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Extracellular Nucleotides Block Bone Mineralization *in Vitro*: Evidence for Dual Inhibitory Mechanisms Involving Both $P2Y_2$ Receptors and Pyrophosphate

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Extracellular nucleotides, signaling through P2 receptors, may act as local regulators of bone cell function. We investigated the effects of nucleotide agonists [ATP, ADP, uridine triphosphate (UTP), and uridine diphosphate] and pyrophosphate (PPi, a key physiological inhibitor of mineralization) on the deposition and mineralization of collagenous matrix by primary osteoblasts derived from rat calvariae. Our results show that extracellular ATP, UTP, and PPi strongly and selectively blocked the mineralization of matrix nodules; ADP and uridine diphosphate were without effect. Significant inhibition of mineralization occurred in the presence of relatively low concentrations of ATP, UTP, or PPi (1–10 μM), without affecting production of fibrillar or soluble collagen. In cultures treated with 10 μM ATP or UTP, the expression and activity of alkaline phosphatase, which promotes mineralization by hydrolyzing PPi, was inhibited. The potent inhibitory actions of ATP and UTP on bone mineralization are consistent pharmacologically with mediation by the $P2Y_2$ receptor, which is strongly expressed by mature osteoblasts. In support of this notion, we found 9–17% increases in bone mineral content of hindlimbs of $P2Y_2$-deficient mice. We also found that osteoblasts express ectonucleotide phosphodiesterase/pyrophosphatase-1, an ectonucleotidase that hydrolyzes nucleotide triphosphates to yield PPi, and that addition of 10 μm ATP or UTP to osteoblast cultures generated 2 μM PPi within 10 min. Thus, a component of the profound inhibitory action of ATP and UTP on bone mineralization could be mediated directly by PPi, independently of P2 receptors. (*Endocrinology* 148: 4208–4216, 2007)

IT IS NOW well recognized that extracellular nucleotides, signaling via the P2 receptors, are involved in a wide number of biological processes in both neuronal and non-neuronal tissues (see reviews in Refs. 1, 2). P2 receptors are subdivided into the P2X ligand-gated ion channels and P2Y G protein-coupled receptors (first proposed by Kennedy and Burnstock in 1985) (3, 4). Currently seven P2X receptors ($P2X_{1–7}$) and eight P2Y ($P2Y_{1,2,4,6,11,12,13,14}$) receptors have been identified; each of these receptors has been cloned, characterized, and displays distinct tissue expression and pharmacology (5, 6). P2 receptors respond to a range of adenine and uridine-containing nucleotides including ATP, ADP, uridine triphosphate (UTP) and uridine diphosphate (UDP).

In recent years the role of extracellular nucleotides in the regulation of bone cell function has received considerable attention (see reviews in Refs. 7 and 8). Osteoblasts, the bone-forming cells, express at least seven different P2 receptor subtypes ($P2X_2$, $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$) in a differentiation-dependent manner (9). Four receptor subtypes have been linked to functional effects in osteoblast-like cells. The $P2X_5$ receptor is thought to stimulate osteoblast proliferation (10). The $P2X_7$ receptor has been implicated in the skeletal response to mechanical loading (11) and, recently in cell membrane blebbing in response to high concentrations of ATP (12). The $P2Y_1$ receptor is reported to play a role in modulating osteoblast responses to systemic factors such as PTH (13, 14). UTP and ATP, at 1–10 μM concentrations, cause strong inhibition of mineralized bone nodule formation by cultured rat osteoblasts, whereas adenosine and ADP are without effect (15). These potent actions of ATP and UTP are consistent pharmacologically with the involvement of either $P2Y_2$ or $P2Y_4$ receptor subtypes. Adenosine 5′-*O*-3-thiotriphosphate (ATPγS), a potent $P2Y_2$ agonist, also inhibits bone formation *in vitro* (16). Recent work in our laboratory has also shown the $P2Y_2$ receptor is strongly up-regulated in mature osteoblasts and that reactive blue 2, a $P2Y_4$ receptor antagonist that is inactive at $P2Y_2$ receptors, fails to prevent the UTP-induced inhibition of bone formation (9). Taken together these data strongly suggest a role for the $P2Y_2$ receptor in the inhibition of bone formation by extracellular nucleotides.

Bone is a composite tissue comprising both organic and mineral phases, and its formation by mature osteoblasts is a two-step process. The first stage is synthesis, exocytotic re-

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Abbreviations: BMC, Bone mineral content; BMD, bone mineral density; COL1, type I collagen; DEXA, dual-energy x-ray absorptiometry; ENPP, ectonucleotide phosphodiesterase/pyrophosphatase; MGP, matrix gla protein; OCN, osteocalcin; OPN, osteopontin; Pi, orthophosphate; PPi, inorganic pyrophosphate; UDP, uridine diphosphate; UTP, uridine triphosphate.

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lease and deposition of the organic matrix (osteoid), consisting mainly of type I collagen, together with many noncollagenous bone proteins. The second step is mineralization of the organic matrix; this normally occurs rapidly in fast-growing juvenile (woven) bone but is delayed for several days in adult (lamellar) bone. Mineralization depends on a constant supply of Ca^{2+} and orthophosphate (Pi) ions, both of which are required for hydroxyapatite crystal formation (see reviews in Refs. 17, 18). One of the main sources of Pi in bone is tissue-nonspecific alkaline phosphatase (ALP), which hydrolyzes a range of phosphate-containing substrates including ATP, inorganic pyrophosphate (PPi), and β-glycerophosphate (19). PPi is a potent inhibitor of mineralization because it antagonizes the ability of Pi to crystalize with Ca^{2+} to form hydroxyapatite and additionally suppresses crystal propagation (20–22). PPi is generated by the hydrolysis of the phosphodiester bond in purine and pyrimidine nucleoside triphosphates (such as ATP), in a reaction catalyzed by members of the ectonucleotide phosphodiesterase/pyrophosphatase (ENPP) family. Osteoblasts express several members of the ENPP family including ENPP-1 (also known as PC-1), which together with ALP is thought to play an important role in the regulation of Pi and PPi levels (23, 24).

