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Effect of Combined Action of Extracellular ATP and Elevated Calcium on Osteogenic Differentiation of Primary Cultures From Rat Calvaria

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ABSTRACT

The in vitro osteogenic differentiation has been intensively studied. However, it is not yet clear precisely how osteogenesis can be optimized. Changes in extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$), as well as modulation of purinergic receptors play an important role in the regulation of osteoblasts differentiation and bone formation. In this study, we investigated the effects of a combined treatment of ATPγ-S and high $[Ca^{2+}]_e$ (5.35 mM) on osteogenic differentiation and function of primary cell cultures from rat calvaria. Our results indicate that ATPγ-S stimulates cell transition from the G_0 to S phase of cell cycle, involving the PI3K signaling pathway. Treatment with 10 or 100 μM ATPγ-S and $[Ca^{2+}]_e$ (ATP-$[Ca^{2+}]_e$) for 48 h increases cell number significantly above the control. ATPγ-S treatment in osteogenic medium containing $[Ca^{2+}]_e$ stimulates the gene expression of BMP-4, BMP-5, and OPN at 16, 48, and 72 h, respectively, above control. In same conditions, treatment for 6 days with 10 μM UTP or 100 μM UDP significantly increased the ALP activity respect to control. Cells grown in osteogenic medium showed a statistically significant increase in calcium deposits at 15 and 18 days, for 10 μM ATPγ-S treatment, and at 18 and 22 days, for $[Ca^{2+}]_e$ treatment, respect to control but ATP-$[Ca^{2+}]_e$ treatment shown a significant greater mineralization at 15 days respect to ATPγ-S, and at 18 days respect to both agonists. In conclusion, we demonstrated that an osteogenic medium containing 10 μM ATPγ-S and 5.35 mM $[Ca^{2+}]_e$ enhance osteogenesis and mineralization by rat primary calvarial cells cultures. J. Cell. Biochem. 117: 2658–2668, 2016. © 2016 Wiley Periodicals, Inc.

KEY WORDS: OSTEOGENESIS; PURINERGIC RECEPTORS; EXTRACELLULAR ATP; CALCIUM

Accumulating preclinical and clinical evidence indicates that mesenchymal stem/stromal cells (MSCs) are good candidates for cell therapy. The use of these cells has great potential for bone reconstruction of congenital malformations and local bone defects caused by trauma, tumor, infection, degenerative joint disease, or periprosthetic bone loss [Pereira et al., 1995; Ahmed and Hincke, 2014; Zhang et al., 2014; Murrell et al., 2015]. MSCs can be isolated from numerous connective tissues but is more common from bone marrow. Under specific conditions, they can be amplified and differentiated in vitro into various cell lineages, osteoblasts (bone forming cells), condrocytes (cartilage), or adipocytes (fat) and then used in tissue repair process [Pittenger et al., 1999; Vater et al., 2011]. The repair of tendons and cartilage by using MSCs has been demonstrated [Awad et al., 2003; Butler et al., 2010; Haleem et al., 2010]. Osteogenic differentiation of MSCs in vitro largely depends on the culture conditions. The employ of different compounds such as dexamethasone, ascorbic acid, β-glycerophosphate, and combination with vitamin D3, transforming growth factor-beta (TGF-β) and bone morphogenetic proteins (BMP) as well as the importance of each of these constituents for induction of in vitro osteogenic

Abbreviations: ATP, adenosine-5′-triphosphate; ATPγ-S, adenosine-5′-O-(thiotriphosphate); ADP, adenosine-5′-(diphosphate); ADPβ-S, adenosine-5′-O-(2-thiodiphosphate); BMPs, Bone Morphogenetic Proteins; UTP, uridine-5′-triphosphate; ALP, Alkaline phosphatase; PI-PLC, phosphatidylinositol-specific phospholipase C; IP3, inositol trisphosphate; DAG, diacylglycerol; PI3K, phosphoinositide 3-kinase; AKT, serine/threonine kinase Akt (also known as protein kinase B or PKB); ERK1/2, extracellular signal regulated kinases 1 and 2; PKC, protein kinase C; TGF-β, transforming growth factor β; BMP-3,-4, and -5, bone mophogenetic protein 3, 4, and 5; BSP, bone sialo protein; OPN/PPS1, osteopontin/ protein phosphatase secreted 1; EDTA, Ethylenediaminetetraacetic acid; PBS, phosphate buffered saline; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

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differentiation have been intensively studied [Vater et al., 2011]. The optimization of culture conditions for osteogenic differentiation in vitro is a prerequisite for improve the obtaining of mature and active osteoblasts with bone forming capacity. However, it is not yet clear precisely how osteogenesis can be optimized.

Extracellular ATP and others nucleotides are known to participate in numerous different metabolic processes of various tissues and cell types through activation of plasma membrane purinergic receptors [Ralevic and Burnstock, 1998; Burnstock and Knight, 2004]. Purinergic receptors are divided in two families, P1 and P2 receptors. The main agonist of P1 receptors is the nucleoside adenosine, whereas the P2 receptors family responds to nucleotides. Moreover, P2 family is subdivided into P2X (P2X1–7), ionotropic receptors (ligand-gated ion channels) which only respond to ATP, and P2Y (P2Y1,2,4,6,11–14), metabotropic/G protein- coupled receptors that are activated by adenine and uridine nucleotides [Ralevic and Burnstock, 1998; Bowler et al., 2001; Burnstock and Knight, 2004; Burnstock, 2007].

Recently, it has been reported that up- or down-regulation of several P2 receptor subtypes, at gene and protein level, occur during human mesenchymal stem cells adipogenic and osteogenic differentiation, and the effect on differentiation is directly influenced by both the application of agonists/antagonists and apyrase-induced nucleotide cleavage [Zippel et al., 2012].

Previously, we demonstrated that activation of PI3K/AKT signaling pathway by extracellular ATP stimulates cell proliferation and alkaline phosphatase (ALP) activity in neonatal rat calvarial cells primary culture, suggesting that purinergic signaling could play a positive role in modulation of the osteoblast maturation [Katz et al., 2011]. In addition, ATP and UTP stimulate cell proliferation involving increases of intracellular calcium concentration ([Ca^{2+}]i), stimulation of PI3K/Akt signaling pathway and members of the MAPKs such as ERK1/2 and JNK1 through activation of P2Y2 receptors in osteoblasts [Katz et al., 2006, 2008, 2011].

Extracellular nucleotides have been shown to induce differentiation of aortic valve cells into osteoblast phenotype [Osman et al., 2006]. ATP and UTP activation of Runx2, a key transcription factor for osteoblasts differentiation, has been observed in the osteoblast-like HOBIT cell line [Costessi et al., 2005]. It has been suggested that the P2Y2 receptors inhibit the mineralization of osteoblasts in vitro [Orriss et al., 2007]. However, our recent studies suggest that stimulation of P2Y2 receptors by ATP, UTP, or ATPγ-S enhances osteoblast differentiation and mineralization of rat calvarial cells primary cultures, which was associated with gene expression increased of alkaline phosphatase (ALP), bone sialo protein (BSP), and bone morphogenetic proteins-2, -4, and -5 (BMP2-4-5) [Ayala-Peña et al., 2013].

