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Osteoblast responses to nucleotides increase during differentiation

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Abstract

Accumulating evidence suggests that extracellular nucleotides, signaling through P2 receptors, play a role in modulating bone cell function. ATP and ADP stimulate osteoclastic resorption, while ATP and UTP are powerful inhibitors of bone formation by osteoblasts. We investigated changes in the expression of P2 receptors with cell differentiation in primary osteoblast cultures. Rat calvarial osteoblasts, cultured for up to 10 days, were loaded with the intracellular Ca^{2+}-sensing fluorophore, Fluo-4 AM, and a fluorescence imaging plate reader was used to measure responses to nucleotide agonists. Peak responses occurred within 20 s and were evoked by ATP or UTP at concentrations as low as 2 μM. Osteoblast number doubled between day 4 and 10 of culture, but the peak intracellular Ca^{2+} response to ATP or UTP increased up to 6-fold over the same period, indicating that osteoblast responsiveness to nucleotides increases as cell differentiation proceeds. The approximate order of potency for the most active nucleotide agonists at day 8 of culture was ATP > UTP and ATPγS > ADP > UDP, consistent with the expression of functional $P2Y_2$, $P2X_2$, $P2Y_4$, $P2Y_1$ and $P2Y_6$ receptors. Smaller responses were elicited by 2-MeSATP, Bz-ATP and α,β-meATP, additionally suggesting the presence of functional $P2X_1$, $P2X_3$, $P2X_5$ and $P2X_7$ receptors. Expression of mRNA for the ATP- and UTP-selective $P2Y_2$ receptor increased strongly between day 6 and 15 in primary rat osteoblasts, whereas mRNAs for the $P2Y_4$ (also ATP/UTP selective) and $P2Y_6$ (UDP/UTP selective) receptors were highly expressed at intermediate time points. In contrast, mRNA for the cell-proliferation-associated $P2X_5$ receptor decreased to undetectable as osteoblasts matured, but mRNA for the cell-death-associated $P2X_7$ receptor was detected at all time points. Similar trends were evident using immunostaining and Western blotting for P2 receptors. Exposure to 10 μM ATP or UTP during days 10–14 of culture was sufficient to cause near-total blockade of the 'trabecular' bone nodules formed by osteoblasts; however, UDP and ADP were without effect. Our results show that there is a shift from P2X to P2Y expression during differentiation in culture, with mature osteoblasts preferentially expressing the $P2Y_2$ receptor and to a lesser extent $P2Y_4$ and $P2Y_6$ receptors. Taken together, these data suggest that the $P2Y_2$ receptor, and possibly the $P2Y_4$ receptor, could function as 'off-switches' for mineralized bone formation.

Introduction

Growing evidence suggests that extracellular nucleotides, signaling through P2 receptors, might play a significant role in bone biology modulating both osteoblast and osteoclast function [1]. The P2 receptors are grouped into the P2X ligand-gated ion channels and P2Y G-protein-linked receptors [2]. Currently, there are seven known P2X subtypes ($P2X_{1-7}$) and eight P2Y subtypes $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$; each of these receptors has been cloned, characterized and displays distinct tissue expression and pharmacology [3].

Studies in the early 1990s showed that extracellular nucleotides act on osteoblast-like cells to induce formation of IP_3 and to elevate intracellular Ca^{2+} transiently; pharmacological profiles were suggestive of the expression of $P2Y_1$- and $P2Y_2$-like receptors (reviewed by [1]). Studies on primary human osteoblasts indicated heterogeneity of P2 receptor expression since all cells exhibited intracellular Ca^{2+} responses to ATP and UTP (suggestive of $P2Y_2$ and/or $P2Y_4$ receptors) but only a sub-population responded to ADP (which is selective for the $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors) [4]. This was taken to indicate that expression of P2 receptors changes during the osteoblast life cycle, depending on the

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differentiation state. Messenger RNA for $P2Y_2$ receptors was localized cytochemically in human osteoblasts [5], and mRNA for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors was detected by PCR in human osteosarcoma cell lines [6]. Evidence for the expression of $P2X_2$, $P2X_5$, $P2Y_1$ and $P2Y_2$ receptors, at the protein or mRNA level, was reported subsequently on rat osteoblasts [7].

Evidence from in vitro studies indicates that relatively low concentrations of extracellular nucleotides exert P2-receptor-mediated effects that would shift the bone remodeling balance strongly in the negative direction [1]. ATP and ADP at concentrations within the approximate range of 0.1 to 10 μM stimulate both the formation and resorptive activity of rodent osteoclasts and resorption in bone organ cultures [8,9]. These findings, together with cytochemical evidence, suggest the involvement of the $P2Y_1$ receptor in mediating the osteolytic effects of ATP [7]. In contrast, both UTP and ATP, at concentrations as low as 1–10 μM, cause strong inhibition of mineralized bone nodule formation by cultured rat osteoblasts, whereas adenosine and ADP were without effect [10]. These potent actions of ATP and UTP point to the involvement of either $P2Y_2$ or $P2Y_4$ receptors. $P2Y_2$ receptors have also been shown to mediate the propagation of intercellular Ca^{2+} waves [11], as well as oscillatory fluid flow-induced Ca^{2+} mobilization in osteoblast-like cell lines [12]. Nucleotide signaling in bone via $P2Y_1$ and $P2Y_2$ receptors could also modulate local remodeling responses to key osteotropic agents such as parathyroid hormone [13,14].

The aim of the present study was to document changes in P2 receptor expression and functional responses to extracellular nucleotides in normal populations of bone-nodule-forming osteoblasts as differentiation and maturation proceed.

Materials and methods

Reagents

All chemicals were purchased from Sigma Aldrich (Poole, Dorset, UK) unless otherwise stated. Fluo-4 AM was supplied by Molecular Probes (Invitrogen, Paisley, UK). Reverse transcriptase, DNA polymerase and other molecular biology reagents were purchased from Promega UK (Southampton, Hampshire, UK), and all primers were from MWG Biotech (Ebersberg, Germany). P2Y primary antibodies were obtained from Alomone (Jerusalem, Israel), P2X antibodies from Roche Bioscience (Palo Alto, CA, USA) and donkey anti-rabbit Cy3-labeled secondary antibodies from Jackson Immuno-research Laboratories (Philadelphia, USA) and Citifluor from Citifluor (London, UK).

Cell culture

Primary rat osteoblast cells were obtained by sequential enzyme digestion of excised calvarial bones from 2-day-old neonatal Sprague–Dawley rats using a 3-step process (1% trypsin in PBS for 10 min; 0.2% collagenase type II in Hanks balanced salt solution (HBSS) for 30 min; 0.2% collagenase type II in HBSS for 60 min). The first two digests were discarded, and the cells resuspended in Dulbecco's Modified Essential Medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin (complete mixture abbreviated to DMEM). Cells were cultured for 2–4 days in a humidified atmosphere of 5% CO_2–95% air at 37°C in 75 cm^2 flasks until confluent. Upon confluence, cells were sub-cultured into 6, 24 or 96-well trays in DMEM supplemented with 2 mM β-glycerophosphate, 50 μg/ml ascorbic acid and 10 nM dexamethasone (mixture abbreviated as 'supplemented DMEM'), with half medium changes every 2–3 days.