The aim of this study was to determine whether the antiosteogenic effects of extracellular nucleotides involve inhibition of organic matrix deposition or mineralization. We also examined the possibility that the actions of ATP and UTP might occur independently of P2 receptors because ectonucleotidases, including ENPP-1, could generate PPi from extracellular nucleotide triphosphates.

Materials and Methods

Reagents

Reagents were purchased from Sigma Aldrich (Poole, UK) unless otherwise stated. All molecular biology reagents were purchased from Promega UK (Southampton, UK) and primers from MWG Biotech (Ebersberg, Germany).

Cell culture

Primary rat osteoblast cells were obtained by sequential enzyme digestion of excised calvarial bones from 2-d-old neonatal Sprague Dawley rats using a three-step process [1% trypsin in PBS for 10 min; 0.2% collagenase type II in Hanks' balanced salt solution for 30 min; 0.2% collagenase type II in Hanks' balanced salt solution for 60 min]. The first two digests were discarded and the cells resuspended in DMEM (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 d in a humidified atmosphere of 5% CO_2-95% air at 37 C in 75-cm^2 flasks until confluent. Upon confluence, cells were subcultured into 24-well in DMEM supplemented with 2 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and 10 nM dexamethasone (mixture abbreviated to supplemented DMEM), with half-medium changes every 3 d. Osteoblasts were cultured in the presence of different nucleotide agonists (ATP, UTP, ADP, UDP, CTP, and GTP) or PPi (sodium salt) at concentrations between 1 and 100 μM for bone nodule formation assays and 10 μM for all other experiments. Fresh nucleotides were added at every medium change. Medium pH, pCO_2 and pO_2 were monitored throughout using a blood gas analyzer (ABL-705; Radiometer, Crawley, UK). All experiments were carefully pH controlled because osteoblast ALP activity and bone mineralization are extremely sensitive to inhibition by acidosis (25).

Bone nodule formation by osteoblasts cultured in 24-well plates was measured using modifications of an assay described previously (15, 25). 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, and then air dried. The plates were imaged at 2000 dots/cm^2 using a high-resolution, flat-bed scanner (Perfection Photo 3200; Epson UK, Hemel Hempstead, UK). Binary images of each individual well were then subjected to automated analysis (Scion Image software; Scion Corp.; http://www.scioncorp.com), using constant threshold and minimum particle levels, to determine the number and plan surface area of mineralized bone nodules.

Determination of ALP and ENPP activity

The ALP activity of cell lysates was determined colorimetrically (Bio-Tek EL_X800 plate reader; Fisher Scientific, Loughborough, UK) using a commercially available kit (Biotron Diagnostics, Hemet, CA); this assay uses *p*-nitrophenyl phosphate as a substrate, which in the presence of ALP, is converted to the yellow chromogen p-nitrophenyl. Osteoblasts were cultured in 24-well trays and enzyme activity measured after 4, 7, 10, and 14 d. To assay ALP activity, cell layers were washed and cells harvested using a scraper (n = 6) followed by sonication at 4 C and centrifugation at 500 × *g*. The supernatant was collected and stored at 4 C until assaying at pH 9.8.

The assay used to measure total ENPP activity was based on the method originally described by Razzell and Khorana (26). Briefly, cells were lysed in a buffer containing 1% Triton X-100 in 0.2 M Tris base with 1.6 mM $MgCl_2$ (pH 8.1). After centrifugation at 500 × *g*, the ENPP activity of collected supernatants was measured using 5 mM *p*-nitrophenylthymidine 5′-monophosphate as a substrate. Total protein in cell lysates was determined using the Bradford assay (Sigma Aldrich).

Measurement of collagen production

Collagen production was determined in osteoblasts after 7 and 14 d of culture; total protein concentration in lysates was determined using the Bradford assay. To measure deposited (fibrillar) collagen, cell layers were washed in PBS, fixed with Bouin's solution for 1 h, and washed extensively in running water. Plates were left to air dry before staining for 1 h with Sirius red dye reagent (Biocolor Ltd., Newtonabbey, UK; 500 μl/well) with gentle agitation. Cell layers were washed extensively with running water, followed by 0.01 M hydrochloric acid to remove unbound dye. The stained cell layers were then digested with 0.1 M sodium hydroxide (500 μl/well) for 30 min. The absorbance of the digests was measured at 570 nm.

To measure soluble collagen production, osteoblasts were transferred to medium containing 5% fetal calf serum, 2 mM β-glycerophosphate, 50 μg/ml ascorbic acid, 10 nM dexamethasone, and the lysyl oxidase inhibitor β-aminopropionitrile (50 μg/ml) for the final 24 h of culture. The concentration of collagen accumulated in the tissue culture medium was assayed using a Sirius red dye-based kit (Sircol soluble collagen assay; Biocolor) according to the manufacturer's instructions.

Total RNA extraction and complementary DNA strand synthesis

Osteoblasts were cultured in 6-well trays for 4–14 d in supplemented DMEM; total RNA was extracted from wells every 2–3 d using TRIZOL reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Before first-strand 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 deoxynucleotide triphosphates, 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.

TABLE 1. Primer sequences and annealing temperatures for RT-PCR

Name	Sequence (5′–3′)	Annealing temperature (C)	Product size (bp)
β-Actin	S, gtt cgc cat gga tga cga t; AS, tct ggg tca tct ttc acg g	53.0	332
OCN	S, gca gac acc atg agg acc ct; AS, gca gct tgt gcc gtc cat ac	59.8	418
ALP	S, ctc att tgt gcc aga gaa; AS, gtt gta cgt ctt gga gac	50.0	238
MGP	S, ccc tgt cgt atg aat ctc; AS, gac tcc gta aca aag cga	50.0	297
OPN	S, cgg tga aag tgg ctg agt; AS, gac tcg gga tac tgt tca	50.0	333
COL1	S, gcg aag aag aca tcc ctg; AS, ctg tcc agg gat gcc atc	50.0	377
ENPP-1	S, gtc agt atg cgt gct aac; AS, tgg cac act gaa ctg tag	51.0	309

S, Sense; AS, antisense.

