Aires, 1113 Buenos Aires, Argentina). The cell line was renamed ROS-A 17/2.8 alluding to our finding that it does express a $P2Y_2$ receptor (see Section 3). It was seeded at a density of 10,000 cells/cm^2 and cultured at 37 °C in Ham's F-12 medium containing 10% FBS under humidified air (5.5% CO_2). After 48 h, the medium was replaced by Ham's F-12 without FBS and the cells were starved for 18–21 h before Western blot analysis. For intracellular calcium measurements and immunocytochemistry, cells were plated onto glass coverslips and allowed to grow until confluence (2–3 days after plating) before use.

2.3. Western blot analysis

ROS-A 17/2.8 cells were treated with ATP, UTP or ADP both in the presence and the absence of the calcium channel blocker Gd^{3+} or inhibitors (suramin, neomycin, compounds Ro318220 and PP1). When the treatment was performed in a Ca^{2+}-free buffer, the medium was replaced by buffer C (see below) 15 min before cell treatment. 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 14,000 × *g* and 4 °C during 15 min the supernatant was collected and proteins were quantified by the Bradford method (Bradford, 1976). Lysate proteins dissolved in 6× Laemmli sample buffer were separated (30 μg/lane) by 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% (or 5%) non-fat milk. After washing, the membranes were incubated with the appropriate dilution of horse radish peroxidase-conjugated secondary antibody in TBST plus 1% (or 5%) 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).

2.4. 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.

2.5. Intracellular calcium measurements

Intracellular Ca^{2+} changes in osteoblastic cells were monitored by using the Ca^{2+}-sensitive fluorescent dye Fura-2 as previously described (Santillán, Baldi, Katz, Vazquez, & Boland, 2004a; Santillán, Katz, Vazquez, & Boland, 2004b). Cells grown onto glass coverslips were loaded with 1 μM of Fura-2/AM in buffer A containing (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 room temperature (20–25 °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 for 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. For measurements in Ca^{2+}-free extracellular medium a buffer C (buffer B without Ca^{2+} plus 0.5 mM EGTA) was used. When purinergic antagonist,

Ca^{2+} channel or PI-PLC inhibitors were used, they were added to the medium 3–5 min before the measurements.

2.6. Mechanical stimulation

Cells grown onto coverslips were subjected to mechanical stimulation by perturbation of the medium during intracellular calcium measurements. For this purpose, an amount of vehicle equivalent to the volume of agonist was added in each case. Mechanical stimulation was performed before and after treatment of the cells with the agonists.

2.7. Immunocytochemistry

ROS-A 17/2.8 cells grown onto glass coverslips were fixed in methanol (at −20 °C) or paraformaldehyde (at room temperature) for 10 min and in the later case, permeabilized with 0.05% Triton X-100. Non-specific sites were blocked with PBS, 5% BSA. Samples were then incubated with the appropriate primary antibody prepared in PBS, 2% BSA (1:50, 1 h, room temperature). After washing with PBS, the samples were incubated with secondary TRITC- (or FITC, not shown) conjugated antibody (1:200, 1 h, room temperature). The samples were examined using a Zeiss LSM 5 Pascal confocal laser microscope.

2.8. Statistical analysis

Statistical significance of data was evaluated using Student's *t*-test (Snedecor & Cochran, 1967) and probability values below 0.01 ($P < 0.01$) were considered highly significant. Quantitative data are expressed as means ± standard deviation (S.D.) from the indicated set of experiments.

3. Results

To determine whether ERK 1/2 and p38 MAPKs are modulated by ATP in ROS-A 17/2.8 osteoblastic cells, we first performed dose- and time-response studies (Fig. 1). Serum-starved osteoblasts were treated with 1–100 μM ATP for 5 min. We found that ERK 1/2 was activated in a dose-dependent manner up to 100 μM ATP, whereas the activation of p38 reached the maximum at 10 μM ATP. The time-response study showed maximum phosphorylation levels of MAPKs within 5 min of treatment with 10 μM ATP.

