

FAST TRACK

# ATP and UTP At Low Concentrations Strongly Inhibit Bone Formation by Osteoblasts: A Novel Role for the P2Y<sub>2</sub> Receptor in Bone Remodeling

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**Abstract** There is increasing evidence that extracellular nucleotides act on bone cells via multiple P2 receptors. The naturally-occurring ligand ATP is a potent agonist at all receptor subtypes, whereas ADP and UTP only act at specific receptor subtypes. We have reported that the formation and resorptive activity of rodent osteoclasts are stimulated powerfully by both extracellular ATP and its first degradation product, ADP, the latter acting at nanomolar concentrations, probably via the P2Y<sub>1</sub> receptor subtype. In the present study, we investigated the actions of ATP, ADP, adenosine, and UTP on osteoblastic function. In 16–21 day cultures of primary rat calvarial osteoblasts, ADP and the selective P2Y<sub>1</sub> agonist 2-methylthioADP were without effect on bone nodule formation at concentrations between 1 and 125 μM, as was adenosine. However, UTP, a P2Y<sub>2</sub> and P2Y<sub>4</sub> receptor agonist, known to be without effect on osteoclast function, strongly inhibited bone nodule formation at concentrations ≥ 1 μM. ATP was inhibitory at ≥ 10 μM. Rat osteoblasts express P2Y<sub>2</sub>, but not P2Y<sub>4</sub> receptor mRNA, as determined by *in situ* hybridization. Thus, the low-dose effects of extracellular nucleotides on bone formation and bone resorption appear to be mediated via different P2Y receptor subtypes: ADP, signalling through the P2Y<sub>1</sub> receptor on both osteoclasts and osteoblasts, is a powerful stimulator of osteoclast formation and activity, whereas UTP, signalling via the P2Y<sub>2</sub> receptor on osteoblasts, blocks bone formation by osteoblasts. ATP, the ‘universal’ agonist, can simultaneously stimulate resorption and inhibit bone formation. These findings suggest that extracellular nucleotides could function locally as important negative modulators of bone metabolism, perhaps contributing to bone loss in a number of pathological states. *J. Cell. Biochem.* 86: 413–419, 2002. © 2002 Wiley-Liss, Inc.

**Key words:** P2 receptors; bone; osteoblast; P2Y<sub>2</sub>; UTP; ATP

Extracellular nucleotides are now recognized as important signaling molecules mediating a wide range of functions; they act via two types of receptors: P2X, which are ligand-gated ion channels and P2Y, which are G protein-coupled receptors raising cytosolic free calcium ([Ca<sup>2+</sup>]<sub>i</sub>) [Ralevic and Burnstock, 1998]. There is increas-

ing evidence that extracellular nucleotides, signalling through P2 receptors, may play a role in modulating the function of the two major bone cell types: osteoclasts and osteoblasts [see Dixon and Sims, 2000]. Osteoclasts are the multinucleated cells responsible for bone resorption and are formed by fusion of mononuclear precursors of the monocyte/macrophage lineage, whereas osteoblasts are mesenchymal stem cell-derived cells responsible for bone formation. As we have reported previously, both cell types express a range of P2X and P2Y receptor subtypes [Hoebertz et al., 2000].

Functional studies on osteoclasts reported that adenosine 5'-triphosphate (ATP), a potent agonist at almost all P2 receptor subtypes, has a stimulatory effect on both recruitment and activation of osteoclasts [Bowler et al., 1998a;

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Morrison et al., 1998]. We have recently shown that adenosine 5'-diphosphate (ADP), the first degradation product of ATP, is a powerful stimulator of osteoclast formation and activation [Hoebertz et al., 2001]. Furthermore, we provided evidence, using P2 receptor subtype-selective agonists and antagonists that these osteolytic effects are most likely mediated via the metabotropic P2Y<sub>1</sub> receptor, linking for the first time a specific P2 receptor subtype to a key functional action of extracellular nucleotides on bone.