In vitro studies have shown that high extracellular Ca^{2+} concentration positively influences osteogenesis of different cell types, such as pre-osteoblasts, osteoblasts [Dvorak et al., 2004; Nakamura et al., 2010; Khoshniat et al., 2011], macrophages [Honda et al., 2006; Kanaya et al., 2010], and rat bone marrow mesenchymal stromal cells [González-Vázquez et al., 2014]. In addition, it has been shown that Ca^{2+}, dose-dependently, enhances proliferation of MC3T3 and human periosteal derived stem cells [Chai et al., 2010]. It also was reported that elevation of extracellular Ca^{2+} stimulates the proliferation of rat primary osteoblasts involving store-operated calcium entry and phosphorylation of ERK1/2 induced by activation of calcium-sensing receptors [Ma et al., 2005; Hu et al., 2014]. ERK1/2 phosphorylation also occurred when MC3T3-E1 cells were treated with PO_4^{3-} in the presence of Ca^{2+}, but not in its absence [Khoshniat et al., 2011]. Recently, it was reported that elevated extracellular calcium increases expression of BMP-2 gene via a calcium channel and ERK pathway in human dental pulp cells [Tada et al., 2010]. Enhanced expression of osteopontin (OPN), osteocalcin (OC), ALP, Col1α 1 and BSP, as well as of BMP-2 by high extracellular Ca^{2+} it was observed in osteoblasts [Dvorak et al., 2004; Honda et al., 2006; González-Vázquez et al., 2014]. As mentioned above, ATP, ATPγ-S, and UTP have been shown to increase [Ca^{2+}]i, stimulate PI3K/Akt signaling pathway and members of the MAPKs such as ERK1/2, and expression of BMP2-4-5 in rat calvarial cells [Katz et al., 2006, 2008, 2011; Ayala-Peña et al., 2013]. Although extracellular ATP and high calcium stimulate various signaling pathways, many of which are shared and both agents have been shown to play a role in osteogenic differentiation, studies on the effect of combined treatment of extracellular ATP and high calcium in the differentiation of osteoblasts are not known yet.

By action of extracellular enzymes, ATP rapidly hydrolyzed to ADP, AMP, and adenosine, which mediate the activation of multiple subtypes of purinergic receptors from P1 and P2 families. Therefore, the effects observed by the action of ATP might be the result of joint activation of several purinergic receptors subtypes. Using a non-hydrolyzable analogue of ATP as ATPγ-S, it helps reduce the spectrum of receptors involved since only some subtypes of the P2 receptor family would be activated by this compound [Ralevic and Burnstock, 1998; Burnstock, 2007]. ATPγ-S is not a good agonist for P2X receptors family and between the P2Y family, only a few receptor subtypes as P2Y2, P2Y4, and P2Y11, prefer nucleotides triphosphate as agonist [Abbracchio et al., 2006; Von Kugelgen, 2006; Burnstock, 2007].

The aim of this study is to determine the effect of treatment with ATPγ-S, a non-hydrolyzable analogue of ATP, in combination with high extracellular calcium concentration (5.35 mM) on growth, osteogenic differentiation, and cell function of primary cultures from neonatal rat calvaria.

MATERIALS AND METHODS

MATERIALS

ATPγ-S, UTP, UDP, Alizarin Red, β-glycerophosphate, and α-MEM (1.36 mM Ca^{2+}; $CaCl_2.2H_2O$ 0.2 g/L) were from Sigma–Aldrich (St. Louis, MO). Ly294002 was from Calbiochem (San Diego, CA). Fetal bovine serum (FBS) was from Natocor (Córdoba, Argentina). Alkaline phosphatase (ALP) activity kit was from Wiener Lab. Rosario, Argentina. Quick-RNA™ Miniprep for RNA isolation, the mix KAPPA SYBR® FAST qPCR Kit, for Real Time Quantitative–PCR (RQ-PCR) and propidium iodide (PI) for flow cytometry were from Biosystems S.A (Buenos Aires, Argentina). High-Capacity cDNA Reverse Transcription Kit and primers for RQ-PCR were from Invitrogen S.A (Buenos Aires, Argentina). All other reagents used were of analytical grade.

CELL ISOLATION

Calvarial cells were obtained from 5-day-old neonatal rats. Animals were sacrificed by fast decapitation. All procedures were carried out in conformity with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) under protocol n° 012/2014 of Institutional Animal Care and Use Committee (CICUAE) of Universidad Nacional del Sur, Argentina. Briefly, calvarias were incubated in phosphate buffer saline (PBS) containing 4 mM EDTA at 37°C for two 10-min periods and the supernatants were discarded. Subsequently, calvarias were rinsed in PBS and submitted to digestion in PBS containing 200 U/ml collagenase 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 15% FBS, 1% penicillin and streptomycin under humidified air (5.5% CO_2). After 24 h, the medium was replaced by α-MEM supplemented with 10% FBS, 1% penicillin and streptomycin and the cells were cultured until ~80% of confluence (2–3 days). Then the cells were frozen in liquid nitrogen until their use.

CELL CULTURE AND TREATMENT

Cells were thawed and seeded into 10 cm diameter glass Petri dishes and allowed to grow to 80% confluence. Then, cells were passed at a density of 10×10^3 cells/cm^2 and cultured for 3–4 days in α-MEM supplemented with 10% FBS, in a humidified atmosphere (5.5% CO_2) at 37°C. For each experiment, the cells underwent two passages, one when they were obtained before being frozen, and the other when they were counted and seeded to the various experimental conditions tested. When cells reached 80% confluence, they were starved in 1% FBS medium for 7–16 h before starting treatment. Treatments were performed by replacing the medium by α-MEM supplemented with 1% FBS or osteogenic medium (α-MEM supplemented with 1% FBS, 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid) containing the indicated agonist amounts (ATPγ-S, UTP, UDP, or the vehicle/water pH:7.2 used in control conditions), in the presence of normal/control (1.36 mM Ca^{2+}) or high (5.35 mM Ca^{2+}) and in the presence or absence of 10 μM Ly294002. The control or treatment medium was renewed every 2–3 days.

FLOW CYTOMETRY

Cells were seeded on 10 cm diameter Petrie dishes and grown until 80–90% confluent. Then treatment with the indicated agonist in the presence or absence of 10 μM Ly294002 in α-MEM supplemented with 1% FBS was performed during ≈16 h. Afterward, the cells were detached with trypsin (0.25% in PBS) and collected by centrifugation. Subsequently, cells were resuspended and permeabilized with 70% ethanol. Finally, cells collected by centrifugation were resuspended with propidium iodide (PI), 2×10^6 cells/ml, to stain nuclei and then, the samples were processed in a flow cytometer.