We next evaluated the participation of extracellular Ca^{2+} in the activation of both MAPKs by ATP. Incubation of cells during 15 min with a calcium-free buffer

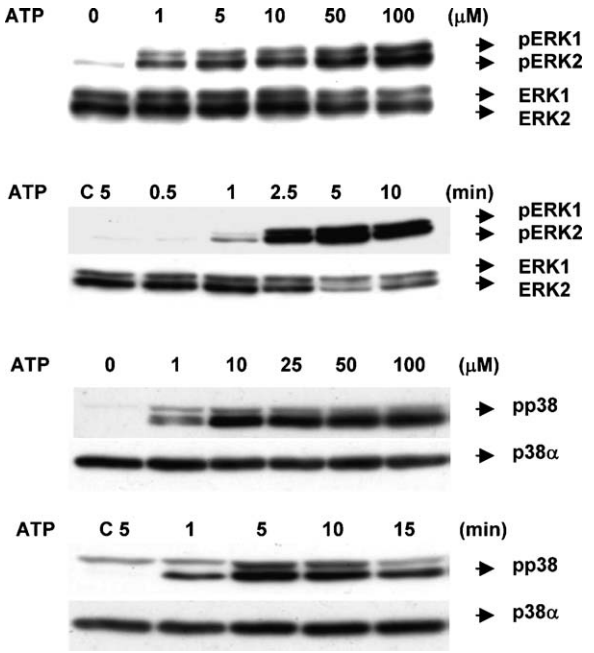

Fig. 1. Dose- and time-response of changes in ERK 1/2 and p38 MAPK activation (phosphorylation) in response to ATP in osteblasts. Serum-starved (21 h) ROS-A 17/2.8 osteoblast-like cells were treated with 1–100 μM ATP during 5 min or with 10 μM ATP during 0.5–15 min. Subsequently, a Western blot analysis was performed as described in Section 2 employing antibodies that recognize the active (phosphorylated) forms of ERK 1/2 (pERK 1/2) and p38 (pp38). Controls using vehicle instead of ATP are indicated as 0 in the dose-response and C5 (control during 5 min) in the time-response study. Antibodies directed against the total form (active plus inactive) of ERK 1/2 and p38 (p38 α) were used as loading control. The results are representative of at least three experiments performed independently.

containing 0.5 mM EGTA caused a strong inhibition of ERK 1/2 and p38 phosphorylation by ATP (Fig. 2). This experiment demonstrates the participation of extracellular calcium in ATP stimulation of MAPKs. Cell viability

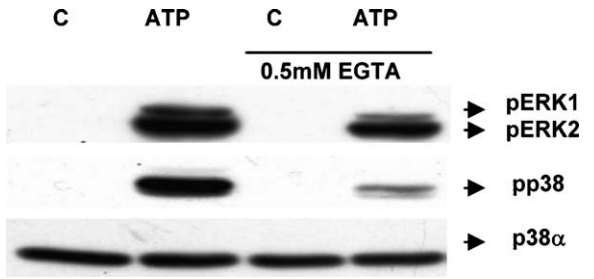

Fig. 2. Participation of extracellular calcium in ATP activation of ERK 1/2 and p38 in osteoblasts. ROS-A 17/2.8 cells were preincubated for 15 min in control medium or calcium-free medium with 0.5 mM EGTA. The cells were stimulated by the addition of either vehicle (C) or 10 μM ATP (ATP) for 5 min. Cell lysate proteins were immunoblotted with pp38 and pERK phospho-specific antibodies as in Fig. 1, with anti-p38 α as a loading control. The immunoblot shown is representative of three separate experiments.

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 further correlate MAPK activation with changes in $[Ca^{2+}]_i$ levels induced by ATP, we studied the effects of ATP on modulation of $[Ca^{2+}]_i$ by spectrofluorimetric measurements in Fura-2 loaded cells. It has been reported that in osteoblast cells, a variety of P2Y purinergic receptor types are expressed (Maier et al., 1997). Thus, to obtain information on the possible subtype of purinergic receptor that activates ERK 1/2 and p38, the action of other purinergic agonists on osteoblast $[Ca^{2+}]_i$ was also evaluated. We found that the cells responded similarly when they were stimulated with 10 μM ATP or UTP, whereas ADP was observed to elicit a weaker response. A rapid and transient increment in $[Ca^{2+}]_i$ was observed upon stimulation with the purinergic agonists (Fig. 3A), thus supporting the notion that more than one P2Y receptor subtype is expressed in ROS-A 17/2.8 cells. Studies on rat osteoblast-like cells have demonstrated that extracellular nucleotides interact with at least two receptor subtypes pharmacologically characterized as $P2Y_1$- and $P2Y_2$-like receptors (Yu & Ferrier, 1993; Sistare, Rosenzweig, Contrera, & Jordan, 1994; Reimer & Dixon, 1992). However, it is generally accepted that ROS 17/2.8 cells do not express a $P2Y_2$ receptor, as reported by Jorgensen, Geist, Civitelli, and Steinberg (1997). The response to purinergic agonists of the ROS 17/2.8 cell line employed in the present study was characteristic of $P2Y_1$, $P2Y_2$ and $P2Y_4$-like receptors. $P2Y_4$ is weakly expressed in rat osteoblasts (Hoebertz et al., 2000), and thus we assume that our stock of ROS 17/2.8 cells expresses at least the $P2Y_1$ and $P2Y_2$ receptor subtypes and we designate it as ROS-A 17/2.8 to differentiate it from $P2Y_2$ receptor-deficient ROS 17/2.8 stocks.