Bone nodule formation by osteoblasts cultured in 6-well plates at an initial density of 10^5/well was measured using modifications of an assay described previously [10]. Briefly, experiments were terminated by fixing cell layers in 2% glutaraldehyde for 5 min; mineralized bone nodules were visualized by staining with alizarin red (1% solution in water) for 5 min, rinsed with 50% ethanol to remove excess stain then air-dried. The plates were imaged at 800 dpi using a high-resolution flat-bed scanner (Epson Perfection Photo 3200). Binary images of each individual well were then subjected to automated analysis (Scion Image software, Scion Corporation; http://www.scioncorp.com), using constant 'threshold' and 'minimum particle' levels, to determine the number and plan surface area of mineralized bone nodules.

Total RNA extraction and complementary DNA strand synthesis

Osteoblasts were cultured in 6-well trays for 6–15 days in supplemented DMEM; every 2–3 days, total RNA was extracted from 2 wells using TRIZOL® reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Before first-strand complementary DNA (cDNA) synthesis, extracted RNA was treated with RNase-free DNase I (35 U/ml) for 30 min at 37°C. The reaction was terminated by heat inactivation at 65°C for 10 min. Total RNA was quantified spectrophotometrically by measuring absorbance at 260 nM. For each sample, 0.5 μg of DNase-treated total RNA was used as a template for first-strand cDNA synthesis in a 20 μl reaction also containing 0.5 μg oligo dT, 3 mM $MgCl_2$, 0.5 mM dNTPs, 20 U recombinant RNasin® ribonuclease inhibitor, ImProm-II® 5× reaction buffer and 200 U ImProm-II reverse transcriptase. The reaction mix was annealed for 5 min at 25°C followed by extension at 42°C for 60 min and inactivation at 70°C for 15 min. cDNA was stored at −20°C until amplification by PCR.

Polymerase chain reaction (PCR)

Rat osteoblast derived cDNA was amplified by PCR in 25 μl reactions containing ~0.5 μg cDNA, 0.2 mM dNTP (10 mM stock), 1.5 mM $MgCl_2$, 0.2 μM of both sense and antisense primer and 1 U Taq DNA polymerase in thermophilic DNA polymerase 10× buffer. PCR was performed according to manufacturer's instructions, with cycles of denaturation at 95°C for 30 s, annealing for 30 s (see Table 1 for annealing temperatures), extension at 72°C for 45 s and reaction termination at 72°C for 5 min. For analysis, PCR products

Table 1
Primer sequences and annealing temperatures for RT-PCR

Protein	Primer sequence (5′–3′)	bp	t_a (°C)
$P2X_2$	S — GAATCAGAGTGCAACCCCAA	357	58.5
	AS — TCACAGGCCATCTACTTGAG		
$P2X_5$	S — GCCGAAAGCTTCACCATTTCCATAA	418	58.5
	AS — CTATCACATCAAAGCGGATGCCGTAGG		
$P2X_7$	S — GTGCCATTCTGACCAGGGTTGTATAAA	354	58.5
	AS — GCCACCTCTGTAAAGTTCTCTCCGATT		
$P2Y_1$	S — ACGTCAGATGAGTACCTGCG	289	57.4
	AS — CCCTGTCGTTGAAATCACAC		
$P2Y_2$	S — CTGGTCCGCTTTGCCCGAGATG	311	58.5
	AS — TATCCTGAGTCCCTGCCAAATGAGA		
$P2Y_4$	S — TGTTCCACCTGGCATTGTCAG	294	58.5
	AS — AAAGATTGGGCACGAGGCAG		
$P2Y_6$	S — TGCTTGGGTGGTATGTGGAGTC	339	60.0
	AS — TGGAAAGGCAGGAAGCTGATAAC		
β-actin	S — GTTCGCCATGGATGACGAT	332	53.0
	AS — TCTGGGTCATCTTTTCACGG		
OCN	S — GCAGACACCATGAGGACC	418	59.8
	AS — GCAGCTGTGCCGTCCATAC		

Abbreviations: OCN, osteocalcin; S, sense; AS, antisense; bp, size of product in base pairs; t_a, annealing temperature.

were loaded on to 1% agarose gels containing 0.3 μg/ml ethidium bromide. Gels were run at ~80 mA and the DNA position visualized by exposure to UV light. To account for differences in original cell number and cDNA quality, all samples were normalized against mRNA for β-actin. The expression of osteocalcin (OCN) and the $P2X_2$, $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors was investigated in osteoblasts cultured for 6, 8, 11, 13 and 15 days. All reactions were carried out in triplicate using cDNAs derived from 3 different osteoblast cultures. Primer sequences and annealing temperatures are shown in Table 1. The identity of PCR products generated by these primers has previously been confirmed by sequencing [15–17].

Immunofluorescence

Rat osteoblastic cells were seeded onto sterile 1 cm diameter discs, cut from Melinex (Du Pont, Dumfries, UK) clear polyester film, in 24-well trays at 5×10^4 cells/disc and cultured in supplemented DMEM for 4–18 days. Every 3 days, discs were removed and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 20 min at room temperature, washed 3 × 5 min with PBS and stored at 4°C in PBS–methiolate until staining. Each disc was incubated with a blocking solution consisting of 10% normal horse serum (NHS), 0.05% methiolate and 0.1% Triton X100 in PBS for 1 h. Rabbit primary antibodies were diluted in blocking solution at 1:200 (P2X receptors) or 1:100 (P2Y receptors). Discs were incubated overnight in the primary antibody solution with gentle agitation. Following removal of the primary antibody solution, cells were subjected to three 5-min washes with PBS before incubation for 1 h with the donkey anti-rabbit Cy3-labeled secondary antibody solution (1:400) and a DAPI counterstain (1:3500), diluted in PBS with 1% NHS and 0.05% methiolate. After three further 5-min PBS washes, discs were mounted on to microscope slides using Citifluor AF2 solution and stored at 4°C or viewed by fluorescence microscopy (Cy3 absorbance and emission at 550 nm and 570 nm, respectively). All images were acquired using constant camera settings to allow comparison of staining intensity. Control experiments were performed with the primary antibody omitted from the staining procedure.