CELL COUNT

After treatments, cells were detached with trypsin and resuspended in α-MEM 10% FBS. Then 0.4% Trypan Blue was added to cell suspension in 1:1 ratio. Follow, a hemocytometer was loaded with the cell suspension and counting was performed to obtain the number of total cells (T)/ml, the number of viable cells (V)/ml and the number of dead cells (D)/ml. Being D = blue cells that incorporated the dye, V = which did not incorporate the dye and T = V + D.

ALKALINE PHOSPHATASE ACTIVITY (ALP)

The ALP activity of cell was colorimetrically determined 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. The ALP activity was measured after the indicated cell treatment. Briefly, cell layers were washed with PBS and then cells were incubated with 50 μl/well of PBS containing 0.2% Triton for 10 min at 37°C. Follow 250 μl of substrate was added to each sample and it was incubated for 10 min (37°C) before the addition of 1.25 ml of color reagent and quantification as indicated above. A blank (B) and standard (S) (200 IU/L phenol) were also processed. Optical density of de samples (D) was measured and ALP activity was calculated as follow: ALP (IU/L) = 200IU/Lx (D − B)/(S − B). Results were expressed in arbitrary units taking the control value obtained at the experiment day as 1.

ALIZARIN RED STAINING

Calvarial osteoblasts seeded in 48-well plates were cultured and treated as above for 15, 18, and 22 days. The cells were then fixed with 2% glutaraldehyde in PBS (pH 7.4) at room temperature for 10 min, washed three times with PBS, and then incubated with 2% Alizarin Red (pH 4.2) for 30 min at 37°C. The cells were washed thoroughly with deionized water. After Alizarin Red staining, the samples were incubated with 1 ml of 0.1 M NaOH and O.D. was measured by spectrophotometer at 548 nm. Results were expressed in arbitrary units as a proportional ratio of the control of day 15, to which value 1 was assigned.

REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION (RQ-PCR)

Total RNA was isolated using Quick-RNA™ Miniprep. The amount of RNA was measured by spectrophotometry. Reverse transcription of total RNA was performed using the High-Capacity cDNA Reverse Transcription Kit. Quantitative PCR was done by SYBR Green real-time PCR methods. PCR was performed according to manufacturer's instructions, with denaturation cycles at 95°C for 3 s, like annealing/extend cycles at 60°C for 30 s. The relative mRNA expression was calculated using the comparative threshold method (ΔΔCt-method) with GAPDH for normalization. The average value of expression level of different genes in control cells was defined as 1. All experimental conditions were processed in triplicate.

RTQ-PCR primers were specifically designed to amplify the following cDNAs (Table I).

STATISTICAL ANALYSIS

Statistical significance of data was evaluated using Student's *t*-test and probability values below 0.05 ($P < 0.05$) were considered

TABLE I. Primers for GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase, rBMP: Rat Bone Morphogenetic Protein, OPN: Osteopontin

Target	Forward	Reverse
GAPDH	5′ GGCAAGTTCAACGGCACAGT 3′	5′ TGGTGAAGACGCCAGTAGACTC 3′
rBMP-3	5′ GGACCCTCCAATCCAACCA 3′	5′ AGGTTTGGCCGTATCTACTGACA 3′
rBMP-4	5′ CCAAGCGTAGTCCCAAGCAT 3′	5′ CGACGGCAGTTCTTATTCTTCTTC 3′
rBMP-5	5′ TCCCTTTGATGGCGTTGGT 3′	5′ AATCTGCCGGTCAGAAGCAA 3′
OPN	5′ TATCAAGGTCATCCCAGTTGCCCA 3′	5′ ATCCAGCTGACTTGACTCATGGCT 3′

significant. Quantitative data are expressed as means ± standard deviation (SD) from the indicated set of experiments.

RESULTS

ATPγ-S STIMULATES THE TRANSITION OF RAT PRIMARY CALVARIAL CELLS FROM G_0/G_1 TO S PHASE OF THE CELL CYCLE INVOLVING PI3 K PATHWAY

In our previous work using the MTT assay, we demonstrated that ATP stimulates the proliferation of rat primary calvarial cells involving the PI3 K/AKT pathway [Katz et al., 2011]. Now, we evaluated the mitogenic effect of ATP by using flow cytometry as indicated in the Materials and Methods section. It was observed that the cell treatment with 10 μM or 100 μM ATPγ-S for 16 h increased (26% and 53%, respectively) the number of cells which reached the S phase of the cell cycle. The PI3K inhibitor Ly294002 (10 μM) completely abolished this effect of ATPγ-S (Fig. 1A and B). These data suggest that ATP stimulates cell transition from the G_0 to S phase of cell cycle and confirm the involvement of PI3K signaling pathway in the mitogenic action of ATP on rat calvarial cells.

EFFECT OF ATPγ-S AND HIGH CALCIUM TREATMENT ON THE PROLIFERATION OF RAT CALVARIAL CELLS

It has been reported that high concentrations of extracellular calcium favor proliferation and osteoblast differentiation [Dvorak et al., 2004; Honda et al., 2006; Kanaya et al., 2010; Nakamura et al., 2010; Khoshniat et al., 2011; González-Vázquez et al., 2014]. In order to evaluate the effect of combined treatment with high calcium and ATP on proliferation of rat calvarial cells, cell counts were performed in a hemocytometer after various treatments as indicated follow. Cells were treated with 10 or 100 μM ATPγ-S in α-MEM 1% FBS, containing normal/control, 1.36 mM ($-Ca^{2+}$), or high, 5.35 mM ($+Ca^{2+}$), concentration of $CaCl_2$, for 24 and 48 h. No significant changes were observed in cell number after treatments with ATPγ-S or high calcium alone respect to control at any of times studied. Whereas, the treatment with 10 or 100 μM ATPγ-S for 48 h in the presence of 5.35 mM $CaCl_2$ significantly increases the cell number respect to control (Fig. 2).