PCR

Rat osteoblast derived cDNA was amplified by PCR in 25-μl reactions containing approximately 0.5 μg cDNA, 0.2 mM deoxynucleotide triphosphate (10 mM stock), 1.5 mM $MgCl_2$, 0.2 μM of both sense and antisense primer, 1 U *Taq* DNA polymerasein thermophilic DNA polymerase 10× buffer. PCR was performed according to manufacturer's instructions, with cycles of denaturation at 95 C for 30 sec, annealing for 30 sec extension at 72 C for 45 sec, and reaction termination at 72 C for 5 min. For analysis, PCR products were loaded on to 1% agarose gels containing 0.3 μg/ml ethidium bromide. Gels were run at approximately 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 ALP, ENPP-1, osteocalcin (OCN), osteopontin (OPN), type I collagen (COL1), and matrix gla protein (MGP) was investigated in osteoblasts cultured for 4, 7, and 14 d. All reactions were carried out in triplicate using cDNAs derived from three different osteoblast cultures. Primer sequences and annealing temperatures are shown in Table 1.

Western blot

Osteoblasts were cultured for 14 d before the monolayers were lysed in ice-cold radio immunoprecipitation lysis buffer [50 mM Tris HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1% sodium dodecyl sulfate, 1 mM phenyl methyl sulfonyl fluoride, 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. Before loading, total protein samples were denatured by incubating at 95 C for 5 min with 5× reducing sample buffer [60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% sodium dodecyl sulfate, 10% β-mercaptoethanol, and 0.1% bromophenol blue]. Protein samples (20 μg/lane) were loaded into SDS-PAGE (8%) gels and transferred onto a polyvinylidenifluoride membrane (Amersham, Buckinghamshire, UK) by the use of a wet tank blotter (Bio-Rad, Hercules, CA) at 150 V for 1 h. The membrane was afterward blocked with 5% nonfat milk and incubated with goat ENPP-1 antibody (Abcam, Cambridge, UK) overnight at room temperature. After washing, blots were incubated in horseradish peroxidase-conjugated secondary antibodies for 1 h. Immunoreactivity was visualized using Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore, Watford, UK). Control experiments were performed by preadsorbing the primary antibody with the immunizing peptide and by omission of the primary antibody.

Measurement of PPi levels

Osteoblasts were cultured until the onset of bone formation before measurement of PPi levels. Culture medium was removed, cell layers

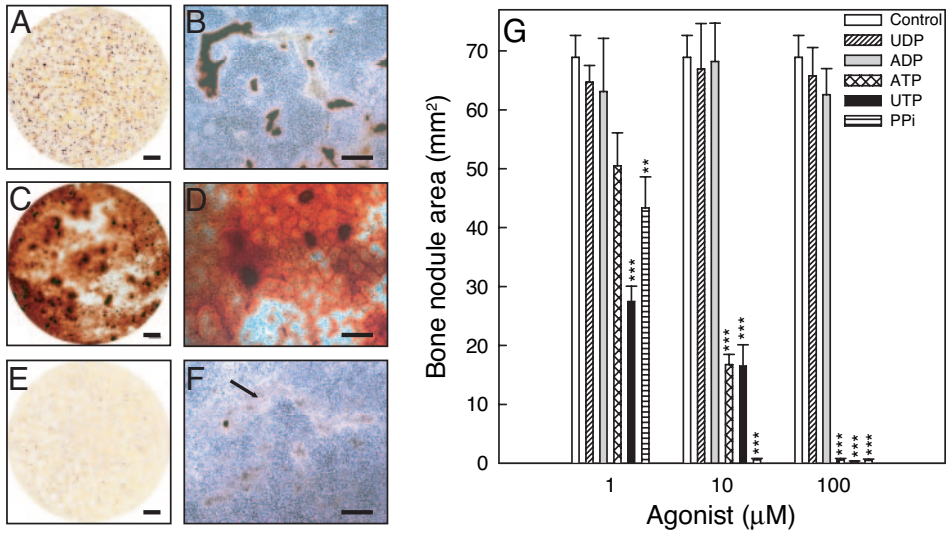


FIG. 1. Inhibition of bone nodule mineralization by PPi and extracellular nucleotides; influence of β-glycerophosphate on mineralization. Rat calvarial osteoblasts were cultured for 14 d before staining with alizarin red to demonstrate mineral deposition. A and B, Bone formed in the presence of 2 mM β-glycerophosphate exhibits typical trabecular morphology with discrete mineralization confined to regions of matrix nodules. C and D, In the presence of 10 mM β-glycerophosphate, widespread, nonspecific (dystrophic) deposition of bone mineral occurs across the cell monolayer, with inhibition of normal matrix deposition. E and F, Inhibition of matrix mineralization in osteoblasts treated with 10 μM UTP (cells cultured with 2 mM β-glycerophosphate). Normal deposition of organic matrix occurs in UTP-treated cultures (*arrow*), but alizarin *red* staining is almost absent. Similar effects were observed cultures treated with 10 μM ATP or 10 μM PPi. A, C, and E are transmitted light scans; B, D, and F are phase-contrast micrographs. *Scale bars*, 0.2 cm (A and E); 0.1 cm (C); 50 μm (B, D, and F). G, Comparison of the actions of nucleotide agonists with that of PPi on mineralization of matrix nodules. ATP, UTP, and PPi were of roughly similar potency, whereas ADP and UDP were without effect. Significantly different from control: **, $P < 0.01$, ***, $P < 0.001$; values are means ± SEM (n = 6). The data shown are representative of three independent experiments.

washed, and cells incubated in 10 mM HEPES buffer containing 0.9% NaCl and 1% BSA (pH 7.4) for 1 h. Extracellular nucleotides (10 μM) were added to the HEPES buffer and samples collected at 2 and 10 min after treatment. PPi levels were measured using an assay that links pyrophosphatase to a phosphate binding colorimetric indicator (PiPer; Molecular Probes Inc., Invitrogen, Paisley, UK). Cell viability was determined by measuring the amount of lactate dehydrogenase in the culture supernatants (CytoTox 96 nonradioactive cytotoxicity assay; Promega).