When the $[Ca^{2+}]_i$ measurements were carried out in a Ca^{2+}-free medium (plus 0.5 mM EGTA) no significant differences were observed (Fig. 3B), indicating that the release of Ca^{2+} from intracellular stores is the main component of the Ca^{2+} response to purinergic agonists. These results are apparently in contradiction with the extracellular calcium-dependent activation of ERK 1/2 and p38 by ATP described before (see Fig. 2). However, in parallel we observed that after ATP stimulation and once $[Ca^{2+}]_i$ returned to basal levels, mechanical perturbation by the addition of vehicle to the recording chamber induced a second $[Ca^{2+}]_i$ transient increase. This second rise in $[Ca^{2+}]_i$ was not observed in a free-calcium buffer (plus 0.5 mM EGTA) suggesting that it is caused by calcium influx from the extracellular space (Fig. 3B). Moreover, the application of such mechanical stress prior to

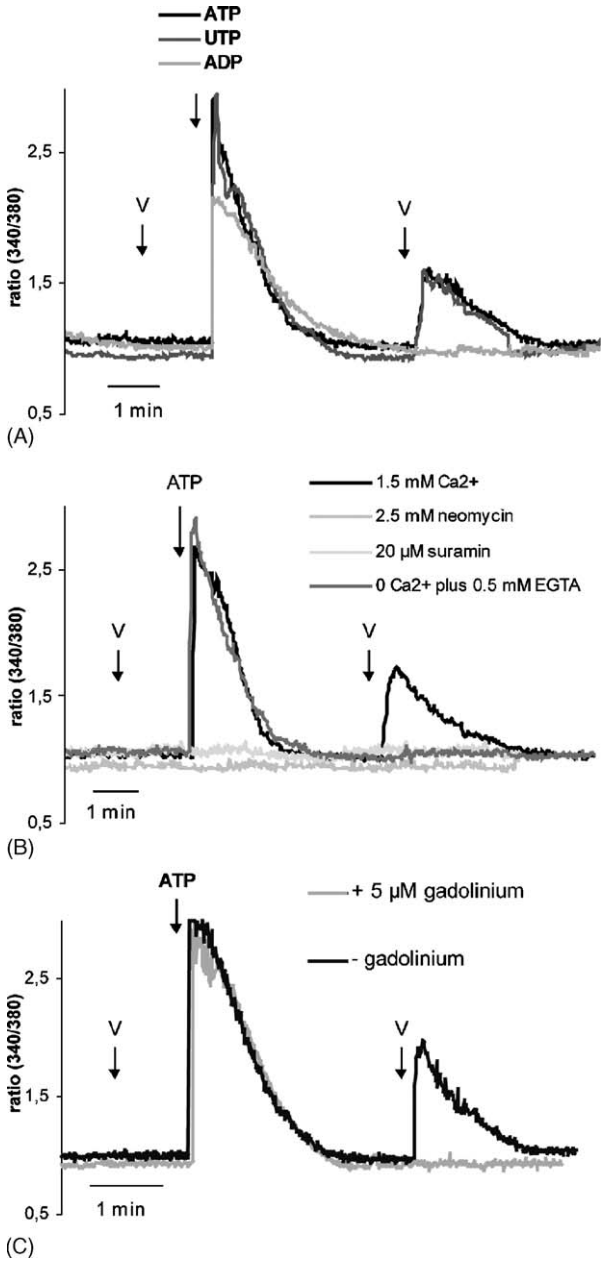

Fig. 3. Effects of extracellular nucleotides ATP, UTP and ADP on osteoblast $[Ca^{2+}]_i$ levels and Ca^{2+} influx stimulation by mechanical stress. Fura-2-loaded osteoblast-like ROS-A 17/2.8 cells incubated in 1.5 mM Ca^{2+} or in a Ca^{2+}-free medium were stimulated with 10 μM of either ATP, UTP or ADP (second arrow), and $[Ca^{2+}]_i$ was measured as described in Section 2. Mechanical stress was induced by addition of vehicle, before (first "V") and after (second "V") purinergic stimulation (A). Alternatively, the cells were preincubated 5–10 min with 20 μM suramin or 2.5 mM neomycin (B), or 5 μM Gd^{3+} (C) followed by nucleotide addition. The figure shows time traces representative of at least five independent experiments, each performed onto no less than 40–50 cells.

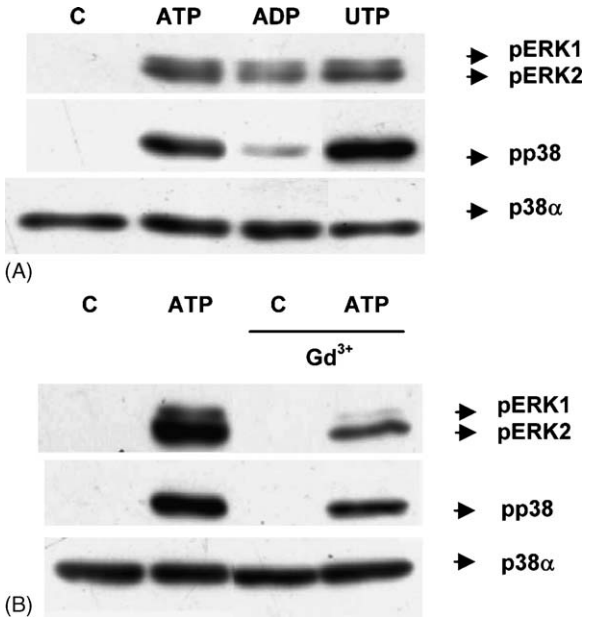