EFFECT OF ATPγ-S AND HIGH CALCIUM TREATMENT ON THE RAT CALVARIAL CELLS VIABILITY AND GROWTH IN OSTEOGENIC MEDIUM

Cells were cultured in osteogenic medium with 1.36 mM ($-Ca^{2+}$l) or 5.35 mM Ca^{2+} ($+Ca^{2+}$) in the presence or absence of 10 or 100 μM ATPγ-S for 24, 48, and 72 h. Then cell number and viability were determined by using Trypan blue as indicated in the Methods section. The osteoblasts treated with 100 μM ATPγ-S as well as those cultured in the presence of 5.35 mM Ca^{2+} during 24 h showed a significantly lower cell count compared to the control condition with normal calcium concentration ($-Ca^{2+}$). However, 10 or 100 μM ATPγ-S with high Ca^{2+} combined treatment not showed significant changes respect to calcium alone at that time of treatment. After 48 h of treatment with 10 or 100 μM ATPγ-S, cell count was significantly reduced respect the control. Treatments with Ca^{2+} alone or ATPγ-S plus Ca^{2+} remained with lower counts, similar to those of 24 h and significantly below control. After 72 h of 100 μM ATPγ-S treatment, cell count remained significantly below the control. By other hand, control condition showed a significant increase in cell counts at 48 and 72 h compared to those of 24 h, whereas treatments with Ca^{2+} with or without ATPγ-S for 48 h showed similar cell counts to those of 24 h and they reached values of the control at 72 h (Fig. 3). Cell viability was maintained above 90% in all conditions without significant changes between treatments (Fig. 3B). These results suggest that cell treatment with osteogenic medium containing high calcium concentration (5.36 mM Ca^{2+}) inhibits cell growth at 24 and 48 h, treatment with 100 μM ATPγ-S produced a similar effect that remains until 72 h, whereas 10 μM ATPγ-S treatment only shows this effect at 48 h.

EFFECT OF ATPγ-S AND HIGH CALCIUM TREATMENT ON RAT CALVARIAL CELL OSTEOGENIC DIFFERENTIATION

Our previous studies show that treatment with 100 μM ATP or UTP enhances the expression of osteogenic differentiation markers ALP, BMP-2, BMP-4, BMP-5, and BSP in rat calvarial cells culture after several days (4–7 days) of treatment [Ayala-Peña et al., 2013]. Now, we evaluated the effect of ATPγ-S in combination with a high concentration of extracellular calcium (5.35 mM) on osteogenic differentiation of the cells. First, we studied the expression of three Bone Morphogenetic Proteins (BMP) genes, BMP-3, BMP-4, and BMP-5, after incubating the cells for 16 and 48 h in osteogenic medium containing 5.35 mM Ca^{2+} in the presence or absence of 10 or 100 μM ATPγ-S. As the figure shows, after cell incubation for 48 h in osteogenic medium with high calcium concentration, it was observed a significant increase in gene expression of BMP-4 respect to cells that were incubated 16 h, while other genes studied in these conditions showed no significant changes (Fig. 4A). Combined treatment of cells with 5.35 mM Ca^{2+} plus 100 μM but not 10 μM ATPγ-S for 16 h significantly increased the expression of BMP-4 around twofold above control. When the cell combined treatment was performed for 48 h, BMP-4 expression also maintained elevated in presence of 10 and 100 μM ATPγ-S respects to control but it was not significant (Fig. 4B). In the case of BMP-5, a significantly increased expression, around 60% above control, after 48 h combined treatment of cells with 5.35 mM Ca^{2+} and 10 μM

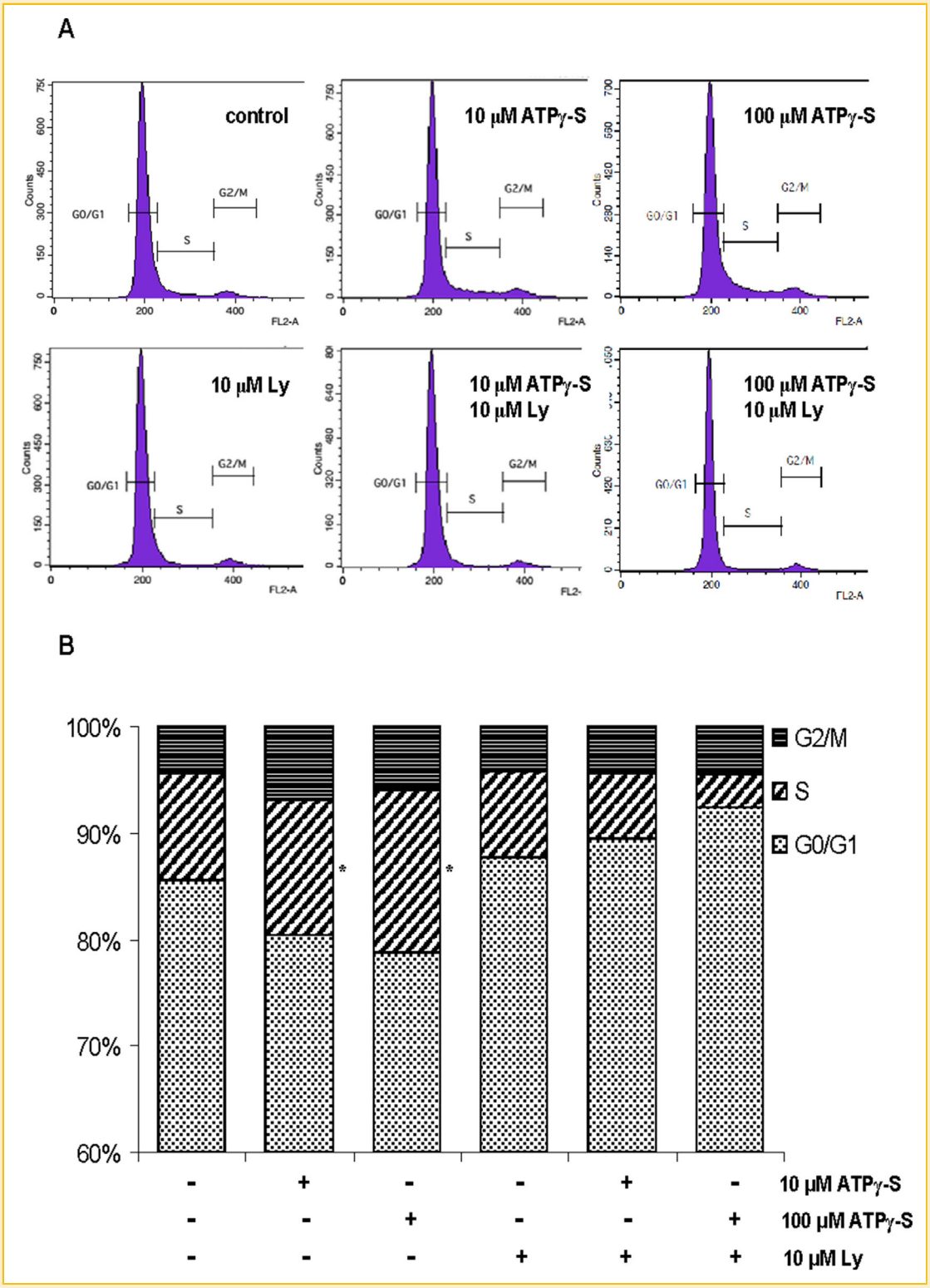

Fig. 1. ATPγ-S activates cell cycle of neonatal rat calvarial cells. Cells were treated with ATPγ-S (10–100 μM) or vehicle (control) in the presence or absence of 10 μM Ly204002 (Ly) for 16 h. They were then processed for flow cytometry as described in the Materials and Methods section. (A) Histograms represent cell number (Counts) versus fluorescence intensity (FL2-A). The graphics are from a representative experiment of three separate experiments. (B) Represents the percentage of the cell population in each stage of the cycle (G_0/G_1, S, and G_2/M). Data from three experiments performed independently (mean ± SD) are shown. $^*P < 0.05$ versus control.