Western blot

Primary rat osteoblasts were cultured for 15 days in supplemented DMEM (differentiation medium) or DMEM (normal medium), and protein was extracted after 4, 8 and 15 days. Osteoblast monolayers were lysed in ice-cold radio immunoprecipitation (RIPA) lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% SDS 1 mM phenyl methyl sulfonyl fluoride (PMSF), 1 mg/ml aprotinin, 1 mM Na_3VO_4 and 2.5 mg/ml deoxicolic acid). Cell homogenates were sonicated for 5 min and stored at −80°C for at least half an hour before use. Protein concentrations from lysates were determined using the Bradford assay (Sigma Aldrich, Dorset, UK). Prior to loading, total protein samples were denatured by incubating at 95°C for 5 min in the presence of 5× reducing sample buffer (60 mM Tris–HCl pH 6.8, 25% glycerol, 2% SDS, 10% β-mercaptoethanol and 0.1% bromophenol blue). Protein samples (20 μg/lane) were loaded into SDS-PAGE (10%) gels and transferred onto a polyvinylidenifluoride (PVDF) membrane (Amersham, Buckinghamshire, UK) by the use of a wet tank blotter (Bio-Rad, Hercules, CA, USA) at 150 V for 1 h. The membrane was afterwards blocked with 5% non-fat milk and incubated with $P2Y_2$ (1:200) or $P2Y_4$ (1:200) antibodies overnight at room temperature. After washing, blots were incubated in horseradish-peroxidase-conjugated secondary antibodies for 1 h at room temperature. The peroxidase detection system (1.25 mM luminol, 0.2 mM coumaric acid, 0.1 M Tris pH 8.5, 0.032% hydrogen peroxide) was used for the visualization of the immunoreactivity. Control experiments were performed with the primary antibody omitted from the Western blot procedure and, for both anti-$P2Y_2$ and $P2Y_4$ antibodies, the primary antibody was preadsorbed with the peptides used to immunize the rabbits.

Measurement of intracellular Ca^{2+}

Osteoblastic cells were seeded into poly-D-lysine-coated black walled, clear-bottomed 96-well trays (BD Biosciences, Oxford, UK) at a density of 5×10^4 cells/well and cultured for 4, 8 or 10 days in supplemented DMEM or DMEM. Cells were twice washed with PBS and loaded for 30 min with the cell-permeant Fluo-4 AM (2 μM) in PBS containing 2.5% pluronic acid in 100 μl DMSO. After removal of the fluorophore loading solution, cell layers were washed twice more and 150 μl of PBS was added per well. The cell plates were then loaded into a fluorescence imaging plate reader (FLIPR, Molecular Devices, Wokingham, UK), together with a separate 96-well plate containing nucleotide agonists and 1 μM ionomycin (the latter as positive control). The agonists were distributed in a randomized pattern to minimize any cell plating effects due to well position. The FLIPR was programmed to transfer the agonists simultaneously to all 96 cell wells 30 s after commencement of recording; fluorescence was excited at 488 nm and emission was measured at 510–560 nm. Duration of recording was typically about 3 min; at the end of experiments, cell layers were fixed with 2.5% glutaraldehyde for 5 min and stained with 1% alizarin red (w/v) to demonstrate the presence of bone nodules. Due to technical constraints (pH, monolayer peeling), it was not possible to culture osteoblasts in the 96-well trays for periods exceeding 10 days, meaning functional responses were only measured for the first 10 days of culture.

Cell proliferation assay

Osteoblast proliferation was measured using the MTT assay. The assay exploits the ability of mitochondrial dehydrogenases in metabolically active cells to reduce the yellow tetrazolium 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT), generating a purple formazan product. Rat calvarial osteoblasts were cultured in standard 96-well plates as described above. MTT reagent (100 μg dissolved in 200 μl DMEM) was added to each cell layer, and plates were incubated in the dark at 37°C for 5 h; culture medium was then removed carefully, and formazan crystals were solubilized by the addition of 200 μl dimethyl sulfoxide (DMSO). Absorbance was read at 550 and 650 nm in a plate reader (EL_X800, Bio-Tek International); a standard curve for determination of cell numbers was constructed using cells seeded at 10^2 to 10^6/well.

Statistical analysis

Statistical comparisons were made by one-way analysis of variance and adjusted using the Bonferroni method. Representative data are presented as means ± SEM for ten to twelve replicates. Results presented are for representative experiments that were each repeated at least three times.

Results

Expression of P2 receptor mRNA in osteoblasts

Osteoblasts were cultured for up to 15 days in 6-well plates, and total cellular RNA was extracted at days 6, 8, 11, 13 and 15 to enable the levels of selected mRNAs to be determined by PCR amplification. Representative results are shown in Fig. 1. RNA levels in individual samples were normalized using β-actin. Osteocalcin mRNA expression, used as a marker of osteoblast differentiation, was barely detectable at day 6 but increased to high levels at days 11–15. Expression of $P2X_2$ and $P2X_5$ receptor mRNAs peaked at day 8 but then declined to undetectable or near-undetectable levels by day 15, however, $P2X_7$ receptor mRNA was detected at all time points between day 6 and 15. $P2Y_1$ mRNA receptor was expressed at relatively constant level until day 15, where a decline was observed. In contrast, $P2Y_2$ receptor mRNA showed a striking, progressive upregulation with time in culture. $P2Y_4$ and $P2Y_6$ receptor mRNAs were most highly expressed between day 8 and 13.

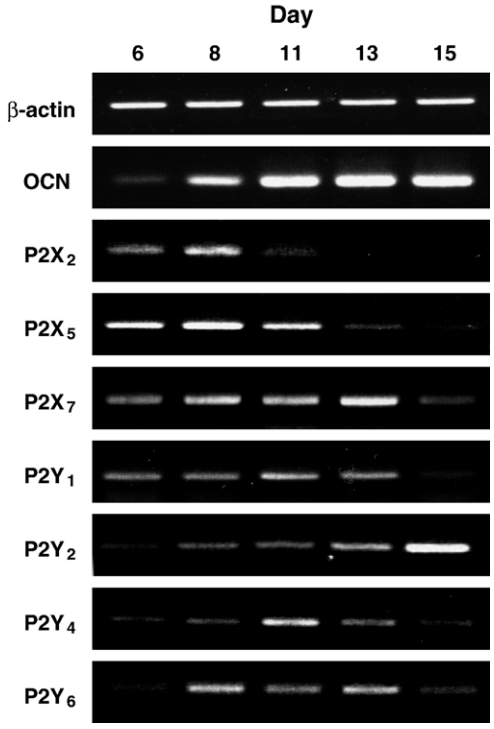

Fig. 1. Expression of P2 receptor mRNA in rat primary osteoblasts. Total RNA was extracted from 6-, 8-, 11-, 13- and 15-day cultures. Samples were normalized with mRNA for β-actin, and osteocalcin (OCN) mRNA was assessed as a marker of osteoblast differentiation. Marked differences in the expression of some P2 receptor mRNAs were evident over time. $P2X_2$ and $P2X_5$ receptor mRNAs were expressed in early cultures, whereas expression of mRNA for the UTP-selective $P2Y_2$ receptor increased strongly in later cultures. Expression of $P2X_7$, $P2Y_1$, $P2Y_4$ and $P2Y_6$ receptor mRNAs showed less time dependence but tended to be greatest between day 8 and 13.

