Archives of Biochemistry and Biophysics 513 (2011) 144–152

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Activation of the PI3K/Akt signaling pathway through $P2Y_2$ receptors by extracellular ATP is involved in osteoblastic cell proliferation

Sebastián Katz, Victoria Ayala, Graciela Santillán *, Ricardo Boland *

Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan 670, (B8000ICN) Bahía Blanca, Argentina

ARTICLE INFO

Article history:
Received 14 April 2011
and in revised form 28 June 2011
Available online 6 July 2011

Keywords:
ATP
$P2Y_2$ receptors
PI3K/Akt
Osteoblast proliferation
Calmodulin
PKC

ABSTRACT

We studied the PI3K/Akt signaling pathway modulation and its involvement in the stimulation of ROS 17/2.8 osteoblast-like cell proliferation by extracellular ATP. A dose- and time-dependent increase in Akt-Ser 473 phosphorylation (p-Akt) was observed. p-Akt was increased by ATPγS and UTP, but not by ADPβS. Akt activation was abolished by PI3K inhibitors and reduced by inhibitors of PI-PLC, Src, calmodulin (CaM) but not of CaMK. p-Akt was diminished by cell incubation in a Ca^{2+}-free medium but not by the use of L-type calcium channel blockers. The rise in intracellular Ca^{2+} induced by ATP was potentiated in the presence of Ro318220, a PKC inhibitor, and attenuated by the TPA, a known activator of PKC. ATP-dependent p-Akt was diminished by TPA and augmented by Ro318220 treatment in a Ca^{2+}-containing but not in a Ca^{2+}-free medium. ATP stimulated the proliferation of both ROS 17/2.8 cells and rat osteoblasts through PI3K/Akt. In the primary osteoblasts, ATP induces alkaline phosphatase activity via PI3K, suggesting that the nucleotide promotes osteoblast differentiation. These results suggest that ATP stimulates osteoblast proliferation through PI-PLC linked-$P2Y_2$ receptors and PI3K/Akt pathway activation involving Ca^{2+}, CaM and Src. PKC seems to regulate Akt activation through Src and the Ca^{2+} influx/CaM pathway.

Introduction

Purine and pyrimidine nucleotides released from dying cells or from living cells via several physiological mechanisms have been shown to regulate bone metabolism through activation of membrane receptors [1–7]. Nucleotide receptors of the P2 family are subdivided into P2X ($P2X_{1-7}$) ionotropic and P2Y ($P2Y_{1,2,4,6,11,12,13,and14}$) metabotropic sub-classes [4,7–10]. $P2Y_{1,\ 2,\ 4,\ 6,\ and\ 11}$ receptors couple to $G_{q/11}$ to activate phosphatidylinositol-specific phospholipase C (PI-PLC)[1] with the consequent rise in inositol trisphosphate (IP_3), diacylglycerol (DAG) and intracellular calcium [8,11,12].

The phosphoinositide 3-kinase (PI3K)/Akt signal transduction pathway plays an important role in mitogenic signaling, cell survival, growth and motility [13]. At the plasma membrane, PI3Ks catalyze the phosphorylation of phosphoinositides on the 3′-OH (D3) position of the inositol ring to generate 3′ phosphorylated phosphoinositides (3-PIs) as second messengers [14–16]. The 3-PIs bind to the pleckstrin homology domain-containing proteins such as Akt in order to activate them [17]. In osteoblasts, the PI3K/Akt signaling pathway has been involved in growth and survival [18]. In addition, it has been reported that Akt participates in IGF-1 stimulated alkaline phosphatase activity in osteoblastic cells [19]. In this cell type, Akt was also activated by cyclic stretch or androgen [20,21]. However, the modulation of the PI3K/Akt signal transduction pathway by extracellular ATP through nucleotide receptors in osteoblastic cells and its role in proliferation has not been reported until the present study.

Class I PI3Ks has been subdivided into two classes (IA and IB) according to their structures and mechanisms of activation. Members of class IA PI3Ks comprise heterodimers of one p85α, p55α or p50α regulatory subunits and one of p110α, p110β or p110δ catalytic subunits that are activated upon receptor tyrosine kinase (RTK)-stimulation [15,16,22]. Class IB PI3K consists of only one regulatory subunit (p101) that has been shown to interact with the p110γ catalytic subunit [23]. This class is primarily regulated by small G-proteins such as Ras and by the βγ subunits of G protein-coupled receptors [22,24]. In addition, it has been reported that p85/p110β PI3K can also be activated by Gβγ subunits [14]. It has been reported that P2Y receptors that couple to Gq/11 can

* Corresponding authors. Fax: +54 291 4595130.
E-mail addresses: gsantill@criba.edu.ar (G. Santillán), rboland@criba.edu.ar (R. Boland).

[1] *Abbreviations used:* PI-PLC, phosphatidylinositol-specific phospholipase C, IP_3, inositol trisphosphate; DAG, diacylglycerol; PI3K, phosphoinositide 3-kinase; 3-PIs, 3′ phosphorylated phosphoinositides; RTK, receptor tyrosine kinase; BSA, bovine serum albumin; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; ALP, alkaline phosphatase activity; PIP_2, phosphatidyl inositol 4,5-bisphosphate; CaM, calmodulin; GPCRs, G protein-coupled receptors; CaMKK, CaM kinase kinase; MAPK, mitogen activated proteinkinase; SAC, stress activated calcium channels.

0003-9861/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2011.06.013

activate the MAPKs through a pathway that involves PI3K stimulation [25–27]. We have previously reported that $P2Y_2$ receptors are involved in the stimulation of p38, ERK1/2 and JNK1 MAPK by ATP in osteoblasts [28]. However, there are no reports investigating the participation of P2Y receptors in the modulation of PI3K in osteoblasts in response to the stimulation by extracellular ATP.

It has been established that Akt is activated by growth factors and other stimuli, through both PI3K-dependent and independent mechanisms [29–31]. In the last case, the involvement of calmodulin in Akt regulation after EGF stimulation has been reported [32]. However, the role of calcium and other calcium-dependent proteins such as PKC or CaM in Akt activation in osteoblastic cells is not clear. In this work, we have obtained evidences on the signaling involved in Akt activation upon G protein-coupled $P2Y_2$ receptor stimulation by ATP. We describe the upstream mediators of Akt activation and the role of calcium in such activation. In addition, we demonstrate the participation of the PI3K/Akt signaling pathway in osteoblastic cell proliferation.

Materials and methods

Materials

ATP, ATPγS, UTP, ADPβS, Wortmannin, neomycin sulfate, U73122, nifedipine, verapamil, fluphenazine dihydrocloride, trifluoperazine, Ham's F-12 medium, α-MEM and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO, USA). Ly294002, Ro318220 and KN62 were from Calbiochem (San Diego, CA, USA). PP2 was from BIOMOL (Plymouth Meeting, PA, USA). Fetal bovine serum (FBS) was from Natocord (Córdoba, Argentina). CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit was from Promega (Madison, WI, USA). Monoclonal anti phospho-Akt (Ser473) (1:1000) antibody, monoclonal antibodies recognizing dually phosphorylated ERK 1/2 (Thr202, Tyr204) (1:1500), polyclonal anti phospho-Src (Tyr416) (1:300) antibody, 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 and polyclonal antibody recognizing total ERK1/ERK2 (1:1000) were from Cell Signaling Technology (Beverly, MA, USA). Polyclonal anti Akt1/2 (H-136) (1:1000) antibody, polyclonal antibodies recognizing dually phosphorylated p38 (Thr180, Tyr182) (1:1000), polyclonal antibody against p38alpha (1:1000), monoclonal anti-c-Src (1:500) antibody, 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 kits 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). Cells were seeded at a density of 10,000 cells per 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 FBS-free medium and the cells were starved for 18–21 h before the experiments.