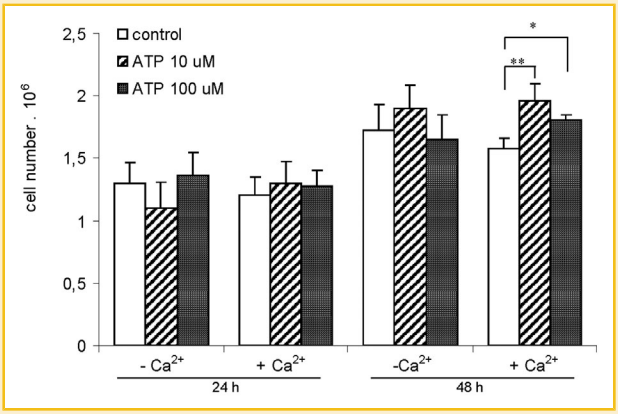

Fig. 2. ATPγ-S plus Ca^{2+} combined treatment stimulates proliferation of neonatal rat calvarial cells. Cells incubated in αMEM 1% SFB were treated with vehicle (control) or the indicated amounts of ATPγ-S in the presence of 1.36 mM Ca^{2+} ($-Ca^{2+}$) or 5.35 mM Ca^{2+} ($+Ca^{2+}$) for 24 and 48 h. Then, number of cells was determined as described in the Materials and Methods section. The data shown are the cell number average ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

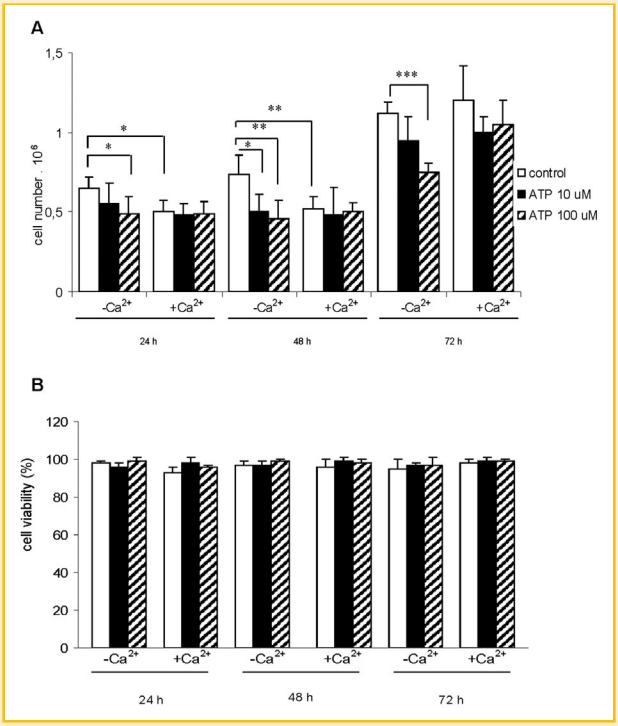

Fig. 3. Effect of ATPγ-S and high Ca^{2+} on growth and viability of neonatal rat calvarial cells in osteogenic medium. Cells incubated in osteogenic medium (αMEM 1% SFB, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid) were treated with vehicle (control) or the indicated amounts of ATPγ-S (ATP) in the presence of 1.36 mM Ca^{2+} ($-Ca^{2+}$) or 5.35 mM Ca^{2+} ($+Ca^{2+}$) for 24, 48, and 72 h. (A) Then, number of cells was determined as described in the Materials and Methods section. The data shown are the cell number average ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.025$; $^{***}P < 0.01$. (B) Cells were treated as indicated above and then, cell viability was determined as described in the Materials and Methods section. The results are expressed as viable cell percentage (mean ± SD) (n = 3).

ATPγ-S was observed (Fig. 4C). No significant changes were observed in BMP3 gene expression relative to control after cell treatment with 10 or 100 μM ATP(gamma)-S for 16 or 48 h (not shown). In addition, the expression of osteopontin (OPN), a marker of osteogenic differentiation, which is expected to express more belatedly than osteogenesis inducer genes such as BMPs, after 72 h of combined treatment with 5.35 mM Ca^{2+} and ATPγ-S was evaluated. Both the stimulation with 10 μM, as well as 100 μM ATPγ-S, significantly increased the expression of OPN over the control (Fig. 5). These results suggest that calvarial cell treatment with ATPγ-S (10–100 μM) in an osteogenic medium containing 5.35 mM Ca^{2+} stimulates the expression of osteogenic genes as BMP-4 and BMP-5 and the OPN, a gene related with extracellular matrix (ECM) production.

In previous work, it was suggested that stimulation of P2Y2 receptor (responsive to ATP, ATPγ-S, and UTP) enhance osteoblasts differentiation [Ayala-Peña et al., 2013]. To check the involvement of this receptor subtype, cells were treated for 4, 5, and 6 days with 10 and 100 μM UTP or UDP in osteogenic medium containing 5.35 mM Ca^{2+} and then, ALP activity was measured as indicated in the Materials and Methods section. The results show that treatment for 6 days with 10 μM UTP and 100 μM UDP significantly increased the ALP activity respect to the control (Fig. 6). The remaining conditions showed no significant differences from controls (data not shown). Thus, this data show that calvarial cell treatment with 10 μM UTP or 100 μM UDP in an osteogenic medium containing 5.35 mM Ca^{2+} stimulates osteoblasts differentiation suggesting the involvement of P2Y2 and P2Y6 receptor subtypes.

EFFECT OF ATPγ-S AND HIGH CALCIUM TREATMENT ON RAT CALVARIAL CELL CULTURES MINERALIZATION

Calvarial osteoblasts reach a mature stage after long incubation times (>15 day) in osteogenic medium and mineralization nodules can be visualized around day 20, or even later. For this reason, the effect of cell treatment with ATP and high calcium on extracellular matrix mineralization after long incubation times was assessed. This was performed by determining the calcium deposits on cell cultures using the Alizarin Red organic dye, as described in the Methods section. Cells were treated with 10 μM ATPγ-S in osteogenic medium with normal (1.36 mM Ca^{2+}) or high (5.35 mM Ca^{2+}) calcium for 15, 18, and 22 days.