Detection of P2 receptor subtypes in osteoblasts by immunofluorescence

For immunocytochemistry, osteoblasts were cultured for 4, 8, 11 and 14 days. Specific primary antibodies directed against individual P2 receptors and a Cy3-labeled secondary antibody enabled the determination of receptor protein by fluorescence microscopy. The development and specificity of the P2X and P2Y polyclonal antibodies has been reported previously [18–20].

Marked changes in immunostaining for P2 receptors with time in culture were observed (Fig. 2). Both $P2X_2$ (Figs. 2A and B) and $P2X_5$ (Figs. 2C and D) receptor staining was strongest at earlier time points and was increasingly confined to sub-populations of cells as cultures progressed; however, $P2X_7$ receptors were present at a relatively constant, low level (Fig. 2E). Staining for $P2Y_2$ (Figs. 2G and H) receptor protein increased strongly with time, whereas $P2Y_1$ receptor protein expression appeared fairly constant (Fig. 2F). $P2Y_4$ (Fig. 2I) and $P2Y_6$ (Fig. 2J) receptor expression tended to be greatest at intermediate time points. Staining for individual receptor proteins was not always uniform across cell layers.

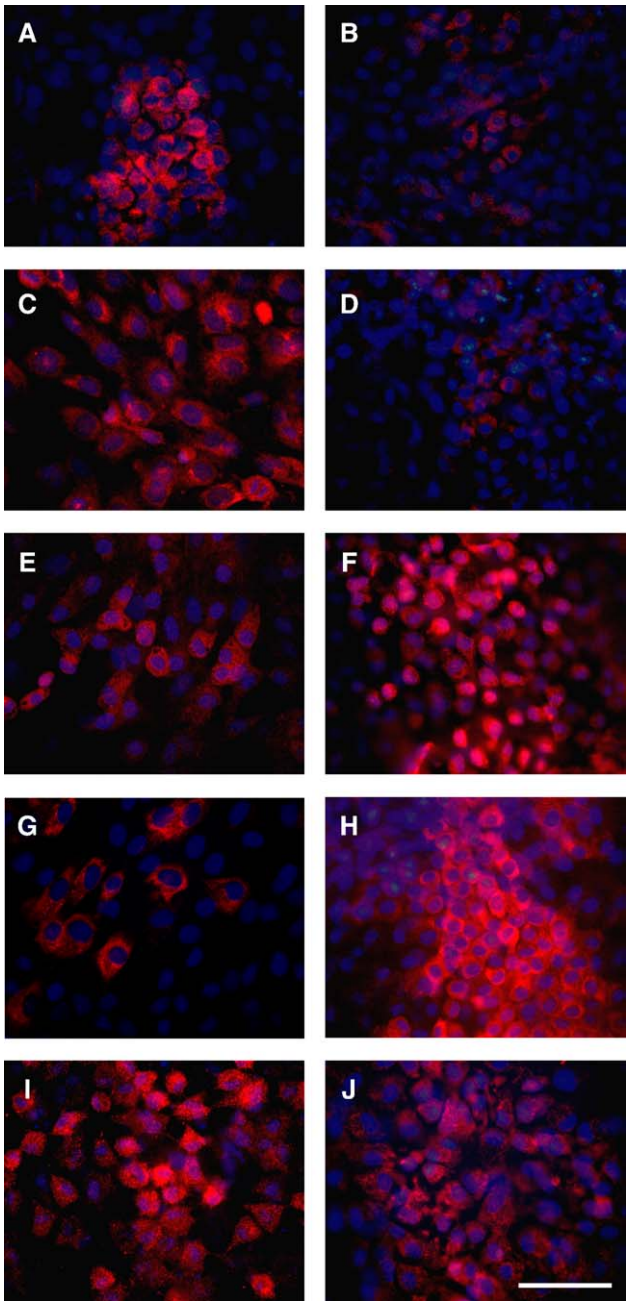

Fig. 2. P2 receptor expression in rat primary osteoblasts. Changes in P2 receptor expression over time in culture were studied by immunofluorescence using specific primary polyclonal antibodies, Cy3-labeled anti-rabbit secondary antibody (red) and DAPI nuclear stain (blue). Photomicroscopy values were the same for each image. (A) Strong $P2X_2$ receptor expression at day 8 of culture; (B) greatly reduced $P2X_2$ receptor expression in mature osteoblasts at day 14. (C) High level of $P2X_5$ receptor expression at day 4; (D) low level $P2X_5$ receptor expression at day 14. (E) Representative micrograph of constant, low-level $P2X_7$ receptor expression in osteoblasts observed at time points from days 4 to 14 of culture. (F) Representative micrograph of $P2Y_1$ receptor expression observed at all time points from days 4 to 14 of culture. (G) Low $P2Y_2$ receptor expression at day 4; (H) striking increase in $P2Y_2$ receptor expression at day 14. (I) $P2Y_4$ receptor expression was greatest between day 8 and 14 (micrograph shows day 11 culture). (J) $P2Y_6$ receptor expression was observed at all time points but was strongest in mature osteoblasts (micrograph shows day 11 culture). Scale bar = 25 μm.

Changes in $P2Y_2$ and $P2Y_4$ receptor protein expression over time

Primary osteoblasts, derived from rat calvaria, were cultured for up to 15 days in DMEM (normal medium) or supplemented DMEM (differentiation medium) and the levels of $P2Y_2$ and $P2Y_4$ receptor protein investigated using Western blotting. Calvarial cells induced to differentiate displayed a striking increase in $P2Y_2$ expression between 4 and 15 days of culture (Fig. 3A), whereas untreated cells only exhibited a slight increase at day 15. Low $P2Y_4$ receptor expression was observed at day 4, with peak levels at 8 days of culture (Fig. 3B) in differentiated osteoblasts. In unstimulated calvarial cells, no $P2Y_4$ receptor expression was observed at day 4, however, small increases in expression were observed after 8 and 15 days of culture. No bands were observed when the anti-$P2Y_2$ and $P2Y_4$ antibodies were preadsorbed with the appropriate immunogenic peptide or when the primary antibody was omitted.