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). After 48 h, the cells were starved in 1% FBS medium for 18–21 h before the experiments.

Western blot analysis

Cells were treated with ATP, ATPγS, UTP or ADPβS in the presence or the absence of various inhibitors (Ly294002, Wortmannin, nifedipine, verapamil, neomycin, fluphenazine, trifluoperazine, KN62, Ro318220, TPA and PP2) 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 14,000*g* and 4 °C during 15 min the supernatant was collected and proteins were quantified by the Bradford method [33]. Lysate proteins dissolved in 6× Laemmli sample buffer were separated (30 μg/lane) on 10% SDS–polyacrylamide gels 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 or overnight 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 Bio-Max 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.

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 [34]. Cells grown onto glass coverslips were loaded with 1 μM 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 the 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 to 380 nm signals were obtained, thus making the measurement independent of variations in cellular dye content, dye leakage or photobleaching. When the PKC inhibitor Ro318220 or the PKC activators TPA were used, they were added to the medium 5–10 min before the measurement. In order to measure the ATP-dependent mechanical stress activated Ca^{2+} influx (SAC influx), cells were subjected to mechanical stimulation by perturbation of the medium during intracellular Ca^{2+} measurements before and after cell treatment with ATP. Mechanical stimulation was performed by addition of 5 µl of vehicle, a volume equivalent to the agonist.

Cell Proliferation Assay (MTS)

The number of viable cells in proliferation was determined employing the commercial CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit from Promega as we have previously described [28]. ROS 17/2.8 cells (1250 cells/well) or rat primary osteoblasts (2000 cell/well) were seeded into 96 multiwell plates and cultured as indicated above. The cells were incubated with Ly294002 or Wortmannin for 30 min and then were treated with the indicated agonists in FBS-free medium (ROS 17/2.8 cells) or in medium containing 1% FBS (rat primary osteoblasts). When the treatment was finished, the medium containing agonist and the inhibitors was replaced by medium containing 20 µL MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)/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 subtract background absorbance from absorbance values of the samples. The results were obtained as absorbance per well and expressed as percentage stimulation with respect to the control.

Alkaline phosphatase activity (ALP)

The ALP activity of cell lysates was determined colorimetrically using a commercially available kit (Wiener Lab., Rosario, Argentina); this assay uses sodium phenylphosphate as a substrate; ALP in the presence of methyl propanol amine (pH 10) releases phenol. The phenol released is combined with a color generating reagent solution of 4-amino-antipyrine and ferrocyanide and quantified at 520 nM. Primary osteoblasts were cultured in 6-well plates (2×10^5 cells/well) in α-MEM supplemented with 10% FBS for 2 days. Then, the medium was replaced by α-MEM containing 1% FBS, 2 mM β-glycerophosphate, 50 µg/ml ascorbic acid and 10 µM ATP or vehicle (control), in the presence or absence of 10 µM LY294002, with total-medium changes every 2–3 days. The ALP activity was measured after 0, 3, 7 and 9 days. To assay enzyme activity, cell layers were washed and cells harvested in 200 µl PBS using a scraper followed by sonication at 4 °C and centrifugation at 500*g*. The supernatant was collected and stored at 4 °C until assaying at pH 10 as indicated above. A blank (B) and standard (S) (200 UI/L phenol) were also processed. Optical density of de samples (D) was measured and ALP activity was calculated as follow: ALP (UI/L) = 200UI/Lx (D − B)/(S − B).

Statistical analysis

Statistical significance of data was evaluated using Student's *t*-test 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

To our knowledge, the modulation of the PI3K/Akt signaling pathway by extracellular ATP in osteoblastic cells has not been investigated until the present paper. Although phosphorylation at Thr 308 partially activates Akt, full activation of Akt requires phosphorylation on Ser 473 located in their regulatory tail [35]. This site-specific phosphorylation of Akt involves the participation of phosphoinositides dependent kinases 1 and 2 in a PI3K dependent manner [36]. Therefore, we first investigated the effects of cell treatment with extracellular ATP on the phosphorylation of Akt at Ser 473. For this purpose, serum-starved ROS 17/2.8 osteoblastic cells were treated with different doses of ATP (1–100 µM) during 5 min. Subsequently, Akt-Ser 473 phosphorylation levels were evaluated by Western blot using a phospho-specific antibody. As shown in Fig. 1A, maximal Akt activation was observed after cell treatment with 10–25 µM ATP and remained activated up to 100 µM ATP, although with a slight diminution. When the cells were treated with 10 µM ATP for different periods of time (1–15 min), a time-dependent activation of Akt was observed. The higher levels of Ser 473 phosphorylation were reached after 2.5–5 min of treatment with ATP (Fig. 1B). These results suggest that ATP modulates Akt in osteoblastic cells, at least in part in a PI3K-dependent manner. Furthermore, the involvement of PI3K in Akt activation induced by ATP was evaluated using Ly294002 (Fig. 2A) and Wortmannin (Fig. 2B), both inhibitors of PI3K. ROS 17/2.8 cells were incubated with 10–50 µM Ly294002 or 100–200 nM Wortmannin during 30 min prior to ATP treatment (5 min-10 µM). As shown in Fig. 2, Akt-Ser 473 phosphorylation induced by ATP was blocked by the PI3K inhibitors. This result also suggests that Akt is activated by extracellular ATP in a PI3K-dependent manner.

To study whether ATP leads to Akt activation through PI-PLC, cells were incubated with 1–5 mM neomycin (Fig. 3A). This inhibitor sequesters phosphatidyl inositol 4,5-bisphosphate (PIP_2)

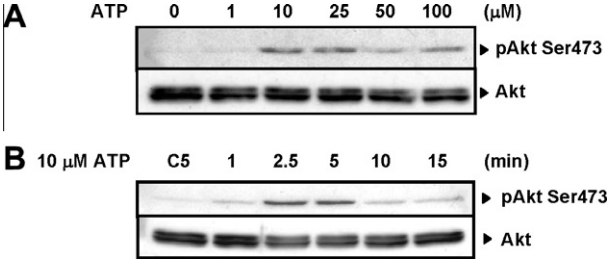

Fig. 1. Dose and time profile of Akt activation in response to ATP in ROS 17/2.8 osteoblast-like cells. Serum-starved (21 h) cells were treated with 1–100 µM ATP during 5 min (A) or with 10 µM ATP during 1–15 min (B). Subsequently, Western blot analysis was performed as described in Methods employing an antibody that recognizes Akt phosphorylated at Ser 473 (pAkt Ser473). Controls using vehicle instead of ATP are indicated as 0 in the dose-response and C5 (control during 5 min) in the time-response studies. An antibody directed against the total form (active plus inactive) of Akt was used as loading control (Akt). The results are representative of at least three experiments performed independently.