Cells grown in osteogenic medium with high Ca^{2+} showed a statistically significant increase in calcium deposits at 18 and 22 days, respect to control. Cell treatment with 10 μM ATPγ-S induced a significant increase in mineralization that was detectable at 15 and 18 days with respect to control. When the treatment of 10 μM ATPγ-S was combined with high Ca^{2+} greater and significant mineralization was observed at 15 and 18 days respect to ATPγ-S alone and both agonist, respectively (Fig. 7). These results suggest that calvarial cell treatment with ATPγ-S (10 μM) in an osteogenic medium containing 5.35 mM Ca^{2+} stimulates the in vitro culture mineralization.

DISCUSSION

In the present study, we show for the first time the up-regulation of BMP-4, BMP-5, and OPN genes as well as the stimulation of

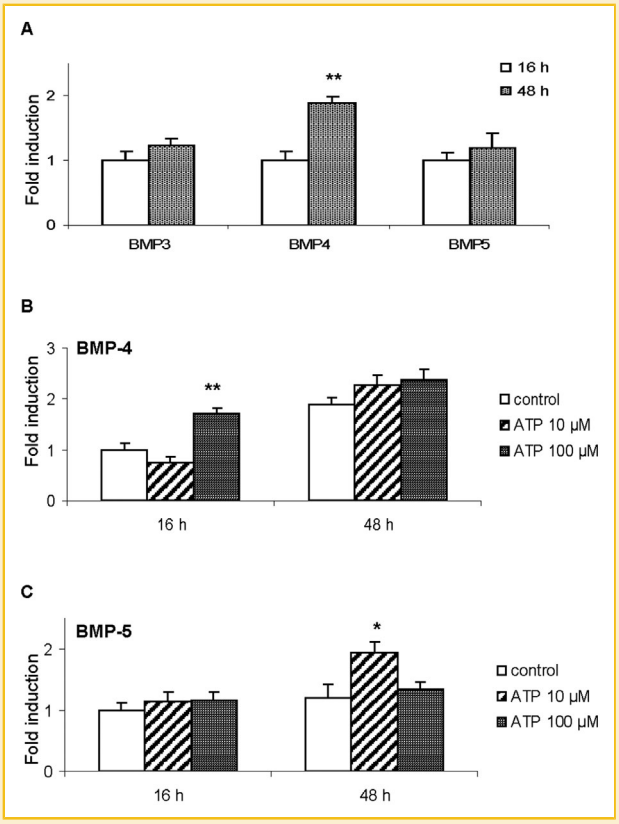

Fig. 4. Effect of high Ca^{2+} and ATPγ-S combined treatment on osteogenic gene expression in neonatal rat calvarial cells: cells were incubated in osteogenic medium containing 5.35 mM Ca^{2+} for 16 and 48 h, and total cellular RNA was extracted. Then, quantification of BMP-3, BMP-4, and BMP-5 mRNA by real-time PCR analysis was performed as indicated in the Materials and Methods section (A). Cells were incubated in osteogenic medium containing 5.35 mM Ca^{2+} in the presence or absence of the indicated amount of ATPγ-S (ATP) for 16 and 48 h, and total cellular RNA was extracted. Then, quantification of BMP-4 (B) and BMP-5 (C) mRNA by real-time PCR analysis was performed as indicated in the Materials and Methods section. The expression levels of target genes were standardized by GAPDH level in each sample and normalized with respect to 16 h control. Results are shown as average value ± SD of data from two separate experiments, each sample processed in triplicate. $^{*}P < 0.025$; $^{**}P < 0.01$ versus control.

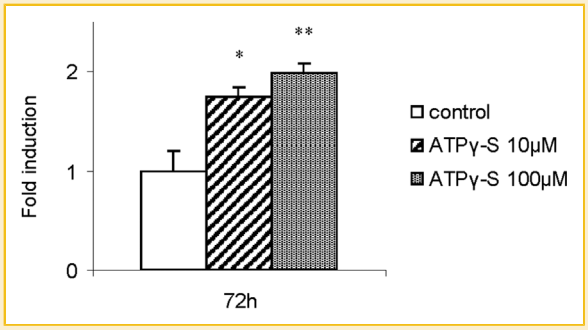

Fig. 5. Effect of ATPγ-S plus high Ca^{2+} combined treatment on OPN gene expression in neonatal rat calvarial cells: cells were incubated in osteogenic medium containing 5.35 mM Ca^{2+} in the presence or absence of the indicated amount of ATPγ-S for 72 h, and total cellular RNA was extracted. Then, quantification of OPN mRNA by real-time PCR analysis was performed as indicated in the Materials and Methods section. The expression levels of target gene was standardized by GAPDH level in each sample and normalized with respect to control. Results are shown as average value ± SD of data from two separate experiments, each sample processed in triplicate. $^{*}P < 0.05$; $^{**}P < 0.025$ versus control.

osteogenic differentiation and mineralization of primary cultures of neonatal rat calvarial cells by an osteogenic medium containing a micromolar concentration of ATPγ-S (10–100 μM) and high concentration of Ca^{2+} (5.35 mM). Neonatal rat calvarial cell cultures are a heterogenic cell population majorly containing mesenchymal stem cells, committed osteoprogenitor cells, preosteoblasts, and osteoblasts. Under appropriate conditions, these cell cultures can differentiate into osteoblasts, condrocytes, or adipocytes as it has been described [Pirih et al., 2008; Weivoda and Hohl, 2012].

Despite known, several osteogenic formulations as well as the importance of each of its constituents for induction of in vitro osteogenic differentiation, this has not yet been fully optimized [Vater et al., 2011]. Furthermore, the conducing mechanism of osteogenesis has not been totally elucidated.

Calcium is a known inducer of cell proliferation and osteogenic differentiation as has been well demonstrated in mesenchymal stem cells, preosteoblasts, and osteoblasts [Eklou-Kalonji et al., 1998; Dvorak et al., 2004; Honda et al., 2006; Chai et al., 2010; Kanaya et al., 2010; Nakamura et al., 2010; Khoshniat et al., 2011; González-Vázquez et al., 2014; Hu et al., 2014]. In the present study, we have detected that cell treatment with ATPγ-S for 48 h in the presence of high Ca^{2+} significantly increase cell number, whereas we did not detect changes in cell treatment with Ca^{2+} or ATPγ-S alone respect to control, this may be due to experimental procedure used, cell counting in hemocytometer, which is less sensitive than MTT assay or flow cytometry to detect changes in proliferation rate [Pozarowski and Darzynkiewicz, 2004; Balu et al., 2009]. Instead, by flow cytometry an important increase in transition from G_0/G_1 to S phase after cell incubation for 16 h in the presence of 10 and 100 μM ATPγ-S respect to control was detected. Effect that was blocked by the use of the PI3K inhibitor Ly294001 confirming the involvement of PI3K pathway in the mitogenic action of ATP as has been previously suggested in osteoblasts and other cell types [Katz et al., 2011; Bilbao et al., 2010]. Thus in together, these results evidence that combined treatment of high Ca^{2+} and ATPγ-S has a positive effect on cell proliferation that was not detected for each compound per separated through cell counting.