Nucleotide-induced increases in intracellular Ca^{2+} in rat primary osteoblasts

Concentrations of ATP, UTP and ADP over the range 0.002 μM to 1000 μM were tested in osteoblasts cultured for 8 days. All three agonists triggered increases in intracellular Ca^{2+}, measured using the intracellular fluorophore, Fluo-4 (Fig. 4A); responses typically peaked 10 s after addition and decayed back to baseline within 2–3 min. ATP was the most potent, eliciting increases in fluorescence at concentrations as low as 0.2 μM, with peak responses between 10 and 20 μM (Fig. 4B). UTP was somewhat less potent than ATP, and ADP was clearly the least potent of the three nucleotides. At 8 days, other P2 agonists, including (in decreasing order of potency) ATPγS, UDP, 2-MeSATP, Bz-ATP and α,β-meATP, also increased intracellular Ca^{2+}, but no responses were observed

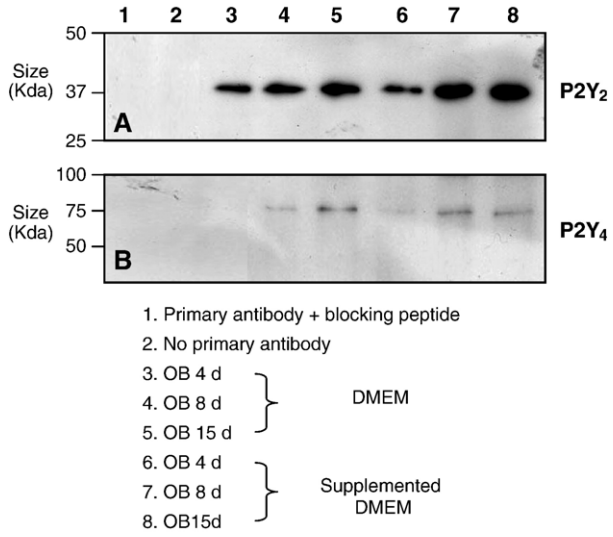

Fig. 3. Changes in $P2Y_2$ and $P2Y_4$ receptor protein expression over time. (A) In osteoblasts (OB) cultured in supplemented DMEM (DMEM plus 10 nM dexamethasone, 50 μg/ml ascorbate and 2 mM β-glycerophosphate), Western blotting revealed large increases in $P2Y_2$ receptor protein at days 8 and 15 compared to day 4. Conversely, cells that were not induced to differentiate (cultured in DMEM) displayed only a small increase in $P2Y_2$ receptor expression at day 15. (B) Osteoblasts cultured in supplemented DMEM displayed maximal $P2Y_4$ receptor protein expression at intermediate time points (day 8). In cells maintained in non-supplemented DMEM, the changes in $P2Y_4$ receptor expression were delayed; no protein was detected at day 4, and peak expression occurred at day 15.

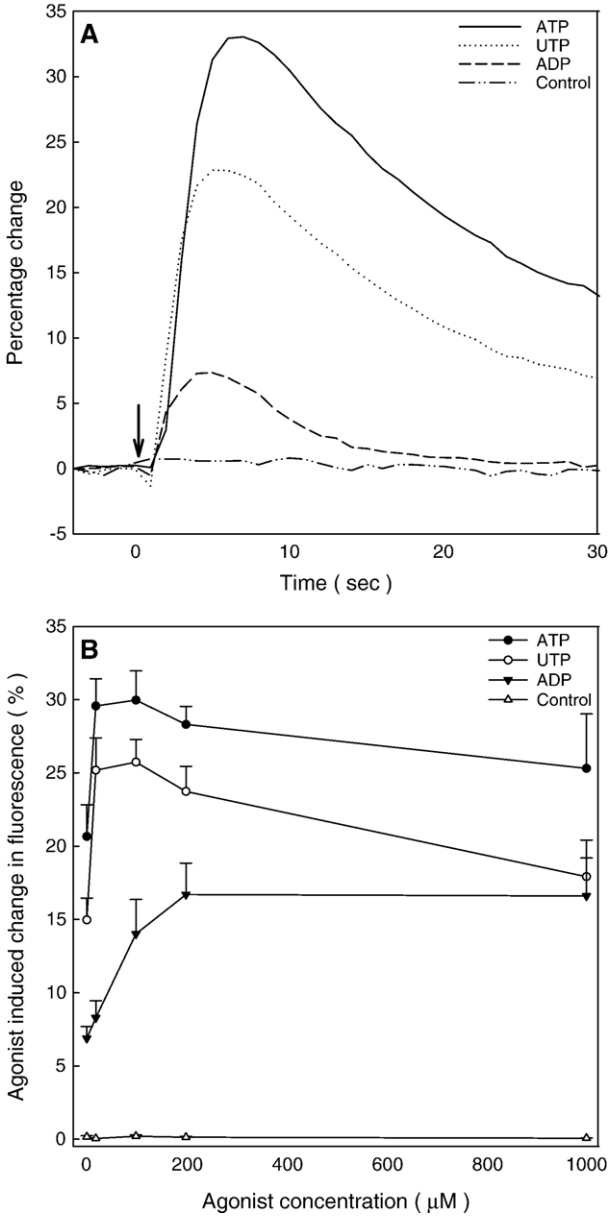

Fig. 4. Nucleotide stimulation of primary rat osteoblasts causes increased intracellular Ca^{2+}. (A) In osteoblasts loaded with the fluorescent dye Fluo-4, 20 μM nucleotide agonists caused a rapid, transient increase in intracellular Ca^{2+} which returns to baseline within ~90 s, with the following order of potency: ATP > UTP ≫ ADP. Traces show averages of data from 10 separate cell wells at day 8 of culture, plotted as percentage deviation from baseline values. The arrow indicates agonist addition at time $t = 0$. (B) Nucleotides at concentrations >0.2 μM elicited increases in intracellular Ca^{2+}. Data plotted represent the average percentage increase in fluorescence following agonist addition for 10 separate cell wells at day 8 of culture (±SEM).

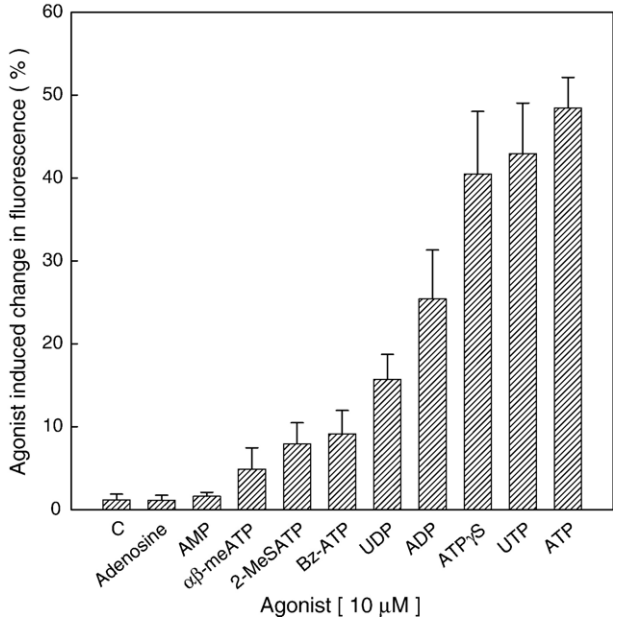

Fig. 5. Comparison of intracellular Ca^{2+} responses of rat osteoblasts to a range of purine and pyrimidine agonists. The responses suggest the presence of functional $P2X_1$, $P2X_2$, $P2X_3$, $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors on rat primary osteoblasts. Data plotted represent the average percentage increase in fluorescence following agonist addition for 10 separate cell wells at day 8 of culture (±SEM).

to the P1 agonists adenosine and AMP (Fig. 5). These functional data are consistent with the expression of various P2 receptor subtypes including $P2X_2$, $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ on osteoblasts.