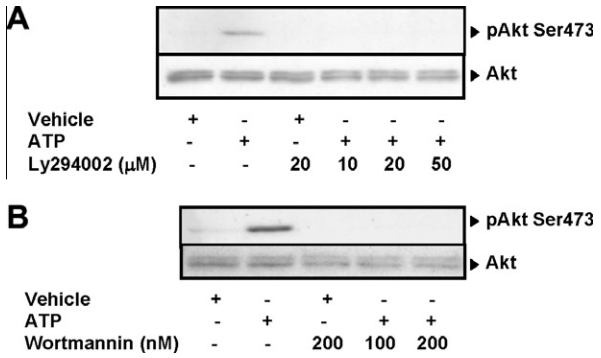

Fig. 2. Participation of PI3K in the activation of Akt induced by ATP in osteoblastic cells. ROS 17/2.8 cells were preincubated with 10–50 μM Ly294002 (A) or 100–200 nM Wortmannin (B) during 30 min. Then, the cells were treated with 10 μM ATP or vehicle for 5 min as indicated. The phosphorylation state of Akt at Ser 473 was evaluated by Western blot. Total Akt antibody was used as loading control. The results are representative of at least three experiments performed independently.

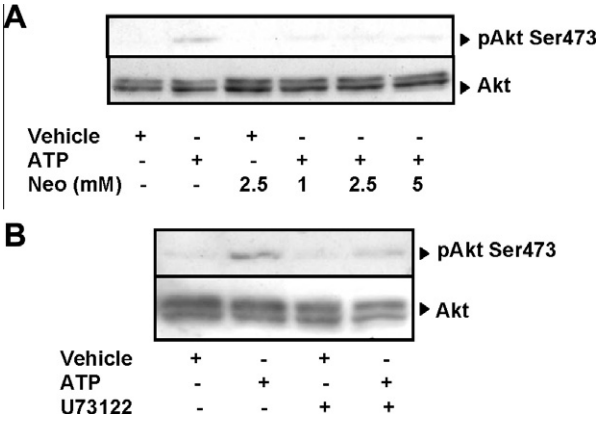

Fig. 3. Participation of PI-PLC in the activation of Akt induced by ATP in osteoblastic cells. ROS 17/2.8 cells were incubated in the presence (+) or the absence (−) of 1–5 mM neomycin (A) or 10 μM U73122 (B) during 15 min. Then, the cells were treated during 5 min with 10 μM ATP or vehicle as indicated. Akt-Ser 473 phosphorylation levels were then evaluated with the phospho-specific antibody as in Fig. 1. Total Akt antibody was used as loading control. The results are representative of at least three experiments performed independently.

which is substrate for both PLC and PI3K. Thus, the PLC-specific inhibitor U73122 was also used (Fig. 3B). The use of these inhibitors, reduced the phosphorylation of Akt at Ser 473 induced by ATP, suggesting that PI-PLC – linked P2Y receptors are involved in such activation.

In order to evaluate the subtype of P2Y receptor that may participate in Akt activation in response to ATP, different purinergic agonists were tested. Akt phosphorylation increased after 5 min treatment of osteoblast-like cells with 5–50 μM ATPγS (a nonhydrolyzable analogue of ATP) or with 10 μM UTP, both known agonists acting on $P2Y_2/P2Y_4$ receptors (Fig. 4A and B, respectively). ATPγS at lower doses (0.5–1 μM) and ADPβS (10–100 μM), a potent agonist of the $P2Y_1$ receptor, were not able to stimulate Akt phosphorylation (Fig. 4A and C, respectively). The biological activity of ADPβS was controlled by using the mouse C2C12 myoblast cell line, known to express the $P2Y_1$ receptor in culture (data not shown). These results suggest that the $P2Y_2$ receptor is the main P2Y receptor subtype involved in Akt stimulation by ATP in osteoblast-like cells as the $P2Y_4$ receptor subtype is not detected in these cells [28].

Some reports have suggested that Ca^{2+} and calmodulin (CaM) may be involved in the mechanism of Akt activation [29–31,37].

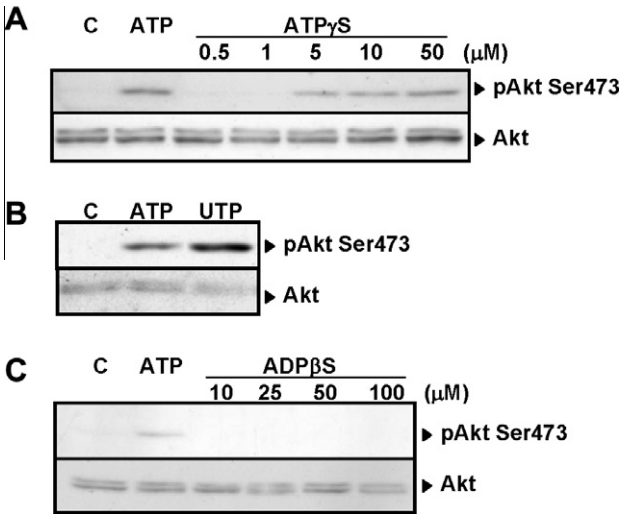

Fig. 4. Pharmacological profile of the P2Y receptors involved in the activation of Akt by ATP in osteoblastic cells. ROS 17/2.8 cells were treated for 5 min with the known agonists of the $P2Y_2/P2Y_4$ receptors: 0.5–50 μM ATPγS (the non-hydrolysable analogue of ATP) (A) or 10 μM UTP (B) and with the potent agonist of the $P2Y_1$ receptor: 10–100 μM ADPβS (C). Controls using vehicle instead of purinergic agonists are indicated as (C). Akt-Ser 473 phosphorylation levels were then evaluated with the phospho-specific antibody as in Fig. 1. Total Akt antibody was used as loading control. The results are representative of at least three experiments performed independently.

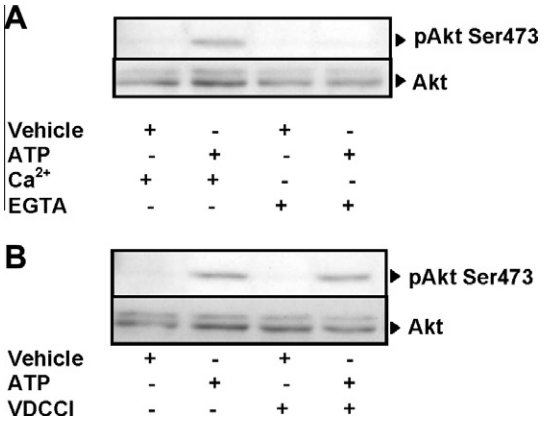

Fig. 5. Participation of extracellular Ca^{2+} and L-type voltage dependent (L-VDCC) calcium influx in the activation of Akt induced by ATP in osteoblast-like cells. ROS 17/2.8 cells were incubated in control medium containing 1.5 mM Ca^{2+} (Ca^{2+}) or calcium-free medium containing 0.5 mM EGTA as indicated (A) or in 1.5 mM calcium medium in the presence (+) or absence (−) of 5 μM nifedipine plus verapamil, L-VDCC blockers (VDCCI) (B). Then, the cells were stimulated by the addition of either vehicle or 10 μM ATP (ATP) for 5 min. Cell lysate proteins were immunoblotted with anti-phospho Akt-Ser473 antibody as in Fig. 1. Total Akt antibody was used as loading control. Representative immunoblots of three independent experiments are shown.

Hence, we evaluated the participation of extracellular calcium in the phosphorylation of Akt at Ser 473 by ATP in the ROS 17/2.8 cell line. Cells were incubated during 15 min in a medium containing Ca^{2+} or in a Ca^{2+}-free medium supplemented with 0.5 mM EGTA prior to the treatment with ATP. The results show that extracellular calcium participates in Akt activation induced by the purinergic agonist (Fig. 5A). In addition, the cells were incubated with ATP in the presence of both nifedipine and verapamil, blockers of L-voltage-dependent calcium channels. Subsequent Western blot analysis of pAkt-Ser 473 levels showed that the inhibitors were not able to impede Akt phosphorylation by ATP (Fig. 5B).