Dual-energy x-ray absorptiometry (DEXA) analysis of $P2Y_2$ knockout mice

Bone mineral density (BMD) and bone mineral content (BMC) of two-month-old male $P2Y_2$ knockout ($P2Y_2^{-/-}$) mice (27) and corresponding wild-type animals was measured by DEXA using a small animal scanner (Lunar PIXImus; GE Healthcare, Chalfont St. Giles, UK). Data were analyzed using the manufacturer's software (version 1.8).

Statistical analysis

Statistical comparisons were made by one-way ANOVA and adjusted using the Bonferroni method. Results shown are for representative experiments that were each repeated at least three times.

Results

Excess β-glycerophosphate causes dystrophic mineralization

Osteogenic growth media are commonly supplemented with β-glycerophosphate, which is hydrolyzed by ALP to yield the phosphate required for bone mineralization. To optimize the bone formation assay, we compared the effects 2 and 10 mM β-glycerophosphate on mineral deposition. Osteoblasts cultured with 2 mM β-glycerophosphate reproducibly formed abundant, discrete nodules with characteristic trabecular morphology; alizarin red staining revealed that mineralization was exclusively confined to defined regions of these matrix structures (Fig. 1, A and B). In contrast, in osteoblasts cultured with 10 mM β-glycerophosphate, widespread dystrophic deposition of mineral occurred. Moreover, generalized mineralization of cells was observed in the presence of 10 mM β-glycerophosphate, resulting in impaired trabecular matrix deposition (Fig. 1, C and D). Therefore, 2 mM β-glycerophosphate was used for all subsequent experiments.

Extracellular nucleotides and pyrophosphate inhibit mineralization of bone matrix nodules

Examination of osteoblast cell layers by light microscopy revealed that in cultures treated with ATP, UTP, or PPi, organic matrix was deposited in the trabecular pattern but that mineralization (as evidenced by alizarin red staining) was selectively inhibited (compare Fig. 1, E and F, with Fig. 1, A and B). ATP, UTP, and PPi were of roughly similar potency, eliciting 30–60%, greater than 75% and greater than 98% inhibitions of alizarin red-stained nodules at concentrations of 1, 10, and 100 μM, respectively, whereas ADP and UDP were without effect (Fig. 1, E–G).

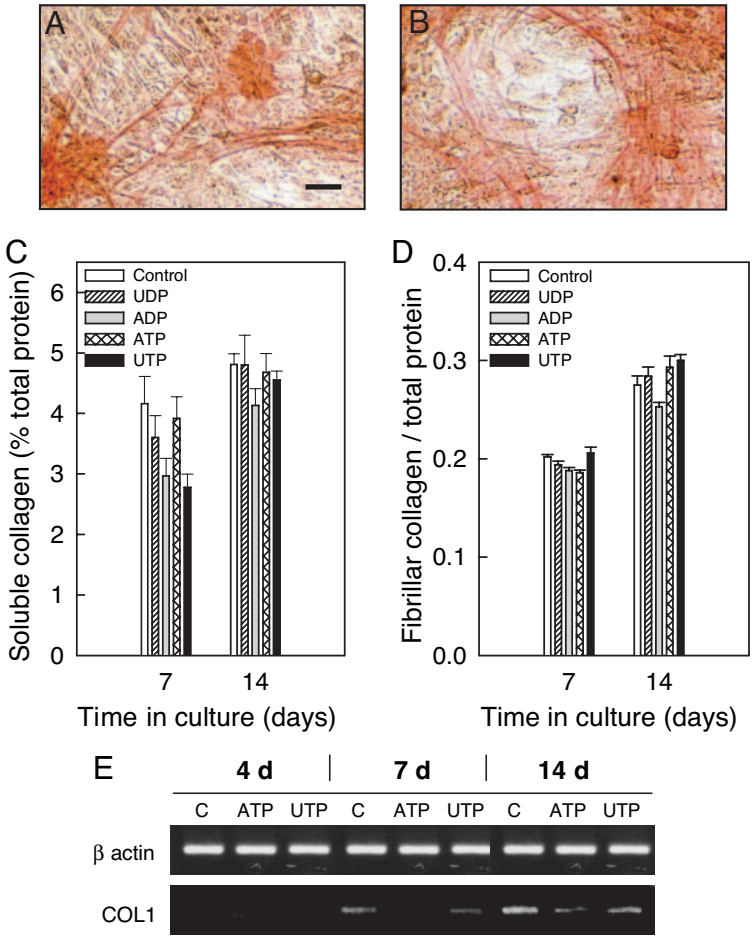


FIG. 2. Nucleotides do not affect collagen formation and deposition by rat primary osteoblasts. Rat calvarial osteoblasts were cultured for up to 14 d in the presence of 10 μM ATP, UTP, ADP, or UDP. A, Representative micrograph showing collagen fibers deposited in Sirius *red*-stained 14-d control culture. B, Representative micrograph showing collagen fibers deposited in Sirius *red*-stained 14-d ATP-treated culture; collagen deposition was similarly unaffected in cultures treated with UTP, ADP, UDP, or PPi. *Scale bar*, 10 μm. Soluble collagen production (C) and collagen deposition (D) were unaffected by treatment with extracellular nucleotides. E, Slight reductions in COL1 mRNA expression were observed in the presence of ATP and UTP after 7 and 14 d of culture. Treatment with ADP, UDP, and PPi did not influence COL1 mRNA expression (not shown). All data are representative of three independent experiments.