Under osteogenic conditions both ATPγ-S and high Ca^{2+} per separated shown to decrease cell number, suggesting inhibition of cell growth but they not have synergist or additive effects when were used in together. This effect could be mediated by a similar mechanism or to involve the same signal transduction pathways. However high ATPγ-S concentration (100 μM) shown a different response after 72 h.

Action of extracellular ATP on in vitro osteoblasts it has been shown mediated by activation of several P2 receptors subtypes. Rat osteoblasts express mRNA and protein for all P2 receptor subtypes, except P2Y11, and P2 receptor pattern expression changes during

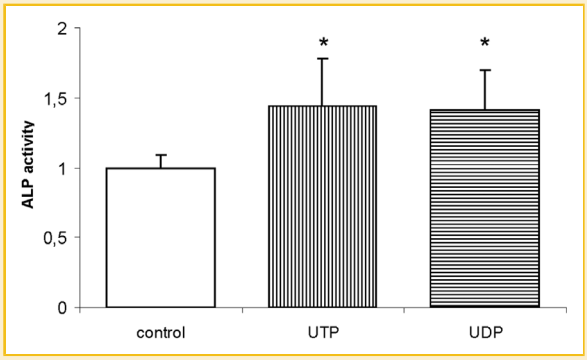

Fig. 6. Effect of UTP or UDP plus high Ca^{2+} combined treatment on ALP activity in neonatal rat calvarial cells: cells were incubated in osteogenic medium containing 5.35 mM Ca^{2+} in the presence or absence of 10 μM UTP or 100 μM UDP for 6 days. Then, quantification of ALP activity was performed as indicated in the Materials and Methods section. Each value is the average ± SD of three independent experiments performed in triplicate. $^{*}P < 0.025$ versus control.

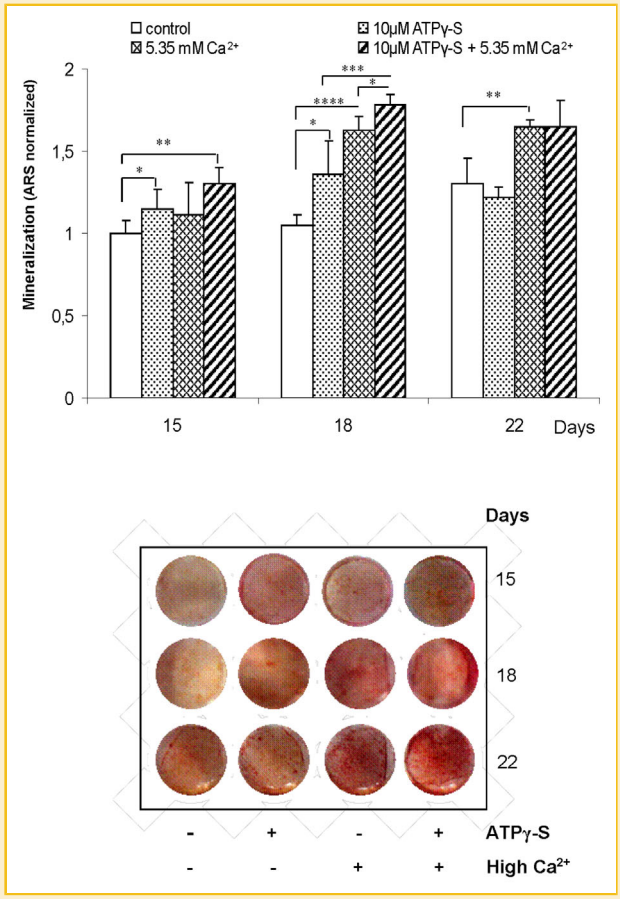

Fig. 7. Effect of ATPγ-S and high Ca^{2+} treatment on neonatal rat calvarial cells mineralization: cells were incubated in osteogenic medium with 1.36 mM Ca^{2+} (control) or 5.35 mM Ca^{2+} (high Ca^{2+}) in the presence or absence of 10 μM ATPγ-S for 15, 18, and 22 days. Then, quantification of mineral matrix deposition after alizarin red staining was performed as indicated in the Materials and Methods section. Each value is the average ± SD of two independent experiments performed in triplicate. Under the graphics a representative photograph of each condition is shown. $^{*}P < 0.05$; $^{**}P < 0.025$; $^{***}P < 0.01$; $^{****}P < 0.005$.

osteoblasts differentiation in vitro [Orriss et al., 2006, 2012]. Between P2Y receptors expressed in osteoblasts, the P2Y1,2,4, and 6 subtypes couple to $G_{q/11}$ protein to activate phosphatidylinositol-specific phospholipase C (PI-PLC), with the consequent rise in inositoltriphosphate (IP_3), diacylglycerol (DAG) and intracellular calcium [Ralevic and Burnstock, 1998; Burnstock, 2007]. The main agonists of P2Y1 receptors are ADP and ADPβ-S, P2Y6 receptors respond to UDP and rat P2Y2 and P2Y4 receptors respond equipotently to ATP and UTP, but ATPγ-S is considered a strong agonist at P2Y2 and weak at P2Y4 receptor [Wildman et al., 2003]. Taking this in consideration, we suggest that the P2Y2 receptor signaling pathway activated by ATPγ-S contribute to the Ca^{2+} effect on osteogenesis observed here. ATP stimulation of P2Y2 receptors activates the ERK1/2, JNK1, and PI3K/AKT pathway through PLC/IP3/Ca^{2+}, PKC, Src, and CaM in an osteoblastic cell line [Katz et al., 2006, 2008, 2011]. Activation of PI3K/AKT signaling pathway by ATP via P2Y2 receptors also stimulates cell proliferation and osteogenic differentiation in rat primary calvarial cells [Katz et al., 2011; Ayala-Peña et al., 2013]. However, other studies shown that extracellular nucleotides block osteoblasts differentiation and bone mineralization in vitro, suggesting a dual inhibitory mechanisms involving both P2 receptors (mainly P2Y2, P2X1, and P2X7 receptors) and pyrophosphate [Orriss et al., 2006, 2007, 2012]. These studies disagree with our work, such discrepancies may be due to different experimental conditions as composition of culture medium, the strain of animals used, differences in ligand binding to specific receptors, changes in affinity, expression levels of the receptors, hydrolysis of agonist, the intracellular calcium concentration, and the expression of ectonucleotidases, between others.