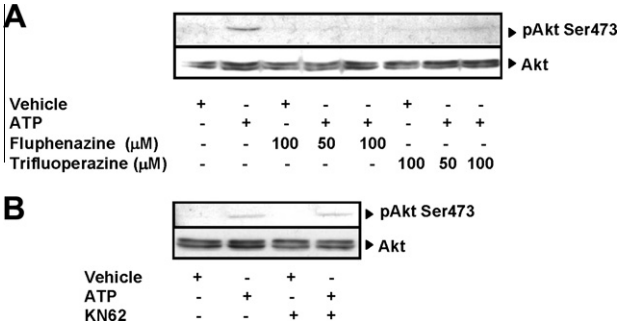

Fig. 6. Involvement of CaM and CAMK family members in the mechanism of activation of Akt by ATP in osteoblast-like cells. ROS 17/2.8 cells were preincubated during 10 min in the presence (+) or absence (−) of 50–100 μM fluphenazine or trifluoperazine (A) or 10 μM KN62 (B) followed by treatment with 10 μM ATP (ATP) or vehicle during 5 min. The levels of Akt-Ser473 phosphorylation were then measured with a phospho-specific antibody as in Fig. 1. Total Akt antibody was used as loading control. Representative immunoblots of at least three independent experiments are shown.

Then, we studied the involvement of CaM and CaMK (Ca^{2+}/CaM-dependent kinase) family members in the stimulation of Akt induced by ATP. The cells were preincubated with 50–100 μM fluphenazine or trifluoperazine, antagonists of CaM, or with 10 μM KN62, a blocker of Ca^{2+}/CaM activation of members of the CaMK cascade [38–40]. Fig. 6A and B show that phosphorylation of Akt by ATP was inhibited by both fluphenazine and trifluoperazine but not by compound KN62, suggesting the participation of CaM but not of CaMK family members in the mechanism of Akt activation by the purinergic agonist.

There is evidence that tyrosine phosphorylation may play a role in Akt regulation [35]. In order to evaluate if members of Src tyrosine kinases are involved in the mechanism of activation of Akt by ATP, cells were preincubated with 25–50 μM PP2, an inhibitor of Src, and the phosphorylation of Akt at Ser 473 was evaluated by Western blot (Fig. 7A). The results suggest that Src participates in the mechanism of Akt activation in response to ATP. We have previously showed that stimulation of Src (Tyr416) phosphorylation by ATP in ROS17/2.8 cells was blocked by Ro318220, a PKC inhibitor, showing that PKC is an upstream mediator in the modulation of this protein [34]. On this basis, it would be expected that PKC inhibition leads to a reduction in Akt (Ser473) phosphorylation in response to the purinergic agonist. However, when the cells were treated with 100 nM Ro318220 there was not inhibition of Akt-Ser 473 phosphorylation. On the contrary, the use of high doses

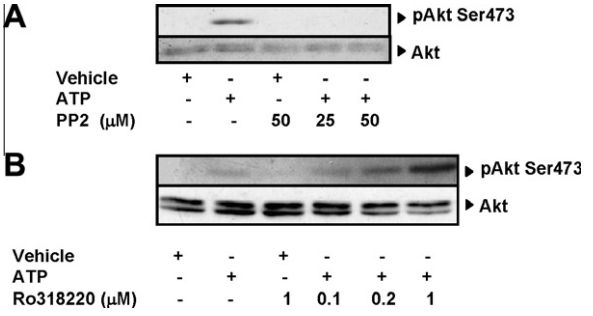

Fig. 7. Participation of Src and PKC in the mechanism of Akt activation by ATP in osteoblast-like cells. ROS 17/2.8 cells were preincubated during 15 min in the presence (+) or absence (−) of 25–50 μM PP2 (A) or 0.1–1 μM Ro318220 (B) followed by treatment with 10 μM ATP (ATP) or vehicle during 5 min. The phosphorylation state of Akt at Ser 473 was evaluated by Western blot. Total Akt antibody was used as loading control. Representative immunoblots of at least three separated experiments are shown.

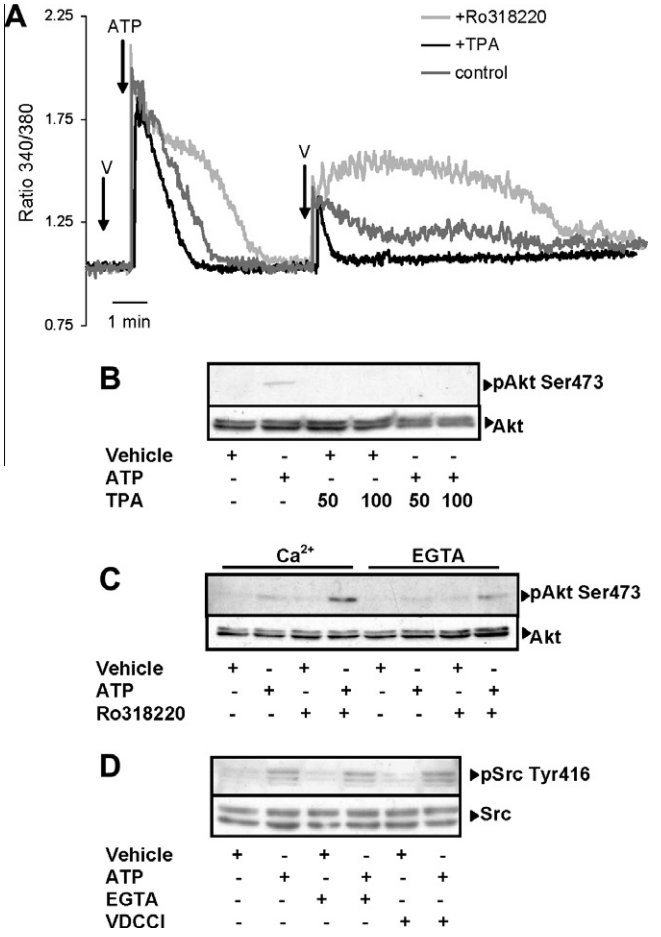

Fig. 8. Modulation of $[Ca^{2+}]i$ levels by PKC as a mechanism that leads to Akt regulation. Fura-2-loaded ROS 17/2.8 cells were incubated in 1.5 mM Ca^{2+} buffer with (+) or without (control) 1 μM Ro318220 or 50 nM TPA and subsequently were stimulated with 10 μM ATP or vehicle (V) as indicated by the arrows. $[Ca^{2+}]i$ was measured as described in Methods. ATP-dependent mechanical stress-activated Ca^{2+} influx (SAC) was induced by the addition of vehicle (V) after the purinergic stimulation (the first "V" corresponds to the control). Time traces representative of at least five independent experiments, each performed onto no less than 40–50 cells are shown (A). ROS 17/2.8 cells were preincubated during 10 min in the presence (+) or absence (−) of 50–100 nM TPA (B), 1 μM Ro318220 (C) or VDCCI (D) in a calcium-containing medium (Ca^{2+}) or a free-calcium medium supplemented with 0.5 mM EGTA (EGTA) as indicated. Then, they were treated with 10 μM ATP (ATP) or vehicle during 5 min. The levels of Akt-Ser473 (B and C) or Src-Tyr416 (D) phosphorylation were then measured with phospho-specific antibodies. Total Akt or Src antibodies were used as loading controls. Representative immunoblots of at least three independent experiments are shown.