Intracellular Ca^{2+} responses of osteoblasts to nucleotides: dependence on time in culture

Osteoblasts cultured for 4, 8 or 10 days in DMEM or supplemented DMEM were challenged with ATP, UTP and ADP over the concentration range 2–200 μM. Intracellular Ca^{2+} responses increased strikingly with time in culture: for example, the response to 20 μM ATP was 6-fold greater in 10-day cultures than in 4-day cultures (Fig. 6A). Similar trends were observed with UTP and ADP, albeit with responses of smaller magnitude, as noted above. To determine whether these observed changes were due to increased culture time and/or cell number, cell proliferation assays were performed in parallel with the FLIPR experiments, and intracellular Ca^{2+} responses were measured in calvarial cells not induced to differentiate. Consistently, differentiated cells were more responsive to stimulation by nucleotide agonists, with only small increases in fluorescence being noted in untreated cells: for example, 20 μM ATP induced a 10-fold higher response in differentiated osteoblasts (Fig. 6B). Furthermore, between day 4 and 10, cell numbers increased only about 2-fold (Fig. 6C). Technical constraints (increasingly rapid exhaustion of culture medium, acidification and monolayer peeling) prevented routine culture beyond day 10 in 96-multiwells.

Late-acting inhibition of bone nodule formation by nucleotides

Rat calvarial osteoblasts were cultured for 17 days; mineralized bone nodules with a characteristic 'trabecular' morphology [10,21] began to form from about day 10 onwards. ATP (10 μM) was added to cultures from days 1, 7, 10 or 14. Addition from 1, 7 or 10 days caused ~18-fold reductions in mineralized bone nodule formation (Fig. 7A). When ATP was present from days 14–17 only, further bone nodule formation by osteoblasts was prevented (although bone already formed was unaffected) (Fig. 7B). In contrast, addition of ATP for the first 7 days of culture had no effect on mineralized nodule formation at day 17 (Fig. 7C). In experiments using UTP, closely similar, late-acting inhibition of bone nodule formation was observed, whereas ADP and UDP consistently failed to block nodule formation. These data indicate that the presence of ATP during early stages of culture does not affect osteoblast function, but application during the later stages of culture can effectively "switch off" bone nodule formation.

Reactive blue 2 does not prevent UTP-induced inhibition of bone nodule formation

ATP and UTP both potently activate rat $P2Y_2$ and $P2Y_4$ receptors. The widespread expression of the $P2Y_4$ receptor observed here questions the findings of Hoebertz et al. [9], which implicated the $P2Y_2$ receptor subtype in the inhibition of bone formation by nucleotides. The purinergic receptor antagonist, reactive blue 2, inhibits $P2Y_4$ receptors but is inactive at $P2Y_2$ receptors; in an attempt to clarify the P2 receptor subtype mediating the inhibition of bone formation, this agent was tested in vitro at a range of concentrations (0.1–100 μM). UTP-treated osteoblasts demonstrated an approximately 85% decrease in bone formation and at 0.1–10 μM, reactive blue 2 failed to prevent this inhibitory effect (Fig. 8A). Reactive blue 2 alone at 0.1–10 μM did not adversely affect osteoblast function (Fig. 8B). Osteoblasts cultured with 100 μM reactive blue 2 (with or without UTP) failed to form bone nodules, suggesting that at high concentrations this agent is toxic.

Currently, there are no antagonists specific for the $P2Y_2$ receptor subtype. Suramin is a non-specific inhibitor acting at multiple P2 receptor subtypes including the $P2Y_2$ receptor, however, it is inactive at the $P2Y_4$ receptor. Osteoblasts were treated with 0.1–100 μM suramin (with or without 10 μM ATP/UTP) and cultured for up to 21 days. Suramin impaired osteoblast proliferation and completely prevented bone nodule formation in the concentration range 0.1 to 1 μM; at concentrations ≥1 μM, it was severely toxic. These data suggest that the effects on osteoblasts of prolonged exposure to suramin were likely to be due to non-selective toxicity.

Discussion

Purinergic signaling appears to have an important role in regulating cellular function in a wide array of tissues and cells [22]. We show here that functional responses of normal