Fig. 4. Involvement of $P2Y_2$ receptor and mechanical stress-activated calcium influx (MSACI) in the activation of ERK 1/2 and p38 MAPKs by ATP in osteoblasts. (A) ROS-A 17/2.8 osteoblastic cells were treated with 10 μM ATP, ADP or UTP, for 5 min, or (B) were first incubated in the absence or presence of 5 μM Gd^{3+} for 2 min before vehicle (C) or 10 μM ATP (ATP) treatment. Levels of pERK 1/2 and pp38 MAPKs were then measured with phospho-specific antibodies as in Fig. 1. Anti-p38 α antibody was used as loading control. The results are representative of at least three experiments performed independently.

purinergic activation did not affect $[Ca^{2+}]_i$ (Fig. 3A–C). So, we hypothesized that this ATP-dependent mechanical stress-activated calcium influx (MSACI) is involved in ATP induced ERK 1/2 and p38 phosphorylation. As shown in Fig. 3A, MSACI was observed only when the cells were prestimulated with 10 μM ATP or UTP but not with ADP. Similar results were obtained with primary cultures of osteoblasts isolated from neonatal rat calvariae (not shown). The use of 20 μM suramin, a non-specific purinergic antagonist, and 2.5 mM neomycin, a PI-PLC inhibitor, suppressed both calcium release from intracellular stores and MSACI by ATP, supporting a role for PI-PLC purinergic activation in MSACI (Fig 3B). The employment of 5 μM Gd^{3+} blocked MSACI induced by ATP (Fig. 3C). Altogether, these results suggest that activation of a $P2Y_2$ receptor subtype sensitizes mechanical stress activated calcium channels leading to calcium influx which participates in the stimulation of MAPKs.

Marked MAPK activation was observed after cell treatment with 10 μM ATP or UTP but no significant p38 and only moderate ERK 1/2 changes in phosphorylation were detected in the case of 10 μM ADP (Fig. 4A). Furthermore, ADPßS (10–100 μM), potent agonist of the $P2Y_1$ receptor, did not stimulate phosphorylation of p38, whereas a very weak signal was observed in the case of ERK 1/2 (data not shown). These results suggest that $P2Y_2$ receptors are the main P2Y receptor subtype involved in MAPK stimulation by ATP.