of the inhibitor (200 nM and 1 μM) led to an increment in Ser473 phosphorylation by ATP, suggesting that PKC could inhibit Akt activation in a way that predominates over PKC/Src modulation (Fig. 7B). Regarding this contention, we have previously reported that $P2Y_2$ receptor stimulation by ATP increased $[Ca^{2+}]i$ through cation release from intracellular stores and sensitizes mechanical stress activated calcium channels leading to calcium influx (SAC influx) [34]. These increments in $[Ca^{2+}]i$ induced by ATP were higher in the presence of 200 nM Ro318220 than in control cells. In addition, the phorbol ester TPA, a known activator of PKC, attenuated the release of Ca^{2+} induced by ATP and strongly reduced the ATP-dependent SAC influx (Fig. 8A). In keeping with these results, to characterize the mechanism of PKC modulation of Akt, cells were incubated with TPA or Ro318220 in a free- or calcium-containing medium as indicated. The Western blot shows that Akt (Ser473) phosphorylation induced by ATP was suppressed by TPA (Fig. 8B) and reduced by Ro318220 in the absence of extracellular calcium

(Fig. 8C). As shown in Fig. 8D, incubation of cells in a free-calcium medium or in a medium containing VDCCI did not influence Src (Tyr416) phosphorylation, showing that it is independent on calcium influx. Altogether, these data suggest that PKC may regulate Akt activation through an extracellular calcium-dependent mechanism in addition to the PKC/Src pathway.

We have previously demonstrated by [^{3}H]-thymidine incorporation and MTS assays that ATP stimulates ROS 17/2.8 osteoblast-like cells proliferation [28]. In the present study, we evaluated the involvement of the PI3K/Akt pathway in this proliferative action of ATP. ROS 17/2.8 cells were incubated in a serum free-medium in the presence or absence of 10 μM Ly294002 or 100 nM Wortmannin and then treated for 24 h with 10 μM ATPγS, the nonhydrolyzable analogue of ATP. In addition rat primary osteoblasts were incubated in α-MEM containing 1% FBS in the presence or absence of 10 μM Ly294002 and then treated for 24, 48 and 72 h with 10 μM ATP or UTP. Subsequently, cell proliferation was evaluated using the MTS assay as described in Methods. Fig. 9A and B shows that purinergic activation stimulated ROS 17/2.8 cells and primary osteoblast proliferation above the control. Moreover, cell proliferation was inhibited in the presence of PI3K inhibitors (Fig. 9A and C). This result suggests that the PI3K/Akt pathway is involved in the proliferative effects of the purinergic agonists in osteoblasts.

As shown in Table 1, treatment of rat primary osteoblasts with 10 μM ATP stimulated 33–39% alkaline phosphatase (ALP) activity at days 7 and 9. This effect was abolished in the presence of the PI3K inhibitor Ly294002 suggesting a role of purinergic signaling through the PI3K/Akt pathway in modulation of osteoblast maturation.

Table 1
Induction of alkaline phosphatase (ALP) activity in osteoblasts by ATP and its suppression by a PI3K inhibitor. ALP activity was measured in rat primary osteoblasts after 0, 3, 7 and 9 days of treatment with 10 μM ATP or vehicle (control) in the absence or presence of PI3K inhibitor LY294002 as indicated in Methods. Values (IU/L) are the average ± SD of two independent experiments performed in triplicate. $^*p < 0.02$, $^{**}p < 0.01$.

Days	−LY294002		+LY294002	
	Control	10 μM ATP	Control	10 μM ATP
0	15.1 ± 0.002	15.2 ± 0.002	nd	nd
3	133.65 ± 14.79	142.22 ± 16.33	nd	nd
7	298.56 ± 53.88	397.76 ± 17.96**	285.88 ± 8.32	270.01 ± 9.15
9	151.37 ± 19.36	211.16 ± 25.28*	nd	nd

We have previously showed that ATP activates p38, ERK1/2 and JNK1 MAPKs in rat osteoblasts [28,34]. In addition, it has been reported that PI3K can regulate the activity of MAPKs [26,41]. Then, the involvement of the PI3K/Akt pathway in MAPK stimulation induced by ATP was studied. As shown in Fig. 10, PI3K inhibitors Ly294002 and Wortmannin did not affect ATP-dependent p38, ERK1/2 and JNK1 MAPK phosphorylation, suggesting that the mitogenic action of ATP in osteoblastic cells is mediated through the direct activation of the PI3K/Akt pathway.

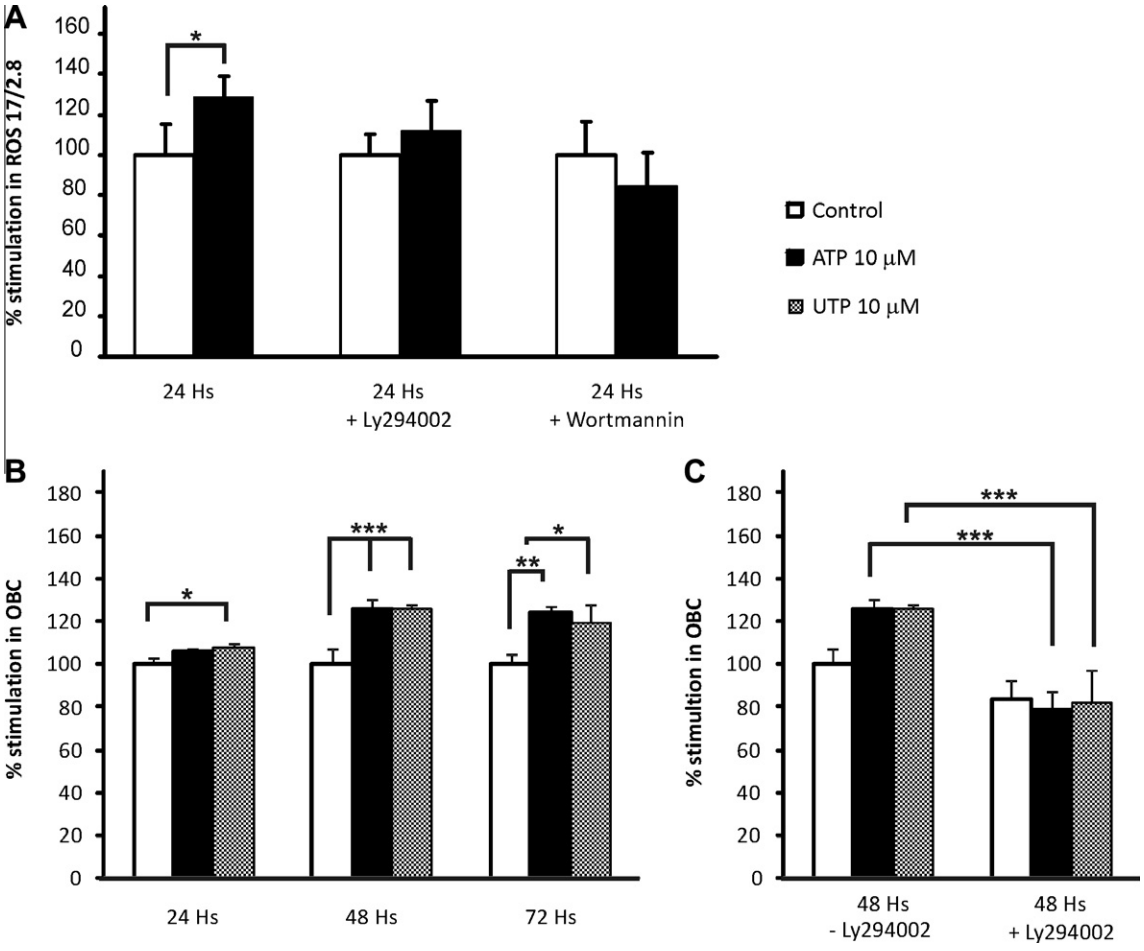

Fig. 9. Participation of the PI3K/Akt pathway in osteoblastic cell proliferation induced by ATP. ROS 17/2.8 cells (A) or rat primary osteoblasts (B and C) preincubated with or without 10 μM Ly294002 or 100 nM Wortmannin were treated with 10 μM ATP, ATPγS, UTP or vehicle (Control) during the indicated times. Then, cellular proliferation was evaluated by the MTS assay as described in Methods. The results are expressed as percentage stimulation with respect to the control (mean ± S.D) ($n = 4$) $^*p < 0.05$, $^{**}p < 0.02$, $^{***}p < 0.001$.