Lack of effect of extracellular nucleotides on osteoblast collagen production

Sirius red staining revealed abundant, organized collagen fiber deposition in both control (Fig. 2A) and 10 μM ATP-, UTP-, ADP-, or UDP-treated osteoblast cultures (Fig. 2B). ATP and UTP (and also ADP and UDP) had no effect on the production of either soluble or deposited collagen by osteoblasts (Fig. 2, C and D), although a slight decrease in COL1 mRNA expression was noted in ATP- and UTP-treated cells (Fig. 2E).

ATP and UTP inhibit ALP expression and activity

ALP plays a key role in mineralization because it hydrolyzes PPi and phosphate esters to yield Pi. The effect of ATP, UTP, ADP, UDP, and PPi (10 μM) on ALP activity was studied at 4, 7, 10, and 14 , of culture. Treatment with 10 μM UTP inhibited ALP activity by 45–65% at all time points; ATP exerted a slightly less potent inhibitory action on ALP activity (Fig. 3A). No effect of ADP, UDP, or PPi on ALP activity was evident at any time point (Fig. 3A).

The effects of nucleotides and PPi (10 μM) on expression of mRNA for ALP at 4, 7, and 14 d of culture were studied by RT-PCR. In control cultures, increasing ALP mRNA expression over time reflected the progression of osteoblast differentiation. In cells treated with ATP or UTP, decreased ALP expression was observed at all time points (Fig. 3B), whereas ADP, UDP, and PPi were without effect (not shown).

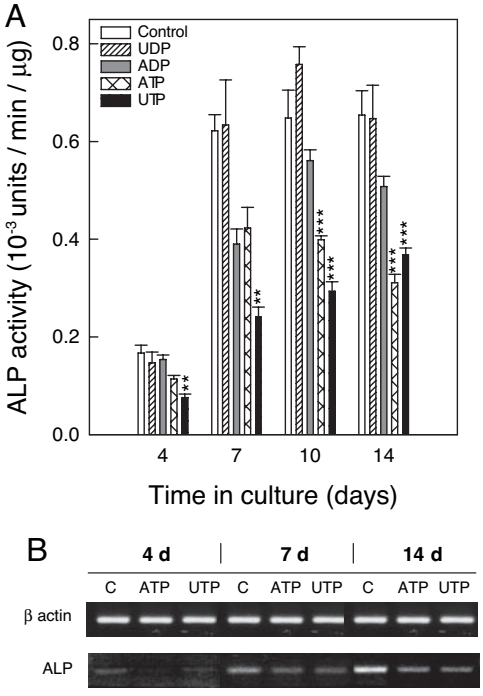


FIG. 3. ATP and UTP inhibit expression and activity of osteoblast ALP. Rat osteoblasts were treated with ATP, UTP, ADP, UDP, or PPi (10 μM) for 4, 7, 10, or 14 d. A, ATP and UTP inhibited ALP activity by approximately 50% at later time points, whereas UDP and ADP were without effect, significantly different from control: **, $P < 0.01$; ***, $P < 0.001$; values are means ± SEM (n = 6). PPi did not influence ALP activity at any time throughout the culture period (not shown). B, ATP and UTP decreased ALP mRNA expression (evident at d 7 and 14), whereas PPi, ADP, and UDP had no effect (not shown). The data shown are representative of three independent experiments.

ATP, UTP, and PPi inhibit expression of ENPP-1 but do not influence total ENPP activity

ENPP-1 hydrolyzes nucleotide triphosphates to produce PPi; thus, its effects on mineralization are antagonistic to those of ALP. ATP, UTP, and PPi (10 μM) each decreased ENPP-1 mRNA expression in rat osteoblasts at 4, 7, and 14 d of culture (Fig. 4A), whereas ADP and UDP were without effect (not shown). Treatment with ATP, UTP, and PPi also decreased ENPP-1 protein expression in 14-d osteoblast cultures (Fig. 4B). However, the total ENPP activity of whole cell lysates was unchanged in osteoblasts cultured for up to 14 d with ATP, UTP, ADP, UDP, or PPi (Fig. 4C).

Effect of extracellular nucleotides and PPi on the expression of OCN, OPN, and MGP by osteoblasts

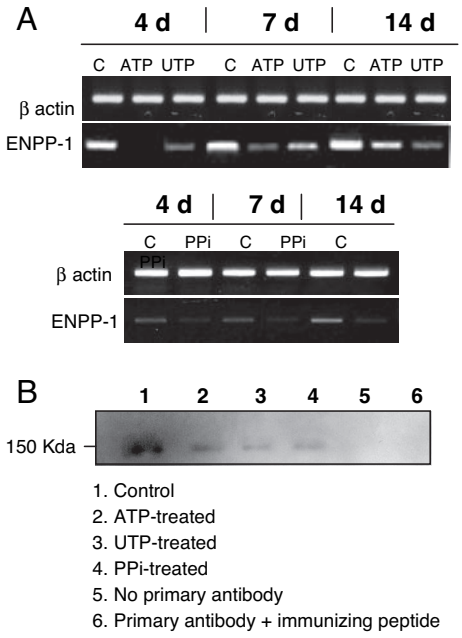
Expression of mRNA for OCN by osteoblasts increased strongly with time in culture. Treatment with ATP or UTP reduced OCN mRNA expression in 14-d cultures (Fig. 5); no effects of ADP, UDP, or PPi on OCN expression were evident (not shown). Expression of mRNAs for OPN and MGP were unaffected by treatment with nucleotides (Fig. 5) or PPi (not shown).