A number of studies have shown that ATP and other nucleotides act through P2Y receptors to transiently elevate  $[Ca^{2+}]_i$  and induce formation of IP<sub>3</sub> in osteoblast-like cells and non-transformed human bone-derived cells [Kumagai et al., 1989, 1991; Schoebl et al., 1992]. Studies on rat osteoblast-like UMR-106 cells demonstrated that extracellular nucleotides interact with at least two receptor subtypes coupled to internal  $[Ca^{2+}]_i$  release. The pharmacological profiles were characteristic of P2Y<sub>1</sub>- and P2Y<sub>2</sub>-like receptors [Reimer and Dixon, 1992; Yu and Ferrier, 1993; Sistare et al., 1994]. Studies on single cells and populations of human osteoblasts revealed a heterogeneity of receptor expression within one cell culture: ATP and UTP induced  $[Ca^{2+}]_i$  rises in every cell tested, whereas only a subgroup of cells responded to ADP or 2-methylthio ATP (2-meSATP). This pattern of receptor expression might reflect changes in the osteoblast differentiation status [Dixon et al., 1997].

The first molecular evidence for the expression of P2Y receptors by osteoblasts came with cloning of the human P2Y<sub>2</sub> receptor and its localization in both osteoclast-like cells and in human osteoblasts derived from different sources [Bowler et al., 1995]. RT-PCR demonstrated the expression of P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, and P2Y<sub>6</sub> receptors in human bone and in osteoblastic cell lines [Maier et al., 1997].

However, the functions of extracellular nucleotides in osteoblast biology are not yet well understood. Both, ATP and adenosine are able to act as mitogens for osteoblastic cells, an effect which might be mediated indirectly through enhancement of prostaglandin E synthesis [Shimegi, 1996; Nakamura et al., 2000]. Additionally, several studies reported that nucleotides can act synergistically on osteoblasts with other factors, both with growth factors such as platelet-derived growth factor (PDGF) and

insulin-like growth factor (IGF) to induce proliferation [Suzuki et al., 1993; Shimegi, 1996], but most notably with parathyroid hormone to potentiate PTH-induced  $Ca^{2+}$  signalling and *c-fos* expression [Kaplan et al., 1995; Sistare et al., 1995; Bowler et al., 1999; Buckley et al., 2001]. Localized release of nucleotides *in vivo* might thus help to modulate or even target responses to systemic factors.

Extracellular nucleotides have also been shown to reduce the amount of bone formed by primary rat osteoblasts in an *in vitro* appositional bone formation model [Jones et al., 1997]. However, the concentrations of ATP used were rather high (50–500  $\mu$ M), and results for the effects of UTP were equivocal. Thus, it was still unclear, which receptor subtypes might be involved in the effects of nucleotides on bone formation, and how this might relate to the role of P2 receptors in bone resorption described above. In this study, we used primary rat calvarial osteoblasts to investigate the effects of low-dose nucleotides on osteoblastic bone formation employing the bone nodule assay.

## MATERIALS AND METHODS

### Bone Nodule Assay

Calvarial bones from 2-day-old neonatal Sprague–Dawley rats were excised and all fibrous tissue removed. Primary rat osteoblastic cells were obtained by sequential enzyme digestion using a three-step process (1% trypsin in PBS for 10 min; 0.2% collagenase type II in HBSS for 30 min; 0.2% collagenase type II in HBSS for 60 min, all at 37°C), rejecting the first two digests. The cells were resuspended in Dulbecco's modified Eagle's medium with Glutamax<sup>®</sup> + 10% FBS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 0.25  $\mu$ g/ml amphotericin B (complete mixture abbreviated 'DMEM'), and cultured in a humidified atmosphere of 5% CO<sub>2</sub>-95% air in 75-cm<sup>2</sup> flasks until confluence (2–4 days). At confluence, cells were subcultured into 24-well plates at a density of 10<sup>4</sup>/well in DMEM supplemented with ascorbic acid (50  $\mu$ g/ml),  $\beta$ -glycerophosphate (2 mM) and dexamethasone (10<sup>-8</sup> M). After a 24-h preincubation period, nucleotides (adenosine, ADP, ATP, UTP at 0.2–125  $\mu$ M) were added to the media. Half of the medium was replaced with fresh test media (with nucleotides double the desired concentration) or control medium every 3 days.