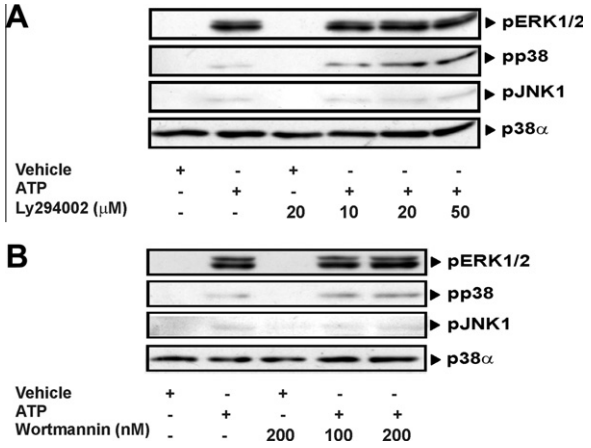

Fig. 10. ROS 17/2.8 cells were incubated with 10–50 μM Ly294002 or 100–200 nM Wortmannin during 30 min. After that, the cells were treated with 10 μM ATP or vehicle for 5 min as indicated. The phosphorylation state of ERK1/2, p38 and JNK MAPKs was evaluated by Western blot. p38α Antibody was used as loading control. The results are representative of at least three experiments performed independently.

Discussion

The present manuscript describes for the first time up-regulation of the PI3K/Akt signal transduction pathway by extracellular ATP in osteoblastic cells and demonstrates its participation in stimulation of cell proliferation by the purinergic agonist. In this work we suggest that stimulation of G protein-coupled $P2Y_2$ receptor activates Akt in a PI3K-dependent manner involving PKC, Src and Ca^{2+}/CaM. For G protein-coupled receptors (GPCRs), conflicting data indicate that these receptors exert diverse effects on the activity of class I PI3Ks and Akt in a cell type-specific manner and by a yet not fully elucidated mechanism. Available evidence indicates that GPCRs activate Akt by a pathway distinct from that utilized by growth factor receptors, as it involves the tyrosine phosphorylation-independent activation of PI3Kβ by G protein βγ dimmers [42,43]. It has been reported that G(q/11)-coupled receptors can mediate mitogenic signaling by promoting PI3K stimulation, acting through Gβγ subunits [44]. In addition, Gαq/Ca^{2+}-dependent activation of the PI3K pathway has also been reported [45]. In agreement with this, the use of neomycin and U73122 to inhibit PI-PLC suggests that P2Y receptors that couple to Gαq activate the PI3K/Akt pathway in osteoblastic cells. In contrast with the results of our study, it has been reported that activation of Gq-coupled receptors inhibits PI3K signaling by direct binding of Gαq-GTP to some isoforms of PI3K [46].

The PI3K products function to tag cytosolic, inactive Akt together with phosphatidylinositol-dependent kinases 1 and 2 (PDK1/2) to the plasma membrane, thereby initiating Akt activation by phosphorylating its Thr308 and Ser473 respectively [47]. The fact that Ly294002 and Wortmannin, pharmacological inhibitors of the class I PI3Ks [48], abolished the ATP-induced Akt-Ser 473 phosphorylation in osteoblastic cells indicates that a PI3K-dependent mechanism is involved in Akt activation. Similarly, in mouse embryonic stem cells, ATP phosphorylated Akt in a time-dependent manner via the P2 purinoceptors [49]. Akt-Ser 473 phosphorylation in ROS 17/2.8 cells was increased after cell treatment with ATP, ATPγS and UTP, a pharmacological profile that is consistent with the activation of the $P2Y_{2/4}$ receptor subfamily [8,50]. However, molecular evidence on the expression of $P2Y_2$ but not of $P2Y_4$ receptor subtype was obtained in ROS 17/2.8 cells [28], suggesting that the $P2Y_2$ receptor subtype is involved in Akt activation by ATP in these cells.

It has been reported that p85 is substrate of Src family tyrosine kinases which relieves the inhibitory activity of p85 on p110 [25,51–54]. In addition, several recent reports that have described the possible role of tyrosine phosphorylation in Akt regulation [55–57] indicate that two tyrosine residues located within the catalytic domain of Akt, Tyr315 and Tyr326, are phosphorylated following receptor activation and are required for its activity. Another report identified Tyr474 as a possible site of phosphorylation in response to insulin and pervanadate which is required for full activation of Akt. The role of this residue in Akt activation seems to depend on Thr308 phosphorylation by PDK1 [55]. Our results suggest that Src family kinases participate in the full activation of Akt, since a reduction in Ser 473 phosphorylation was observed in the presence of PP2, an inhibitor of Src kinases. Since Ser 473 phosphorylation is dependent on PI3K activity, this result suggests that Src contributes to Akt activation by relieving p110 catalytic subunit from the inhibition exerted by p85. However, we could not rule out a direct involvement of Src in Akt phosphorylation that may contribute to its full activation after P2Y stimulation.

It has been reported that activation of CaM by Ca^{2+} influx can contribute to the activation of PI3K/Akt pathway. This mechanism involves Ca^{2+} influx and Ca^{2+}/CaM association with the 85 kDa regulatory subunit of PI3K [37]. In addition, a PI3K-independent mechanism of Akt activation involves Ca^{2+} influx, CaM (which interacts with Akt mediating their translocation to the plasma membrane) and CaM kinase kinase (CaMKK) that directly phosphorylates Akt [29–31]. Our results show that Akt activation after cell stimulation with ATP depends on calcium influx through calcium-permeable channels that are different from VDCC. The identity of such channels remains to be elucidated. It has been reported that Ca^{2+} influx is involved in the $P2X_7$ receptor-mediated phosphorylation of Akt in astrocytes [58]. That study suggests that the contribution of $P2X_7$ receptors to the phosphorylation of Akt is low as compared to other P2X and P2Y receptors. In addition, stimulation of P2X receptors was achieved by using a high dose of ATP (100 μM). Although in the present study we employed a low ATP concentration (10 μM), we could not rule out the participation of P2X channels in Akt activation in osteoblastic cells. Danciu et al. [20] suggest that cyclic stretch activates the PI3K/Akt pathway with the contribution of calcium influx in osteoblasts. As described above, the prime candidate calcium-dependent protein in mediating PI3K/Akt activation is CaM, which can associate with the 85 kDa regulatory subunit of PI3K or directly with Akt [29,37]. Consistent with this, our experiments suggest that there is a calcium-dependent mechanism involved in Akt activation with the participation of CaM. The family of CAMK seems not to participate in the modulation of Akt.