In order to establish a stronger link between MSACI and the activation of ERK 1/2 and p38 by ATP, we studied the effect of a MSACI blocker on MAPK activation by ATP. Pretreatment of ROS-A 17/2.8 cells with 5 μM Gd^{3+} strongly inhibited the phosphorylation of ERK 1/2 although it had a lesser effect on phosphorylation of p38 induced by ATP (Fig. 4B), suggesting again the participation of MSACI in the activation of both MAPKs by ATP.

At all conditions tested, the cells were subjected to the same mechanical stress due to medium perturbation as a consequence of vehicle or agonist addition to culture dishes. In control conditions the levels of phosphorylated ERK 1/2 and p38 were negligible, suggesting that mechanical stress alone does not activate MAPKs.

The use of 2.5 mM neomycin also inhibited the activation of ERK 1/2 and p38 by ATP, a more robust inhibition being observed in the case of ERK 1/2 (Fig. 5A). We then studied the participation of upstream modulators of MAPK signaling pathways in the activation of ERK 1/2 and p38 by ATP. The PKC inhibitor Ro318220 (1 μM) suppressed ERK 1/2 and p38 activation by ATP (Fig. 5A), involving PKC in purinergic activation of MAPKs in osteoblasts. When PP1 (50 μM), a potent and selective inhibitor of the Src-family tyrosine kinases was employed, a complete inhibition of ATP-activated ERK 1/2 was observed, whereas phosphorylation of p38 was affected to a lesser extent (Fig. 5A). Thus, this result suggests the involvement of Src in MAPK activation by ATP. In addition, the phosphorylation state of Src (Tyr416), involved in Src activation, increased after cell treatment with ATP. The stimulation of Src (Tyr416) phosphorylation by ATP was blocked by Ro318220 (Fig. 5B), showing that PKC is an upstream mediator in the modulation of MAPK signaling cascade by ATP in osteoblasts.

Immunocytochemical studies employing an antibody that recognizes members of the Src-family tyrosine kinases (actives and inactives), showed that ATP causes an increase in the amounts of Src with perinuclear and nuclear location and a decrease in the cytoplasm (Fig. 6). Again, this result involves the participation of Src in the activation of ERK 1/2 and p38 and, interestingly, suggests its translocation to the nucleus in mediating the signal initiated by ATP.

It is well established that MKK3/6 is the MAPKK implicated in the activation of p38, therefore we evalu-

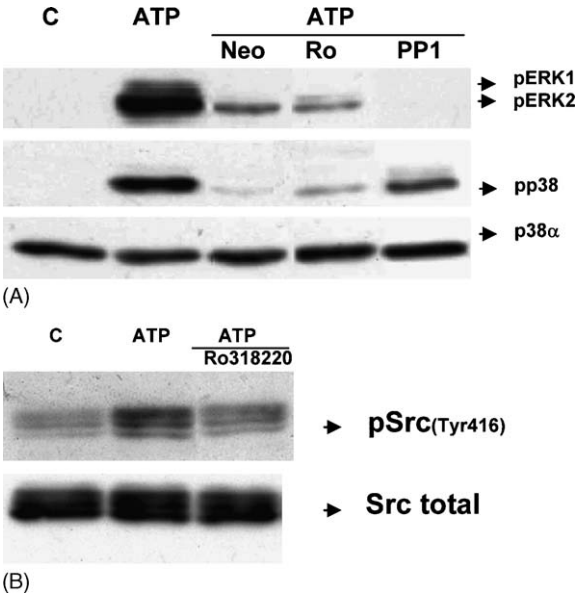

Fig. 5. Participation of PLC, PKC and Src in MAPK modulation by ATP in osteoblasts. (A) ROS-A 17/2.8 cells were incubated without (C) or with 10 μM ATP (ATP) for 5 min. Alternatively, osteoblasts were preincubated during 10 min with 2.5 mM neomycin, 1 μM Ro318220 or with 50 μM PP1, followed by treatment with 10 μM ATP (ATP). Levels of pERK 1/2 and pp38 MAPKs were then measured with phospho-specific antibodies as in Fig. 1. Anti-p38 α-antibody was used as loading control. (B) A 1 μM Ro318220 was added 10 min before cell treatment with 10 μM ATP (ATP). Src activation was evaluated using anti-pSrc (Tyr416) antibody. Anti-Src total was used as loading control. A representative immunoblot from at least three separate experiments is shown.

ated whether MKK3/6 is activated by phosphorylation after ATP treatment of osteoblastic cells. As shown in Fig. 7, MKK3/6 was phosphorylated by the purinergic agonist. Moreover, the use of PP1 reduced the MKK3/6 phosphorylation induced by ATP.