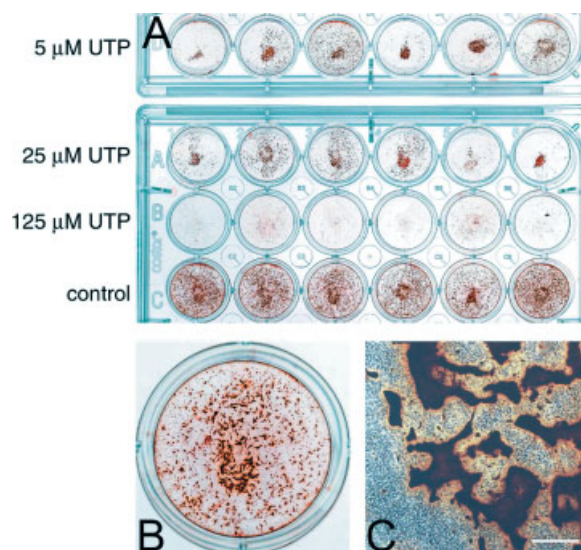
Cultures were examined daily using an Olympus IMT-2 inverted microscope with phase-contrast optics (Olympus Optical Company Ltd., Japan) and continued for 15–21 days, depending on the onset of cell cluster and nodule formation, which varied from culture to culture. At the end of the experiment, cultures were washed three times with PBS, fixed in 2% glutaraldehyde for 5 min, washed again with PBS, and after three washes with 70% ethanol, left to air-dry for 30 min. Mineralized bone nodules were stained with alizarin red (1% solution w/v in water) for 5 min, and excessive stain rinsed three times with 50% ethanol, before plates were left to air-dry completely. Number of bone nodules and total area of bone nodules/well were analyzed using the image analysis programme 'Scion Image' (Version Beta 4.02). To that end, low magnification micrographs of each well, illuminated on a light-box, were taken with a Leica high-resolution digital camera (Leica DC 200, software Version 2.51), converted to a greyscale image using 'Adobe Photoshop' (version 5.0, Adobe Systems, Inc.), and then to a binary image using 'Scion Image' image processing software (with the same settings, i.e., threshold level and minimum particle size for every picture), and quantitated for both total number of nodules/well and total area covered by nodules/well. Data were analyzed by 'Excel' and statistical differences assessed by one-way analysis of variance using 'InStat' (Version 1.13, Graph-PAD Software).

## RESULTS

Adenosine, ADP, ATP, and UTP, at concentrations between 0.1 and 500  $\mu\text{M}$ , were tested for their effects on bone nodule formation.

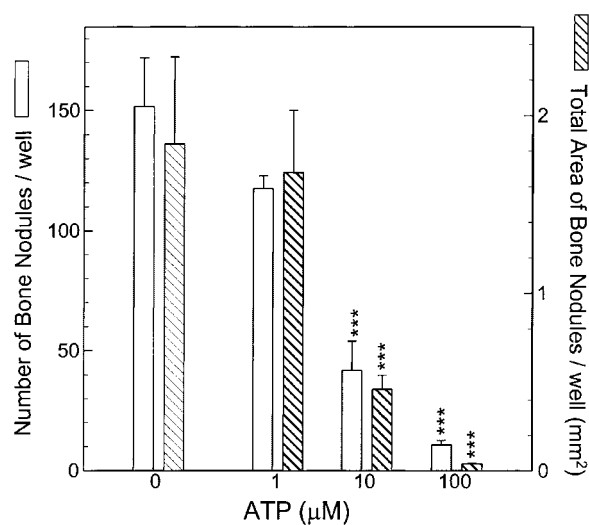
Cell clusters typically started to appear first at around day 3–6 of culture in multiwell plates; these clusters developed progressively into mineralized nodules after 16–20 days culture. Examples of the appearance of bone nodules during culture and stained with alizarin red are illustrated (Fig. 1).

ATP and UTP had a strongly inhibitory effect on bone nodule formation, both on the number of nodules and the total area of nodules per well. These inhibitory effects were easily visible by naked eye (Fig. 1A). The inhibitory effect of ATP was highly significant at 10  $\mu\text{M}$ , and was almost complete at 100  $\mu\text{M}$  (Fig. 2). In the case of UTP,