Few previous reports have shown that PKCs mediate Akt activation [59,60]. Other studies have suggested that specific isoforms of PKC negatively regulate Akt in different cell types [61,62], and that PKC inhibitors enhance Akt Ser473/Thr308 phosphorylation in breast cancer cells and keratinocytes [62,63]. In agreement with this, the results of the present study show that in osteoblastic cells increasing doses of the PKC inhibitor Ro318220 enhanced Akt-Ser473 phosphorylation induced by ATP, suggesting that PKC may negatively regulate Akt in response to ATP in ROS17/2.8 cells. This effect may be due to the inhibitory action of PKC on [Ca^{2+}]i, which could be mediated by desensitization of $P2Y_2$ receptors and/or by inhibition of calcium channels [64,65]. Therefore, a greater contribution of the Ca^{2+}/CaM-dependent mechanism to Akt phosphorylation after PKC inhibition would be expected. These data suggest that PKC could regulate the phosphorylation of Akt by activating their downstream mediator Src and through modulation of calcium influx/CaM activation. The identification of the PKC isoform/s involved in the regulation of Akt activation will be the aim of future studies.

The observation that increased levels of 3-phosphorylated phosphoinositides have been found in transformed and/or mitogen-stimulated cells involves PI3K in oncogenic and mitogenic signal transduction [14,66]. Our findings show that the PI3K/Akt pathway is involved in cell proliferation induced by extracellular ATP in both ROS 17/2.8 cells and rat osteoblasts. Our data also show increased ALP activity after stimulation by ATP, suggesting that ATP promotes osteoblast differentiation. In agreement with these results, in the human osteoblastic cell line HOBIT, ATP has been shown to activate Runx2, a transcription factor essential in controlling osteoblast differentiation [67]. In addition, we obtained preliminary results by flow cytometry using propidium iodide which suggest that ATP induces an increment in the S phase of the cell cycle which was abolished by the use of PI3K inhibitor Ly294002 (data not given). Consistent with this observation, it has been reported that ATP stimulates mouse embryonic stem cell proliferation through PKC, PI3K/Akt, and MAPKs via the P2 purinoceptors [49]. Also, stimulation of purinergic receptors in U138-MG cells leads to cell proliferation mediated by PI3K/Akt, ERK and PKC signaling [68]. Differently, in ROS17/2.8 cells we found that ERK1/2, p38 and JNK1 MAPKs activation induced by ATP was independent of the PI3K pathway. Furthermore, our results are in agreement with those suggesting that in rat osteoblasts, LY294002-mediated inhibition of PI3K activity triggered growth inhibition. In addition, the osteoblastic dysfunction stimulated by LY294002 was accompanied by inactivation of Akt. These data suggested that the PI3K/Akt pathway is involved in the growth and survival of rat osteoblasts [18]. In addition, it has been reported that osteoblastic cell stimulation by growth factors such as IGF-1 and PDGF, mechanical stress or androgens induce activation of Akt, stimulating cellular proliferation [20,21,69]. Classically, Akt activation has been implicated in preventing cells from undergoing apoptosis with the consequent increment in cell survival. Based on this assumption, Akt may contribute to the mitogenic actions of ATP by the activation of anti-apoptotic mechanisms. However, we have previously demonstrated that the process of apoptosis was not induced in ROS 17/2.8 cells under the same experimental conditions assayed here [28]. The effect of ATP in cells induced to undergo apoptosis should be then evaluated in future studies.

Altogether, our results suggest that Akt is modulated by ATP through G protein-coupled $P2Y_2$ receptors in a PI3K-dependent manner which involves the participation of Ca^{2+}, CaM and Src kinases. PKC seems to up-regulate Akt through the activation of Src and the inhibition of Ca^{2+} influx/CaM. The proliferative effect of ATP involves the participation of the PI3K/Akt pathway in rat osteoblasts.

Acknowledgments

This research was supported by Grant PIP 112-200801-02199 from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) to R. Boland and PIP 112-200901-00551 to G. Santillán.