ATP and UTP are rapidly hydrolyzed by osteoblasts to yield PPi

To investigate whether ENPP activity could generate significant concentrations of PPi, primary osteoblasts were cultured until the onset of bone formation at 10 d. The basal accumulation of PPi, measured 1 h after replacement of culture medium with buffered saline, was consistently in the range 2–3 μM. This relatively high background level of PPi could reflect breakdown of nucleotides released from osteoblasts in response to culture medium replacement. Exogenous ATP or UTP (10 μM) was then added to cultures. After 10 min, PPi levels were approximately 2 μM higher in ATP/UTP-treated osteoblasts, compared with vehicle controls, providing further evidence for the rapid hydrolysis of extracellular nucleotides by ENPP-like activity (Fig. 6). Cell viability was unaffected by medium changes or nucleotide addition.

Inhibition of bone nodule mineralization by GTP and CTP

To test whether a component of the inhibition of bone nodule mineralization could occur independently of P2 receptors, we investigated the action of the nucleotide triphosphates, GTP and CTP (which do not activate P2 receptors but can be hydrolyzed by ENPPs to yield PPi) in 14-d osteoblast cultures. Both GTP and CTP elicited dose-dependent inhibitions of matrix mineralization, with 98% reductions in the presence of 10 μM nucleotide and complete blockade at 100 μM (Fig. 7A). However, in contrast with the inhibitory actions of ATP/UTP (Fig. 3) both GTP and CTP were without effect on ALP activity (Fig. 7B).



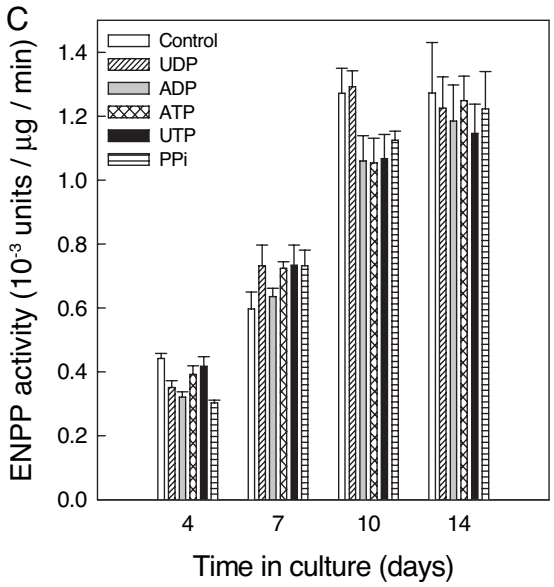


FIG. 4. Effect of extracellular nucleotides and PPi on osteoblast ENPP expression and activity. A, ATP, UTP, and PPi decreased ENPP-1 mRNA expression throughout, whereas ADP and UDP were without effect (not shown). B, ENPP-1 protein expression was decreased in osteoblasts treated with ATP, UTP, and PPi. C, Total ENPP activity in osteoblast lysates increased with time in culture but was not affected by treatment with extracellular nucleotides or PPi (10 μM) at any time point. All data are representative of three independent experiments.

Increased limb BMC in P2Y$_2$ knockout mice

Two-month-old, male P2Y$_2^{-/-}$ mice and wild-type controls were studied using DEXA. Significant differences were observed in limb bones: BMC was increased by 9% in femora of P2Y$_2^{-/-}$ animals (Fig. 8A) and 17% in tibia/fibula (Fig. 8B). Whole-body BMC was 7% greater in knockouts, reflecting modest increases in spine, although these differences were not significant. Significant (8%) increases in femoral

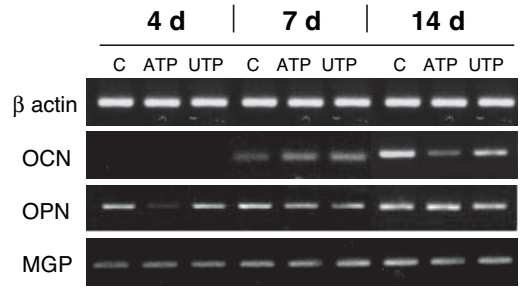


FIG. 5. The effect of extracellular nucleotides and PPi on OCN, OPN, and MGP mRNA expression. Rat osteoblasts were treated with ATP, UTP, ADP, UDP, or PPi (10 μM) for 4, 7, and 14 d of culture. No effects on mRNA expression were evident except for reduced OCN expression in cultures treated for 14 d with ATP or UTP. The data shown are representative of three independent experiments.

BMD were also observed in knockouts, together with modest, nonsignificant increases in femoral and spinal BMD. Body weight and fat content were unchanged in the P2Y$_2^{-/-}$ animals, compared with controls.

Discussion

The primary calvarial osteoblast culture system used here offers the opportunity to study separately the independent processes of bone matrix deposition and mineralization. Our results show that the key functional effect of extracellular ATP and UTP in cultures of primary rat osteoblasts is to block the mineralization of matrix nodules. Significant inhibition of mineralization occurs in the presence of relatively low concentrations of nucleotide (1–10 μM), without affecting production of fibrillar or soluble collagen (although nucleotide

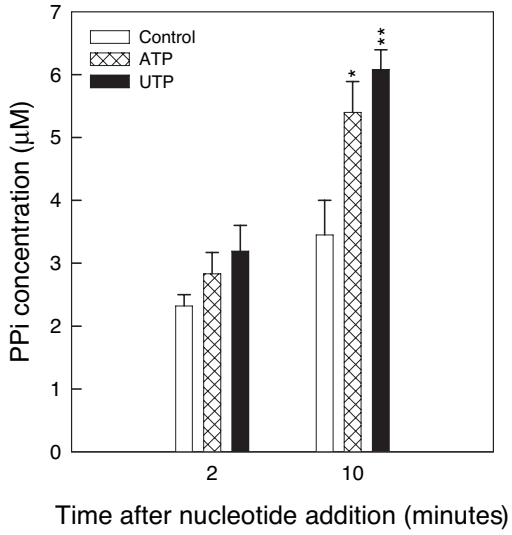


FIG. 6. Generation of PPi after addition of ATP or UTP to rat osteoblasts. PPi levels were measured in osteoblast cultures after addition of 10 μM ATP or UTP. Consistent with the rapid hydrolysis of extracellular nucleotides, culture medium PPi levels increased by approximately 2 μM 10 min after addition of ATP or UTP. Cell viability was not affected by nucleotide addition at either time point. Significantly different from control: *, $P < 0.05$; **, $P < 0.01$; values are means ± SEM (n = 6). All data are representative of three independent experiments.