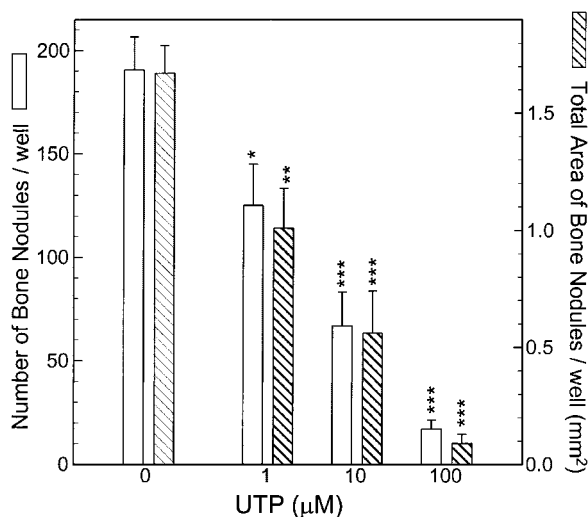


**Fig. 1.** Appearance of alizarin red-stained bone nodules in culture, illustrating inhibitory effect of UTP. (A) Rat calvarial osteoblasts cultured in 24-well plates for 15 days and stained with alizarin red to demonstrate mineralized nodules. Shown are one control row and three rows treated with UTP from 5 to 125  $\mu\text{M}$ ; inhibitory effects of UTP on bone nodule formation are clearly visible by naked eye. (B) Appearance of a single control well, as photographed before image analysis. (C) Detail of control well shown in (B), viewed by phase contrast microscopy; scale bar = 0.5 mm.

significant reduction of bone nodule formation was observed at concentrations as low as 1  $\mu\text{M}$ , again with near-complete inhibition at 100  $\mu\text{M}$  (Fig. 3).



**Fig. 2.** Inhibitory effect of ATP on bone nodule formation. Concentrations of ATP  $\geq 10$   $\mu\text{M}$  significantly inhibited number of bone nodules and total area of bone nodules/well. Values are means  $\pm$  SEM (n = 6). Significantly different from control: \*\*\*\* $P < 0.001$ .



**Fig. 3.** Inhibitory effect of UTP on bone nodule formation. Concentrations of UTP  $\geq 1$   $\mu\text{M}$  significantly inhibited number of bone nodules and total area of bone nodules/well. Values are means  $\pm$  SEM ( $n=6$ ). Significantly different from control: \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

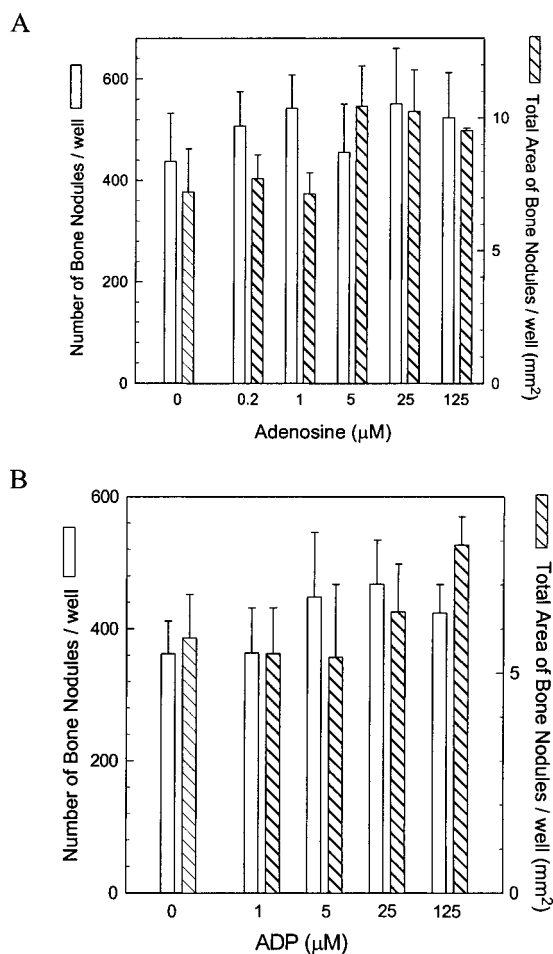
In contrast, neither adenosine (Fig. 4A) nor ADP (Fig. 4B) were found to have an effect on bone nodule formation, indicating that the inhibitory effect of ATP was indeed due to ATP itself, and not its degradation products.

## DISCUSSION

Purinergic signalling in osteoblasts has been investigated for more than a decade. The present study has clarified the actions of nucleotides on the ultimate function of osteoblasts, namely bone formation.