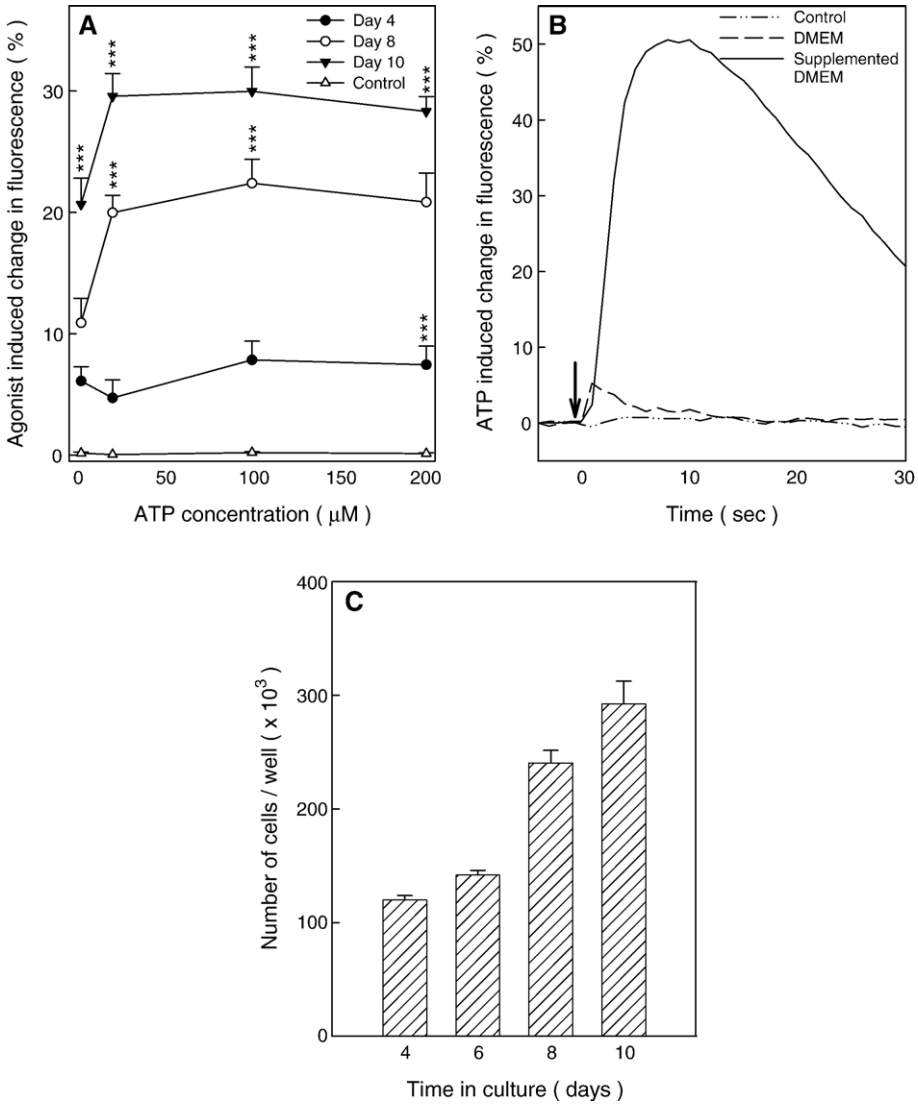

Fig. 6. Osteoblast responses to ATP increase strongly with time in culture. (A) Primary rat osteoblasts were cultured in 96-well trays for 4, 8 or 10 days prior to loading with the Ca^{2+}-sensing fluorophore, Fluo-4 AM and challenge with ATP. At all concentrations tested, ATP elicited significantly increased (up to 6-fold) responses at days 8 and 10 of culture, compared with day 4. Data plotted represent the average percentage increase in fluorescence following ATP addition for 10 separate cell wells. Significantly different from respective value at day 4: ***$P < 0.001$. (B) The responses to nucleotide agonists of calvarial cells stimulated to differentiate in supplemented DMEM (DMEM plus 10 nM dexamethasone, 50 μg/ml ascorbate and 2 mM β-glycerophosphate) were compared with responses of cells in which differentiation was prevented by culture in non-supplemented DMEM. Application of 20 μM ATP elicited a 10-fold greater response in differentiated cells. Traces show averages of data from 10 separate cell wells at day 8 of culture, plotted as percentage deviation from baseline values. The arrow indicates ATP addition at time $t = 0$. (C) Cell numbers, assessed by MTT assay, increased only 2.4-fold between day 4 and 10 of culture, suggesting that increased osteoblast responsiveness to ATP at later time points is related to cell differentiation rather than to proliferation.

osteoblasts to the nucleotides ATP and UTP increase during differentiation with time in culture; these changes were notably accompanied by an increase in the expression of the P2Y receptors particularly the UTP-sensitive $P2Y_2$ receptor and to a lesser extent the $P2Y_4$ and $P2Y_6$ receptors.

We used a fluorescence imaging plate reader (FLIPR) system that enables efficient quantitative screening of intracellular Ca^{2+} responses of cell populations to agonists to study the acute actions of ATP and other nucleotides on primary osteoblasts maintained in long-term cultures. Several nucleotide agonists elicited rapid increases in intracellular Ca^{2+}; the most potent of these were ATP, the "universal agonist" at P2 receptor subtypes, followed by UTP, which is selective for $P2Y_2$ and $P2Y_4$ receptors. Peak intracellular Ca^{2+} responses to ATP and UTP occurred in the range 2–200 μM, consistent with the blockade of bone nodule formation caused by the nucleotides at the same concentrations [10]. Smaller intracellular Ca^{2+} responses were also evoked by several other nucleotide agonists, consistent with the presence of functional $P2X_2$, $P2Y_6$, $P2Y_1$, $P2X_5$ and $P2X_7$ receptors on primary osteoblasts (for reviews of P2 receptor pharmacology, see [1,3]). P1 receptors couple to adenylate cyclase and either increase or decrease cAMP levels; as expected, when tested in the FLIPR, adenosine and AMP did not increase intracellular Ca^{2+}. Whether osteoblasts possess functional P1 receptors remains unclear as there is evidence for [23] and against [24] their expression.

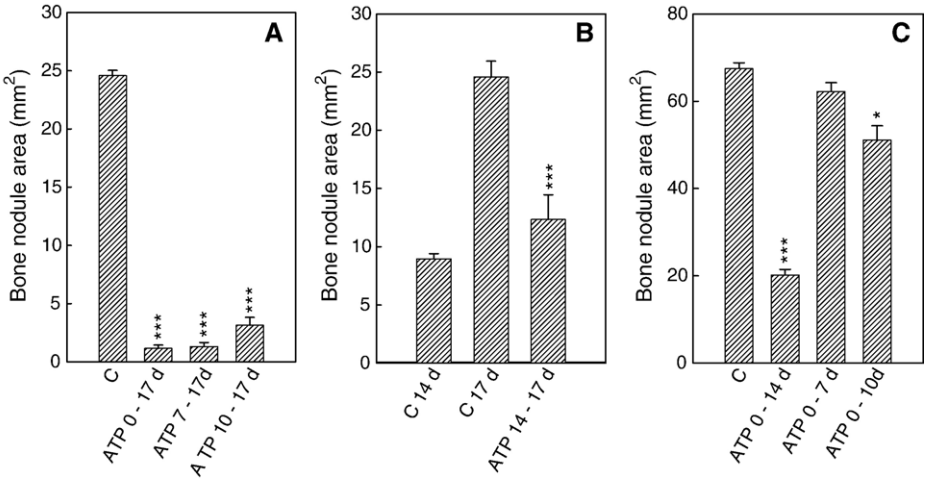

Fig. 7. Late-acting inhibition of mineralized bone nodule formation by ATP. Primary rat calvarial osteoblasts were cultured for up to 17 days. (A) ATP (10 μM) was added from days 1, 7 or 10. Data indicate that the presence of ATP even from day 10 was sufficient to cause a maximal 18-fold inhibition of mineralized bone nodule formation. (B) Addition of 10 μM ATP from day 14 prevented further bone formation but did not affect nodules that were already formed. (C) The presence of 10 μM ATP during the first 7 days of 14-day cultures did not affect subsequent bone nodule formation. Values are means ± SEM ($n = 6$), significantly different from controls: ***$P < 0.001$, *$P < 0.05$.

Sharp augmentations (~6-fold) in the intracellular Ca^{2+} responses of osteoblasts to ATP and UTP occurred as cultures progressed towards bone nodule formation which could not be accounted for by the modest increases (~2-fold) in cell number that were noted. Furthermore, when rat calvarial cells were not induced to differentiate, the responses to nucleotide agonists remained small, being up to 10-fold lower than differentiated osteoblasts after 8 days of culture. These observations suggested that osteoblast differentiation was accompanied by changes in expression of P2 receptors. PCR and immunocytochemical analysis revealed a strong differentiation dependence of P2 receptor expression in long-term osteoblast cultures, characterized by a shift from early P2X expression to P2Y expression at later stages. Most notable was the striking increase in levels of the $P2Y_2$ subtype during the second week of culture, coinciding with the onset of bone nodule formation. In some cases, receptor protein expression appeared to persist beyond that of the mRNA; this could be due to differences between cultures and/or because the protein signal is sustained after the mRNA has been degraded. Expression of the $P2Y_4$ receptor which, like the $P2Y_2$ subtype, is UTP-selective, also increased during the second week of culture, but the mRNA then declined while the protein persisted albeit at a lower level. Western blot analysis of $P2Y_2$ and $P2Y_4$ receptors confirmed the trends observed in the immunofluorescence. Furthermore, as calvarial cells not stimulated to differentiate only showed small much