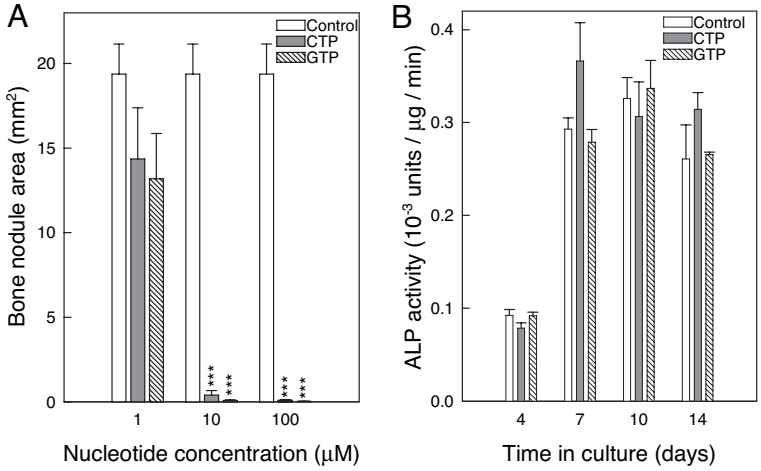


FIG. 7. Inhibition of bone nodule mineralization by CTP and GTP. The action of the nucleotide triphosphates, GTP and CTP (which do not activate P2 receptors but can be hydrolyzed by ENPPs to yield PPi), was investigated in 14-d osteoblast cultures. A, Both CTP and GTP elicited dose-dependent inhibitions of matrix mineralization, evidenced by alizarin red staining, with 98% reductions in the presence of 10 μM nucleotide and complete blockade at 100 μM. B, In contrast with the inhibitory actions of ATP and UTP, both CTP and GTP were without effect on alkaline phosphatase activity. Significantly different from control: **, $P < 0.01$; ***, $P < 0.001$; values are means ± SEM (n = 6). The data shown are representative of three independent experiments.

treatment did elicit small decreases in COL1 mRNA expression).

Several lines of evidence suggest that the inhibitory actions of UTP and ATP on bone mineralization are mediated, at least in part, via the ATP/UTP-selective $P2Y_2$ receptor. Functional expression of this receptor is strongly up-regulated in mature osteoblasts (9). Involvement of the $P2Y_4$ receptor (which is also activated by ATP and UTP) in the control of mineralization is less likely because reactive blue 2, a selective $P2Y_4$ receptor antagonist, fails to block the effects of UTP on osteoblasts (9). It was not possible to determine whether suramin (an antagonist with selectivity for the $P2Y_2$ receptor) could block the inhibitory action of ATP/UTP on mineralization because it is rapidly toxic to primary osteoblasts at the required concentrations (>1 μM) (Orriss, I. R., unpublished results). The lack of effect of ADP and UDP indicates that the ADP-selective $P2Y_1$ and UDP-selective $P2Y_6$ receptors are unlikely to play a significant role in regulating mineralization. Our findings also suggest that PPi, an extracellular breakdown product of nucleotide triphosphates and a potent physicochemical inhibitor of mineralization (20, 21), could contribute to the observed effects of ATP and UTP on bone formation in a nonreceptor mediated manner.

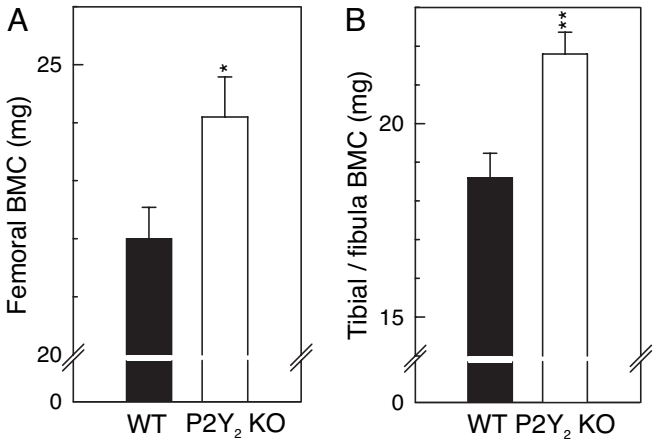


FIG. 8. Increased BMC in long bones of mice deficient in the $P2Y_2$ receptor. Two-month-old, male $P2Y_2^{-/-}$ (KO) and wild-type (WT) mice were studied by DEXA. A, Femoral BMC was increased by 9% in $P2Y_2$ KOs, compared with WTs. B, Tibia/fibula BMC was increased by 17% in KOs. Significantly different from WT control: *, $P < 0.05$; **, $P < 0.01$; values are means ± SEM (n = 10).

Extracellular nucleotides are rapidly metabolized by enzyme cascades that generate both Pi and PPi. The ectoenzymes involved in these reactions are of broad specificity and can be subdivided into four main families: 1) ectonucleoside triphosphate diphosphohydrolases; 2) ALPs; 3) ENPPs; and 4) ecto-5′-nucleotidase (or CD73) (28). Nucleotide triphosphates such as ATP and UTP can be hydrolyzed to produce Pi by ectonucleoside triphosphate diphosphohydrolases and ALPs or by ENPPs to produce PPi. ENPP-1 and ALP are thought to act in opposition to regulate PPi and Pi levels and thus mineralization (24, 29). We found that ATP and UTP (but not ADP and UDP) inhibit the activity and expression of ALP, in agreement with the observed effects of these nucleotides on bone nodule mineralization, and consistent with involvement of the $P2Y_2$ receptor. Despite its potent inhibitory action on matrix mineralization, PPi did not influence ALP activity or mRNA expression, suggesting a differing mode of action from ATP/UTP.