Both ATP and UTP, but not adenosine or ADP, significantly and consistently inhibited bone nodule formation by cultured rat osteoblasts. The potent inhibitory actions of ATP and UTP point to the involvement of either  $\text{P2Y}_2$  or  $\text{P2Y}_4$  receptors, since ATP and UTP are potent agonists at these two receptor subtypes [King et al., 1998]. However, since we have recently studied the expression of P2 receptors in bone cells using in situ hybridization, and found no evidence for the expression of the  $\text{P2Y}_4$  receptor subtype, it seems likely that the  $\text{P2Y}_2$  receptor expressed on osteoblasts is responsible for mediating the inhibitory effects we describe [Hoebertz et al., 2000].

The inhibitory effects of UTP and ATP were evident at concentrations as low as 1 and 10  $\mu\text{M}$ , respectively, a finding which differs from an



**Fig. 4.** Lack of effect of adenosine and ADP on bone nodule formation. Adenosine (A) and ADP (B) at concentrations between 0.2–1.25  $\mu\text{M}$  had no effect on number of bone nodules and total area of bone nodules/well. Values are means  $\pm$  SEM ( $n=6$ ).

earlier study on the effects of nucleotides on appositional bone formation [Jones et al., 1997]. This earlier study employed a different in vitro model of bone formation, using the same type of primary rat calvarial cells, but seeding them on dentine slices with cut-in grooves, on which bone could be deposited. At the end of the experiment, number, length, and area of bone loci were analyzed. However, in this system, significant inhibition of bone formation was only observed with very high concentrations of ATP (500  $\mu\text{M}$ ), although slight stimulation of bone formation was noted at lower concentrations (50  $\mu\text{M}$ ). Lower inhibitory concentrations (20  $\mu\text{M}$ ) were reported for 2-meSATP and the non-hydrolyzable ATP analogue  $\text{ATP}\gamma\text{S}$ , whereas, consistent with our studies, adenosine

had no effect. ADP was not tested, and the effects of UTP were equivocal: in one experiment, 2  $\mu$ M UTP stimulated bone formation, whereas in another experiment, 2–20  $\mu$ M of UTP inhibited bone formation. Thus, the results of the present study appear to provide a more conclusive result: both ATP and UTP, in the low micromolar range, are strong inhibitors of bone formation. The earlier observation by Jones et al. [1997] that ATP $\gamma$ S, a potent agonist at the P2Y<sub>2</sub> receptor, was also inhibitory is consistent with our conclusion that the effect is mediated via the P2Y<sub>2</sub> receptor.

The finding that UTP and ATP, but not ADP, modulate osteoblast function is of particular interest in the context of our recent study on the role of the P2Y<sub>1</sub> receptor in bone resorption [Hoebertz et al., 2001]. Taken together, our results indicate that the low-dose effects of extracellular nucleotides on bone resorption and formation are mediated via different P2 receptor subtypes. ADP, signalling through the P2Y<sub>1</sub> receptor, is a powerful stimulator of osteoclast formation and activity [Hoebertz et al., 2001], whereas UTP, which does not affect osteoclast function [Bowler et al., 1998a], signalling via the P2Y<sub>2</sub> receptor, seems to play a role as an inhibitor of bone formation. A mechanism for the differential purinergic regulation of osteoblasts and osteoclasts has been suggested before, based on [Ca<sup>2+</sup>]<sub>i</sub> responses of osteoclasts and osteoblastic cells to nucleotides: application of extracellular ATP (50 or 100  $\mu$ M) inhibited the Ca<sup>2+</sup> response to a subsequent application of ATP in rat osteoblastic UMR-106 cells, but not in rabbit osteoclasts, suggesting that osteoclasts can adapt to the extracellular ATP, whereas osteoblastic cells cannot adapt and desensitize [Luo et al., 1997].