References

[1] I.R. Orriss, J.C. Utting, A. Brandao-Burch, K. Colston, B.R. Grubb, G. Burnstock, T.R. Arnett, Endocrinology 148 (2007) 4208–4216.
[2] I.R. Orriss, G.E. Knight, J.C. Utting, S.E. Taylor, G. Burnstock, T.R. Arnett, Journal of Cellular Physiology 220 (2009) 155–162.
[3] I.R. Orriss, G. Burnstock, T.R. Arnett, Current Opinion in Pharmacology 10 (2010) 322–330.
[4] G. Burnstock, Cellular and Molecular Life Sciences 64 (2007) 1471–1483.
[5] G. Burnstock, A. Verkhratsky, Evolutionary origins of the purinergic signalling system, Acta Physiology (Oxf) 195 (2009) 415–447.
[6] G. Burnstock, Brazilian Journal of Medical and Biological Research 42 (2009) 3–8.
[7] M.P. Abbracchio, G. Burnstock, A. Verkhratsky, H. Zimmermann, Trends in Neurosciences 32 (2009) 19–29.
[8] V. Ralevic, G. Burnstock, Pharmacological Reviews 50 (1998) 413–492.
[9] M.P. Abbracchio, G. Burnstock, J.M. Boeynaems, E.A. Barnard, J.L. Boyer, C. Kennedy, G.E. Knight, M. Fumagalli, C. Gachet, K.A. Jacobson, G.A. Weisman, Pharmacological Reviews 58 (2006) 281–341.
[10] G. Burnstock, Pharmacological Reviews 58 (2006) 58–86.
[11] W.B. Bowler, C.J. Dixon, C. Halleux, R. Maier, G. Bilbe, W. Fraser, J. Gallagher, R. Hipskind, Journal of Biological Chemistry 274 (1999) 14315–14324.
[12] L. Erb, Z. Liao, C.I. Seye, G.A. Weisman, Pflugers Archiv. European Journal of Physiology 452 (2006) 552–562.
[13] M.A. Krasilnikov, Biochemistry (Moscow) 65 (2000) 59–67.
[14] P.M. Wymann, L. Pirola, Biochimica et Biophysica Acta 1436 (1998) 127–150.
[15] J.A. Engelman, J. Luo, L.C. Cantley, Nature Reviews Genetics 7 (2006) 606–619.
[16] P.T. Hawkins, K.E. Anderson, K. Davidson, L.R. Stephens, Biochemical Society Transactions 34 (2006) Part 5.
[17] J.R. Woodgett, Current Opinion in Cell Biology 17 (2005) 150–157.
[18] Y. Zhang, L. Zhang, M. Yan, X. Zheng, Biomedicine and Pharmacotherapy 61 (2007) 277–284.
[19] T. Noda, H. Tokuda, M. Yoshida, E. Yasuda, Y. Hanai, S. Takai, O. Kozawa, Hormone and Metabolic Research 37 (2005) 270–274.
[20] T.E. Danciu, R.M. Adam, K. Naruse, M.R. Freeman, P.V. Hauschka, FEBS Letters 536 (2003) 193–197.
[21] H.Y. Kang, C. Cho, K. Huang, J. Wang, Y. Hu, H. Lin, Journal of Bone and Mineral Research 19 (2004) 7.
[22] T.F. Franke, Oncogene 27 (2008) 6473–6488.
[23] L.R. Stephens, A. Eguinoa, H. Erdjument-Bromage, M. Lui, F. Cooke, J. Coadwell, A.S. Smrcka, M. Thelen, K. Cadwallader, P. Tempst, P.T. Hawkins, Cell 89 (1997) 105–114.
[24] M. Lopez-Ilasaca, P. Crespo, P.G. Pellici, J.S. Gutkind, R. Wetzker, Science 275 (1997) 394–397.
[25] M. Montiel, Cellular Physiology and Biochemistry 18 (2006) 123–134.
[26] A. Muscella, M.G. Elia, S. Greco, C. Storelli, S. Marsigliante, Journal of Cellular Physiology 195 (2003) 234–240.
[27] L.I. Santiago-Pérez, R.V. Flores, C. Santos-Berríos, N.E. Chorna, B. Krugh, R.C. Garrad, L. Erb, G.A. Weisman, F.A. González, Journal of Cellular Physiology 187 (2001) 196–208.
[28] S. Katz, R. Boland, G. Santillán, Archives of Biochemistry and Biophysics 477 (2008) 244–252.
[29] S. Yano, H. Tokumitsu, T.R. Soderling, Nature 396 (1998) 584–587.
[30] N. Filippa, C.L. Sable, C. Filloux, B. Hemmings, E. Van Obberghen, Molecular and Cellular Biology 19 (1999) 4989–5000.
[31] T.B. Deb, C.M. Coticchia, R.B. Dickson, Journal of Biological Chemistry 279 (2004) 38903–38911.
[32] C.M. Coticchia, C.M. Revankar, T.B. Deb, R.B. Dickson, M.D. Johnson, Breast Cancer Research and Treatment 115 (2009) 545–560.
[33] M.M. Bradford, Annals Biochemistry 72 (1976) 248–254.
[34] S. Katz, R. Boland, G. Santillán, International Journal of Biochemistry & Cell Biology 38 (2006) 2082–2091.
[35] G. Song, G. Ouyang, S. Bao, Journal of Cellular Molecular Medicine 9 (2005) 59–71.
[36] C. Kroner, K. Eybrechts, J.W.N. Akkerman, Journal of Biological Chemistry 275 (2000) 27790–27798.
[37] M.J. Pérez-García, V. Ceña, Journal of Biological Chemistry 279 (2004) 6132–6142.
[38] G.A. Wayman, Y. Lee, H. Tokumitsu, A. Silva, T.R. Soderling, Neuron 59 (2008) 914–931.
[39] H. Enslen, T.R. Soderling, Journal of Biological Chemistry 269 (1994) 20872–20877.
[40] H. Mochizuki, T. Ito, H. Hidaka, Journal of Biological Chemistry 268 (1993) 9143–9147.
[41] M.G. Elia, A. Muscella, S. Romano, S. Greco, B. Di Jeso, T. Verri, C. Storelli, S. Marsigliante, Cellular Signalling 17 (2005) 739–749.
[42] C. Murga, S. Fukuhara, J.S. Gutkind, Journal of Biological Chemistry 275 (2000) 12069–12073.
[43] H. Kubo, K. Hazeki, S. Takasuga, O. Hazeki, Biochemical Journal 392 (2005) 607–614.
[44] K.C. Kong, C.K. Billington, U. Gandhi, R.A. Jr Panettieri, R.B. Penn, FASEB Journal 20 (2006) 1558–1560.
[45] P. Wang, K.A. DeFea, Biochemistry 45 (2006) 9374–9385.
[46] L.M. Ballou, M. Chattopadhyay, Y. Li, S. Scarlata, R.Z. Lin, Biochemical Journal 394 (2006) 557–562.
[47] M.M. Hill, J. Feng, B.A. Hemmings, Current Biology 12 (2002) 1251–1255.
[48] K.E. Anderson, S.P. Jackson, International Journal of Biochemistry and Cell Biology 35 (2003) 1028–1033.
[49] J.S. Heo, H.J. Han, Stem Cells 24 (2006) 2637–2648.
[50] D. Communi, R. Janssens, N. Suarez-Huerta, B. Robaye, J.M. Boeynaems, Cellular Signalling 12 (2000) 351–360.
[51] B.D. Cuevas, Y. Lu, M. Mao, J. Zhang, R. LaPushin, K. Siminovitch, G.B. Mills, Journal of Biological Chemistry 276 (2001) 27455–27461.
[52] M.P. Haynes, L. Li, D. Sinha, K.S. Russell, K. Hisamoto, R. Baron, M. Collinge, W.C. Sessa, J.R. Bender, Journal of Biological Chemistry 278 (2003) 2118–2123.
[53] W. Jin, C. Yun, A. Hobbie, M.J. Martin, P.H. Sorensen, S.J. Kim, Cancer Research 67 (2007) 3192–3200.
[54] M. Ciccarelli, E. Cipolletta, G. Santulli, A. Campanile, K. Pumiglia, P. Cervero, L. Pastore, D. Astone, B. Trimarco, G. Iaccarino, Cellular Signalling 19 (2007) 1949–1955.
[55] N.M. Conus, K.M. Hannan, B.E. Cristiano, B.A. Hemmings, R.B. Pearson, Journal of Biological Chemistry 277 (2002) 38021–38028.

[56] T. Jiang, Y. Qiu, Journal of Biological Chemistry 278 (2003) 15789–15793.
[57] R. Chen, O. Kim, J. Yang, K. Sato, K.M. Eisenmann, J. McCarthy, H. Chen, Y. Qiu, Journal of Biological Chemistry 276 (2001) 31858–31862.
[58] M.C. Jacques-Silva, R. Rodnight, G. Lenz, Z. Liao, Q. Kong, M. Tran, Y. Kang, F.A. Gonzalez, G.A. Weisman, J.T. Neary, British Journal of Pharmacology 141 (2004) 1106–1117.
[59] W. Li, J. Zhang, L. Flechner, T. Hyun, A. Yam, T.F. Franke, J.H. Pierce, Oncogene 18 (1999) 6564–6572.
[60] G. Gliki, C. Wheeler-Jones, I. Zachary, Cell Biology International 26 (2002) 751–759.
[61] W. Zheng, S. Kar, R. Quirion, Journal of Biological Chemistry 275 (2000) 13377–13385.
[62] L. Li, K. Sampat, N. Hu, J. Zakari, S.H. Yuspa, Journal of Biological Chemistry 281 (2006) 3237–3243.
[63] M. Mao, X. Fang, Y. Lu, R. Lapushin, Biochemical Journal 352 (2000) 475–482.
[64] R.V. Flores, M.G. Hernández-Pérez, E. Aquino, R.C. Garrad, G.A. Weisman, F.A. Gonzalez, Molecular and Cellular Biochemistry 280 (2005) 35–45.
[65] J. Soboloff, M. Spassova, T. Hewavitharana, L.P. He, P. Luncsford, W. Xu, K. Venkatachalam, D. van Rossum, R.L. Patterson, D.L. Gill, Handbook of Experimental Pharmacology 179 (2007) 575–591.
[66] K.R. Auger, L.A. Serunian, S.P. Soltoff, P. Libby, L.C. Cantley, Cell 57 (1989) 167–175.
[67] A. Costessi, A. Pines, P. D'Andrea, M. Romanello, G. Damante, L. Cesaratto, F. Quadrifoglio, L. Moro, G. Tell, Bone 36 (2005) 418–432.
[68] M.C. Jacques-Silva, A. Bernardi, R. Rodnight, G. Lenz, Oncology 67 (2004) 450–459.
[69] P. Borgatti, A.M. Martelli, A. Bellacosa, R. Casto, L. Massari, S. Capitani, L.M. Neri, FEBS Letters 477 (2000) 27–32.