Altogether, these results suggest that activation of P2Y receptors, presumably of the $P2Y_2$ type, sensitizes mechanical stress-activated channels in a PI-PLC-dependent manner, leading to calcium influx and activation of ERK 1/2 and p38. In addition, they involve the PKC and Src family kinases (also MKK3/6 in the case of p38) as upstream mediators in the modulation of MAPKs by ATP in osteoblasts.

4. Discussion

The present work investigated the modulation of the ERK 1/2 and p38 MAPK pathways by ATP in osteoblast-like ROS-A 17/2.8 cells, which exhibit purinergic agonist responses characteristic of $P2Y_1$ and $P2Y_2$ subtype receptors. The major finding of our study is that activation of P2Y receptors, presumably the $P2Y_2$ subtype, sensitizes mechanical stress-activated Ca^{2+} channels in a PI-PLC dependent manner, leading to calcium influx and stimulation of ERK 1/2 and p38 MAPK.

As in other cell types, we found that ROS-A 17/2.8 cells responded to extracellular nucleotides with an elevation of $[Ca^{2+}]_i$ due to stimulation of specific purinergic membrane receptors. This receptor specificity was supported by the fact that cells responded to ATP = UTP > ADP and by blocking the purinergic Ca^{2+} response with neomycin, a PI-PLC inhibitor, and suramin, a nonspecific purinergic antagonist which also disrupts receptor-G protein coupling by blocking the association of G protein alpha and beta-gamma subunits (Chung & Kemode, 2005). These patterns of responses and their pharmacological profile are consistent with activation of the P2Y receptor subfamily (Communi, Janssens, Suarez-Huerta, Robaye, & Boeynaems, 2000; Ralevic & Burnstock, 1998). We observed similar increments of $[Ca^{2+}]_i$ that reached a peak and then returned to basal levels after ROS-A 17/2.8 cell stimulation with ATP both in Ca^{2+}-free (plus 0.5 mM EGTA) or in 1.5 mM Ca^{2+} medium. These data suggest that Ca^{2+} release from inner stores as a consequence of PI-PLC activation is the main component in the Ca^{2+} response to ATP, this agrees with previous results obtained with rat osteoblastic UMR-106 cells in which ATP elevates $[Ca^{2+}]_i$, through mobilization of Ca^{2+} from intracellular compartments but not through Ca^{2+} influx (Kumagai, Sakamoto, Guggino, Filburn, & Sacktor, 1989). Moreover, we observed that mechanical stimulation after ATP or UTP, but not ADP, treatment of osteoblasts activates Ca^{2+} influx from the extracellular space. This is in line with our observation, expressed above, that stimulation of purinergic receptors from the $P2Y_2$ subtype sensitizes mechanical stress-activated Ca^{2+} channels. In agreement with our data, it has been previously reported that activation of $P2Y_2$ receptors is responsible for oscillatory fluid flow-induced changes in intracellular Ca^{2+} (You et al., 2002). However that study did not elucidate whether the inner stores or the extracellular calcium compartment are involved in the response. Thus, our result clarifies this aspect, showing that mechanical perturbation after $P2Y_2$ activation induces calcium influx through gadolinium sensitive stretch channels. Considering that expression of multiple P2Y receptor subtypes in human bone and in osteoblastic cell lines has been reported (Bowler, Gallagher, & Bilbe, 1998; Maier et al., 1997), definite characterization of the subtype of receptor involved will be greatly improved upon cloning of purinergic receptor isoforms expressed in osteoblast-like ROS-A 17/2.8 cells.