The local levels of PPi in bone extracellular fluid are not known but plasma concentrations in humans are in the range 2.1–3.6 μM (30, 31). In our osteoblast culture system, PPi inhibited nodule mineralization at concentrations of 1 μM or greater. This observation raises an important question: is the inhibitory effect of ATP and UTP on matrix mineralization entirely P2 receptor mediated, or could a component be due to PPi generated by ENPP hydrolysis of nucleotide triphosphates?

Compared with other tissues, bone and cartilage exhibit high levels of ENPP activity (32), most of which is attributed to ENPP-1 (33) and ENPP-3 (or B10) (23). The expression and specific activities of ENPP-1 and ENPP-3 in primary osteoblasts are reported to be approximately equal, but ENPP-1 may play a more important role in the regulation of mineralization because it is preferentially targeted to the plasma membrane and matrix vesicles after translation, whereas ENPP-3 remains intracellular (34). Our results confirm functional ENPP expression in rat osteoblasts and show that ATP, UTP, and PPi decreased expression of ENPP-1 mRNA and protein (although total osteoblast ENPP activity was unaf-

fected). Consistent with functional ENPP expression, we found that a single application of 10 μM ATP or UTP to mature osteoblast cultures increased PPi levels by approximately 2 μM after 10 min. This suggests that extracellular nucleotides cause biologically significant elevations of PPi that could potentially contribute to the inhibition of mineralization.

Although the present experiments provide no insight into the persistence of the PPi generated after nucleotide application, they do suggest that exogenous nucleotides are hydrolyzed rapidly by osteoblasts. This observation is in line with the *in vitro* results of Buckley *et al.* (35) and is supported by unpublished results from our own laboratory showing that a medium change causes a 1- to 2-μM surge in ATP release from cultured osteoblasts that returns near to baseline (~300 nM) within 30 min. It is worth noting that ATP could potentially accumulate to higher concentrations in bone tissue *in vivo*, in which extracellular fluid volumes adjacent to osteoblasts are much lower than those in tissue culture.

In this study, exogenous nucleotides were replenished every 3 d and thus would be available to act at P2 receptors for only a tiny fraction of that time. Therefore, it seems reasonable to suppose that the long-lasting inhibitory effects of ATP/UTP on mineralization could involve some kind of triggering response at P2 receptors. Additionally (or alternatively), PPi generated by ENPP hydrolysis of exogenous ATP/UTP may persist somewhat longer in the extracellular milieu, owing to slower subsequent degradation by ALP (which is inhibited by ATP/UTP).

Our observation that the nucleotide triphosphates, GTP and CTP, caused dose-dependent inhibitions of bone nodule formation at low micromolar concentrations also supports the notion that the inhibitory effects of ATP/UTP could occur independently of P2 receptors. GTP and CTP do not activate P2 receptors but can be hydrolyzed by ENPPs to yield PPi. Moreover, both GTP and CTP were without effect on ALP activity, in contrast with the inhibitory actions of ATP and UTP. It is unclear why GTP and CTP exerted such large inhibitory effects (comparable with UTP and ATP) on mineralization; one potential explanation might be that these nucleotides are preferentially hydrolyzed to PPi by osteoblasts.

The results of our DEXA analysis of the skeletons of $P2Y_2$-deficient mice could also be interpreted as consistent with the existence of dual mechanisms for the nucleotide inhibition of mineralization. The moderate increases in bone mineral content and density that we observed in knockouts suggest that $P2Y_2$ receptor-mediated mechanisms may indeed have some restraining influence on mineralization *in vivo*. However, the relatively modest scale of the changes seen in $P2Y_2$-deficient mice emphasizes that skeletal mineralization is controlled by multiple mechanisms, which could include PPi generated from extracellular nucleotides.

In conclusion, this study highlights the potential importance of extracellular nucleotides as paracrine signaling agents in bone. We provide evidence that ATP exerts both receptor-mediated and nonreceptor-mediated inhibitory effects on bone mineralization (Fig. 9).

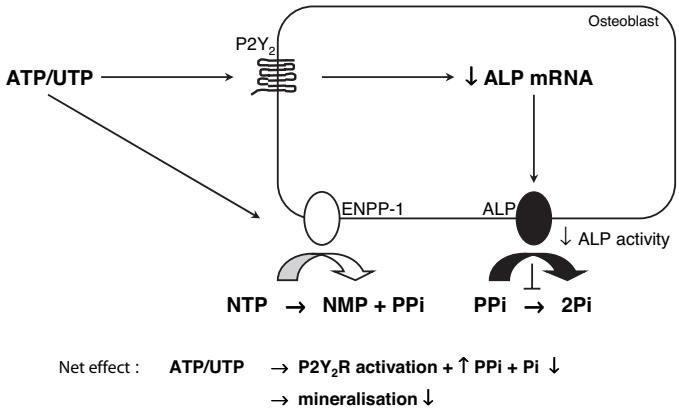


FIG. 9. The effect of ATP and UTP on extracellular PPi and mineralization. Schematic diagram of the potential mechanism by which extracellular nucleotides inhibit bone mineralization. ATP and UTP, acting via the $P2Y_2$ receptor (and possibly also the $P2Y_4$ receptor) on mature, bone-forming osteoblasts, cause decreased expression/activity of ALP. This in turn would lead to an increase in extracellular PPi (a key physiological inhibitor of mineralization) and a decrease in local Pi levels. Concurrently, nucleotide triphosphates (NTP) such as ATP and UTP can also be hydrolyzed by osteoblast ENPP-1 to generate PPi directly. The combined effect is a net increase in extracellular PPi concentration, leading to a decrease in mineralization. NMP, Nucleotide monophosphate.

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