In several cell types, including osteoblasts, increases in intracellular calcium concentration after stimulation of P2Y2 receptors by ATP, at a range of concentration similar to the used here (10–100 μM), have shown to be mediated by calcium release from intracellular stores, induced by PLC/IP3 pathway activation, and calcium influx through different plasma membrane calcium channels such as store depletion operated channels (SOC), capacitative calcium entry (CCE), voltage-dependent channels (VDCC), mechanical stress sensitive channels (SAC) [Seiler et al., 1999; Katz et al., 2006, 2008; Scodelaro Bilbao et al., 2007; Dong et al., 2009; Ryu et al., 2010]. Several of these signal transduction pathway were also found to be activated by changes of extracellular calcium concentration in bone cells. In osteoblastic cells, extracellular calcium activates phospholipase C and Protein Kinase C (PKC) [Godwin and Soltoff, 2002; Pierre, 2010], extracellular signal-regulated kinases (ERK1/2) [Huang et al., 2001; Dvorak et al., 2004], JNK [Chattopadhyay et al., 2004], cAMP/protein kinase A (PKA) [Choudhary et al., 2004] and PI3 K/Akt [Marie, 2012] pathways, some of which are gated by calcium-induced activation of calcium sensing receptor (CaSR), calcium influx through voltage-dependent calcium channels (VDCC), and/or capacitative calcium entry (CCE) between others [Abed et al., 2009; Wen et al., 2012; Hu et al., 2014]. Abolition of function of the CaSR, also a Gq protein coupled receptor, decreases

osteoblast gene expression of murine calvarial osteoblastic cells [Yamauchi et al., 2005]. Of relevance, bones from P2Y2 receptor knockout mice have significantly decreased volume and thickness. In addition, P2Y2 receptor deficiency reduces the osteogenic differentiation and mineralization of bone marrow cells [Xing et al., 2014]. In agreement with this, we show here that the incubation of rat calvarial cells with an osteogenic medium containing 5.35 mM Ca^{2+} rapidly incremented (within 36 h) the expression of BMP-4, a specific gene related with osteogenesis. Moreover, the incorporation of 10–100 μM ATPγ-S into the osteogenic treatment medium containing high Ca^{2+} produced an additional increase in the expression of the mentioned gene and also increases BMP-5. The fact that BMP-4 increased first, suggests that they could upregulate the expression of other genes implicated in osteogenesis, such as BMP-5. In agreement with this, it has been suggested that BMP-4 is required for the osteogenic activity of osteoblasts. In addition, it was reported the requirement of a BMP for osteoblast differentiation and function and that a BMP can functionally substitute another BMP in an autocrine/paracrine manner or mediate a response to an endocrine action on osteoblasts [Martinovic et al., 2006].

Other effect of the combined treatment of high Ca^{2+} plus the purinergic agonist, was enhance the gene expression and activity of proteins required for extracellular matrix (ECM) production, such as OPN and ALP. OPN is an early marker of osteoblasts development and plays a role in bone regeneration and bone remodeling. In addition, it is proposed that OPN may be involved in inhibition of the pathologic calcification of the bone [Jahnen-Dechent et al., 2008; Saad et al., 2008]. The observation that OPN was significantly up-regulated by ATPγ-S in the presence of 5.35 mM Ca^{2+} suggests that signaling pathways associated to purinergic receptors and the calcium messenger system regulate bone matrix synthesis and mineralization. Interestingly, up-regulation of OPN expression in osteoblastic cell monolayer cultures has also been shown by mechanical stress as compressive forces, which also stimulates ATP release from these cells [Rath et al., 2008; Sawada et al., 2008]. Our findings show increased ALP activity after cell stimulation with low concentration of UTP (10 μM) or high concentration of UDP (100 μM) in osteogenic medium containing 5.35 mM Ca^{2+}, suggesting that these conditions promote osteogenic differentiation.

Data obtained in this study suggest that calvarial cells cultured in osteogenic medium supplemented with low ATPγ-S concentration (10–100 μM) and high Ca^{2+} (5.35 mM) upregulates osteogenic genes BMP-4 and BMP-5 stimulating osteoblasts maturation. Consistent with our results, exogenous BMP-4 and BMP-5 have been shown to stimulate the differentiation of primary murine bone marrow cell and human mesenchymal stem cells into fully mature osteoblasts in vitro [Paul et al., 2009; Cordonnier et al., 2011].

Our data also show that osteogenic medium containing 5.35 mM Ca^{2+} and ATPγ-S acts as an expression potent activator of osteogenic genes, accelerating the differentiation process. This is sustained by the fact that cell incubation in same osteogenic medium with normal calcium concentration (1.36 mM) required more than 3–4 days and stimulation with high concentration of ATP or UTP (100 μM) to show changes in BMP-4 and BMP-5 gene expression as we have previously showed [Ayala-Peña et al., 2013].

Recently, it was suggested that P2 receptor activation modulates the differentiation of osteoblasts in a manner dependent upon the stage of differentiation. Thus, ATP treatment of preosteoblasts cells increased the expression of transcription factors associated with osteoblast differentiation, such as Runx2, SP7, and Dix5, whereas SP7 and Dix5 expression was reduced by ATP in differentiated osteoblasts. Furthermore, treatment with ATP modulates the expression of the P2 receptors, increase expression of P2X4, reduces P2Y6 and P2X7 but does not modify the P2Y2 expression, suggesting an important role for the latter in osteogenic differentiation [Rodrigues-Ribeiro et al., 2015]. In agreement with this, our results suggest that high extracellular calcium concentration acting together with ATPγ-S may control in vitro osteoblast differentiation in part through activation of the P2Y2 receptor subtype. This is in agreement with the fact that UTP, which is an equipotent agonist at P2Y2 and P2Y4 receptors, stimulates ALP activity. As UDP, the main agonist of P2Y6 receptor, also increases de ALP activity in the condition employed in this study, the involvement of P2Y6 is not ruled out. Future research, addressed to determine the expression of the different P2 receptors under the conditions tested in this study are necessary to confirm the involvement of each P2 receptor subtypes. Probably, several second messengers induced by extracellular calcium activated signaling may contributing to the ATPγ-S osteogenic action in osteoblasts.

Thus, our results suggest that both agents, ATPγ-S and Ca^{2+}, acting in combined form have a synergistic effect. In relation with this, calcium deposition presented a major increase with the combined treatment than the treatment with the separated agonists, suggesting that an in together action of ATPγ-S and calcium can favor the mineralization process.

In conclusion, in this investigation we demonstrated that osteogenic medium supplemented with 10 μM ATPγ-S acts synergistically with high Ca^{2+} concentration (5.35 mM) to enhance osteogenesis and mineralization by rat primary calvarial cells cultures. This know may be of potential application in the development of new study methods in tissue engineering of mineralized bone in vitro, could offer an alternative approach to osseous regeneration by shortening the obtaining time of mature osteoblasts capable for to synthesize new bone in vitro, and also to address commitment of progenitor cells into osteoblast linage for to implant in bone tissue reconstitution in vivo.

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