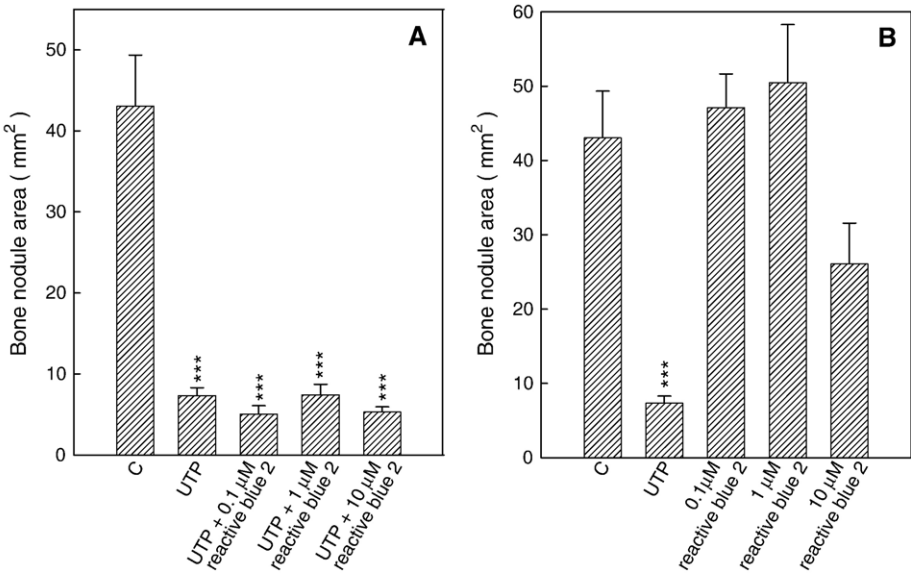

Fig. 8. Reactive blue 2 does not prevent UTP-mediated inhibition of bone nodule formation. (A) Addition of 0.1 μM–10 μM reactive blue 2 did not have any effect on the inhibition of bone nodule formation induced by 10 μM UTP. (B) Reactive blue 2 (0.1–10 μM) did not influence mineralized bone nodule formation by rat osteoblasts. Quantification was by image analysis of alizarin-red-stained cultures. Values are means ± SEM ($n = 6$); significantly different from control: ***$P < 0.001$.

delayed changes in receptor protein expression, the trends observed here are likely due to osteoblast differentiation rather than increased culture time. Together, these data provide good evidence for significant upregulation of UTP-selective receptors as osteoblast differentiation proceeds towards bone formation.

Over the same time period, expression of the $P2X_5$ receptor, which is associated with the proliferation of osteoblasts and other cells [1,25,26], decreased to near-undetectable, whereas mRNA for osteocalcin, a marker of osteoblast differentiation, increased massively. Our results also provide confirmatory evidence for the expression of the $P2X_7$ receptor in osteoblasts [27,28], albeit at relatively low levels. The function of this receptor in bone remains enigmatic. At high concentrations of extracellular ATP, the $P2X_7$ receptor mediates the formation of cytolytic pores [28–30]. Activation of osteoclast $P2X_7$ receptors has been reported to facilitate intercellular calcium signaling between osteoblasts and osteoclasts [32], while blockade of this receptor inhibits osteoclast formation [31]. Deletion of $P2X_7$ receptors in mice leads to deficient periosteal bone formation together with excessive trabecular bone resorption [27]. Despite the clear link between the $P2X_7$ receptor and cell death [33], the low prevalence of apoptosis in osteoblasts [34] suggests that this is unlikely to be a major function for $P2X_7$ receptors in bone cell metabolism. An alternative possibility is that pores induced by transient activation of $P2X_7$ receptors could play a role in the controlled release of certain cytoplasmic components [35,36]. We also found that osteoblast $P2X_2$ receptor expression decreased markedly with time in culture. Information about the physiological role, if any, of this receptor subtype in bone is still limited: it has been implicated in mediating activation of osteoclastic resorption by acid and ATP [8], and mice lacking the receptor exhibit small increases in bone mass during the first few months of life [37].

In agreement with earlier findings [1,6,7,38], we found strong evidence for the expression of the $P2Y_1$ receptor on osteoblasts, together with significant intracellular Ca^{2+} responses to ADP (the natural ligand selective for this receptor as well as $P2Y_{12}$ and $P2Y_{13}$), particularly at higher concentrations. However, ADP does not inhibit bone formation in vitro [10], suggesting that stimulation of intracellular Ca^{2+} does not automatically lead to decreased bone formation. The $P2Y_6$ receptor had previously been identified only in transformed osteoblast-like cell lines [6]. Our data show clearly that the $P2Y_6$ receptor is expressed by normal rat osteoblasts and that UDP, the agonist selective for this receptor, evokes significant intracellular Ca^{2+} responses in osteoblasts. However, UDP, like ADP, does not inhibit bone formation in the low micromolar range. Little else is known about the $P2Y_6$ receptor or the role, if any, that it might play in bone cell metabolism.

The $P2Y_4$ receptor had also only previously been identified in transformed osteoblast-like cell lines and not in normal osteoblasts [6]. Here, we demonstrated clear expression of $P2Y_4$ receptor mRNA and protein in rat osteoblasts. ATP and UTP equipotently activate both the rat $P2Y_2$ and $P2Y_4$ receptors, while UTP is more potent than ATP at the human $P2Y_4$ receptors [1], raising the question of whether the $P2Y_4$ receptor could be involved in mediating the inhibitory effects of nucleotides on rat osteoblasts. In other cell types such as airway epithelial cells, the predominant $P2Y_2$ receptor has been shown to mask the effects of $P2Y_4$ and $P2Y_6$ receptors [39] and this may have occurred here. In an attempt to address this question, we tested the $P2Y_4$ receptor antagonist reactive blue 2, which is inactive at $P2Y_2$ receptors. We found that reactive blue 2 failed to have any effect on the inhibition of bone nodule formation caused by UTP, suggesting that the $P2Y_2$ receptor is indeed the chief mediator of the anti-osteogenic action of extracellular nucleotides.

The present study suggests that ATP and UTP act via the $P2Y_2$ receptor to inhibit bone nodule formation. In accordance with the large increases in intracellular Ca^{2+} responses to UTP and ATP in late stage osteoblast cultures, together with increased $P2Y_2$ receptor expression, the application of ATP or UTP during the final days of culture can effectively switch off bone formation.

Taken together, these results suggest that the P2Y receptors, particularly the $P2Y_2$ subtype, expressed in differentiated, functional osteoblasts, could play an important role in the skeleton by acting as an 'off-switch' for bone formation. In view of the diversity of P2 receptors expressed by bone cells, it is possible that some redundancy of function may exist, with some inhibitory effects mediated additionally, for example, by the $P2Y_4$ or $P2Y_6$ receptor subtypes. The notion that ATP, the "universal agonist" at P2 receptors, could play a physiological role in regulating osteoblast function is supported by the recent observation that it is released from primary osteoblast cultures in amounts that could equate with local concentrations in the high nanomolar or even low micromolar range in vivo [40]. Detailed analysis of the skeletal phenotypes of knockout mice deficient in single or multiple P2Y receptors [41,42] is now required.

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