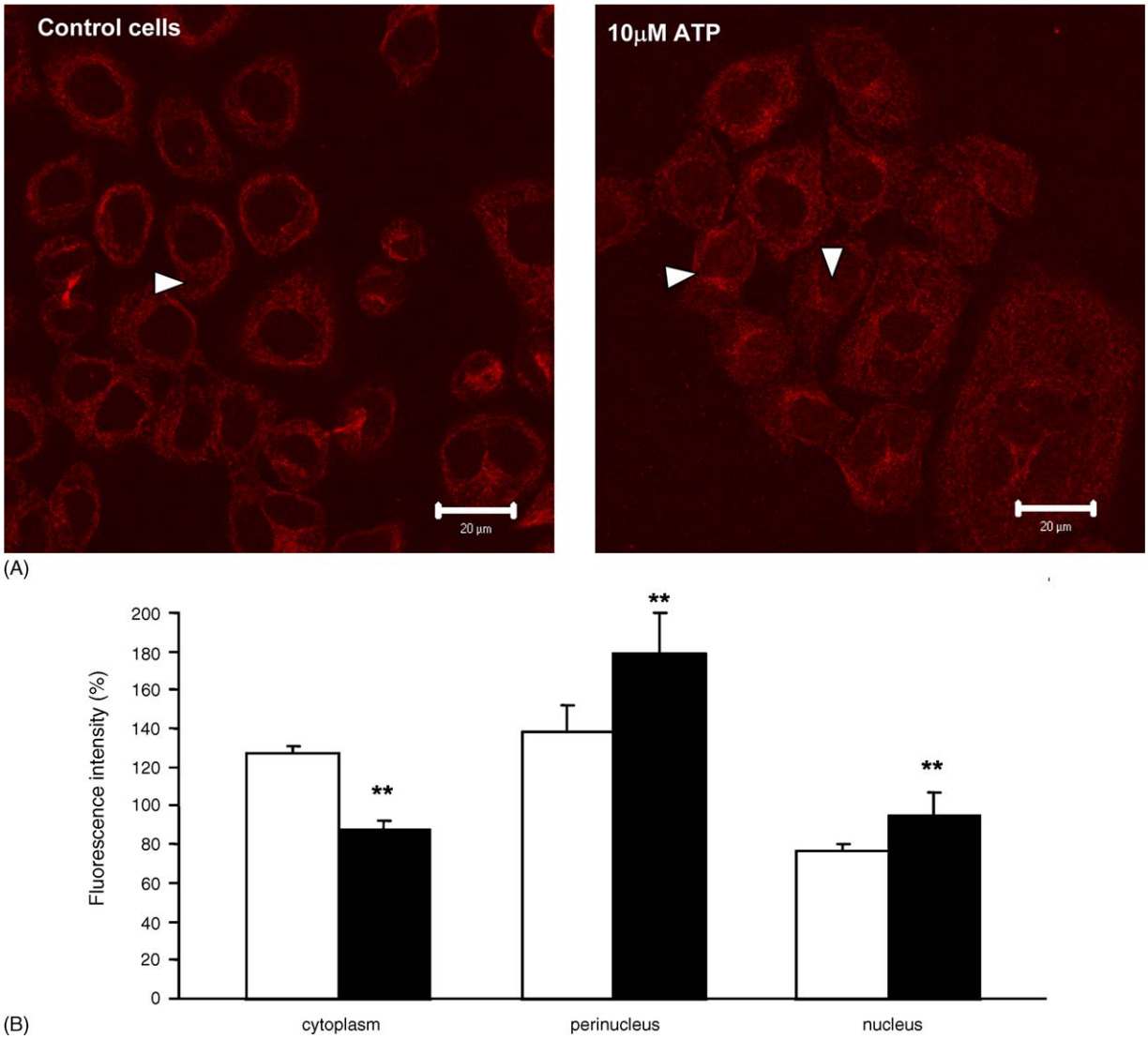

Fig. 6. Effects of ATP on the subcellular distribution of Src in osteoblasts. (A) Immunocytochemical studies in ROS-A 17/2.8 cells were performed employing an antibody that recognizes members of Src-family tyrosine kinases (active and inactive forms). Control cells treated only with vehicle show a clear cytosolic distribution of Src indicated by the arrow (left image). Osteoblasts treated with 10 μM ATP for 5 min show a condensed perinuclear and nuclear distribution of Src as indicated by the arrows (right image). (B) The vertical bar chart shows fluorescence quantification of nuclear, perinuclear and cytoplasmic regions of control (white bars) and ATP-treated cells (black bars). To quantify fluorescence, the summed pixel intensity was calculated by delimiting each region per cell using ImageJ software. Relative values of fluorescence were obtained and expressed as a percentage of fluorescence intensity. The results are shown as mean ± S.D. ($n = 20$). $^{**}P < 0.01$, with respect to the control.

MAPK activity plays a pivotal role in a variety of cell functions. It has been shown to be modulated by diverse external stimuli such as growth factors, cytokines and physical stresses (ultraviolet radiation, hyperosmolarity, hypoxia and mechanical stress). Our study on ERK 1/2 and p38 MAPKs modulation by ATP showed that purinergic activation rapidly stimulates MAPK phosphorylation in osteoblasts. ATP-induced MAPK activation reached a peak at 5 min and remained elevated for at least 15 min. In our report we suggest for the first time that this activation is correlated with ATP-dependent mechanical stress-activated Ca^{2+} influx. We found that gadolinium and extracellular Ca^{2+} chelation strongly inhibited ATP-dependent ERK 1/2 and p38 MAPK activation. This result supports the participation of Gd^{3+}-mechanosensitive Ca^{2+} channels in MAPK activation by ATP. In agreement with these data, several studies have reported ERK 1/2 and p38 MAPK activation upon mechanical stimulation of the cells, including osteoblasts (Inoue, Kido, & Matsumoto, 2004; Kumar,

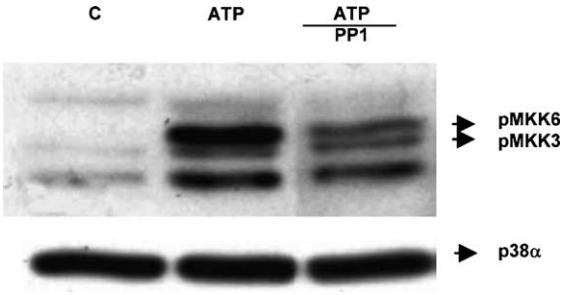

Fig. 7. Involvement of MKK3/6 in p38 activation by ATP in osteoblasts. Phosphorylation levels of MKK3/6 (pMKK3/6) were evaluated after treatment of ROS-A 17/2.8 cells with vehicle (C) or 10 μM ATP (ATP) for 5 min by Western blot analysis using a specific antibody against pMKK3/6. Additionally, 50 μM PP1 was used for 10 min before ATP treatment and then pMKK3/6 was studied. Anti-p38 α antibody was used as loading control. A representative immunoblot from at least two separate experiments is shown.

Knox, & Boriek, 2003; You et al., 2001; Yuge et al., 2003). In some of these investigations and in accordance with our work, stretch-activated, gadolinium-sensitive Ca^{2+} channels (Kamkin et al., 2003), have been implicated (Inoue et al., 2004; Kumar et al., 2003). However, a link between MAPK modulation by ATP and MSACI dependence has not been shown until the present work. An explanation for mechanical stress-induced cellular responses has been the release of nucleotides from the cells acting as mediators of mechanotransduction signaling. However, when mechanical stress was applied in the absence of ATP (control condition), we noted that MAPK phosphorylation was negligible, suggesting that under our experimental conditions the release of nucleotides to the medium was not sufficient to achieve an effect on MAPK activation. As the results show, extracellular Ca^{2+} dependency of ATP-induced MAPK activation was not complete (Fig. 2), suggesting that intracellular Ca^{2+} release or Ca^{2+}-independent mechanisms may also be involved.

The use of Ro318220, a selective PKC inhibitor, blocked MAPK as well as c-Src (Tyr_{416}) and MKK3/6 phosphorylation (not shown) induced by ATP, supporting the involvement of PKC as an upstream mediator in MAPK cascade activation. Interestingly, and as a novel finding, our data showed ATP-induced translocation of c-Src from cytosol to nucleus, which correlates with the main intranuclear location of p-p38 upon ATP cell stimulation (not shown).

In summary, we hypothesize that under certain stress conditions that lead to release of nucleotides from the cells, the stimulation of specific purinergic receptors such as $P2Y_2$, as postulated here, sensitizes mechanical stress activated Ca^{2+} channel through a mechanism that involves PI-PLC activation. Thus, our results provide fundamental information on the signaling mechanism that leads to ERK 1/2 and p38 MAPK activation by ATP in osteoblasts, and establish a possible molecular link between mechanical stress and the osteoblast response.

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