ATP at much higher, millimolar concentrations has long been known to play a role in the mineralization process. ATP (> 5 mM) inhibits Ca<sup>2+</sup> deposition because it is a potent stabilizer of amorphous calcium phosphate, thus, inhibiting its conversion to hydroxyapatite *in vitro*. However, ATP at 1–5 mM can promote bone formation by providing pyrophosphate for the mineralization process by matrix vesicles [Hsu and Anderson, 1977]. The inhibitory effects of ATP and UTP on bone nodule formation in the present study were observed in the low micromolar range. Thus, an indirect, non-receptor-mediated effect on mineralization through the mechanism described above can probably be

excluded. Additionally, negative effects on bone nodule formation, as observed here for ATP and UTP, are most likely due to inhibitory effects on cell aggregation and/or collagen/matrix deposition in the earlier stages of the culture, prior to mineralization, and not to inhibition of mineral deposition.

Interestingly, it has recently been shown that induction of osteoblast differentiation by addition of ascorbic acid led to three- to fourfold increases in mitochondrial ATP production through the respiratory chain, resulting in a fivefold increase in cellular ATP content compared with that in immature, undifferentiated cells [Komarova et al., 2000]. This finding demonstrated that progressive osteoblast differentiation coincides with changes in cellular metabolism and mitochondrial activity, which are likely to play key roles in osteoblast function. An enlarged pool of ATP might be stored in mature osteoblasts for later release in response to appropriate stimuli, so that ATP can act as a paracrine agonist at P2 receptors, and based on our results, potentially serve as a 'stop signal' for bone formation.

The finding that ATP and UTP might play a role in osteoblastic activity agrees with a number of earlier studies. Kumagai et al. first reported in 1989 that ATP elevates cytosolic Ca<sup>2+</sup> through mobilization of [Ca<sup>2+</sup>]<sub>i</sub>, but not through Ca<sup>2+</sup> influx, in rat osteoblastic UMR-106 cells [Kumagai et al., 1989]. In a follow-up study, a two-receptor model was suggested because ADP and UTP, in addition to ATP, also elicited a rapid transient increase in [Ca<sup>2+</sup>]<sub>i</sub> at 1–100  $\mu$ M, followed by an increase in IP<sub>3</sub> and IP<sub>4</sub> [Kumagai et al., 1991]. The presence of P2Y<sub>1</sub> and P2Y<sub>2</sub>-like receptors on osteoblasts was confirmed by further experiments [Reimer and Dixon, 1992; Yu and Ferrier, 1993; Sistare et al., 1994, 1995; Jørgensen et al., 2002]. Reimer and Dixon [1992] reported a time-dependent inactivation of the Ca<sup>2+</sup> signalling pathway with continued receptor occupation by ATP.

Kumagai et al. [1991] also observed a potentiated Ca<sup>2+</sup> response to nucleotides in the presence of PTH. This is consistent with later studies showing that activation of P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors potentiates subsequent PTH receptor-mediated Ca<sup>2+</sup> signalling [Kaplan et al., 1995; Sistare et al., 1995; Bowler et al., 1999; Buckley et al., 2001]. It has been suggested that PTH receptors are capable of activating adenylate cyclase through G<sub>s</sub>

proteins, but might be unable to activate PLC until cells receive a signal as a consequence of P2 receptor activation [Sistare et al., 1995]. These synergies suggest a potentially highly targeted mechanism through which systemic PTH could initiate bone remodeling at specific sites in the skeleton by cooperating with the localized release of nucleotides. A recent study suggests an additional mechanism by which P2-receptor-mediated signalling between bone cells may control bone remodeling: after mechanical stimulation, the P2Y<sub>2</sub> receptor is required for the propagation of intercellular calcium waves between osteoblasts, whereas, surprisingly signalling to osteoclasts appears to require the P2X<sub>7</sub> receptor [Jørgensen et al., 2002].

In addition, ATP and adenosine (10–100 μM) have previously been shown to stimulate proliferation of cloned osteoblast-like mouse MC3T3-E1 cells, whereas UTP had no effect [Shimegi, 1996]. Mitogenic actions of ATP, possibly mediated via the P2X<sub>5</sub> receptor, were also suggested in a recent study on human osteoblast-like MG-63 cells [Nakamura et al., 2000].

Our observation that the functionally effective concentrations of ATP and UTP in osteoblasts are in the low micromolar range could be relevant to the bone microenvironment, where low-level fluctuations of extracellular nucleotide concentrations are likely to occur. Osteoblasts are capable of releasing ATP, resulting in nanomolar concentrations in the medium [Bowler et al., 1998b]; however, concentrations measured in the entire medium are unlikely to accurately reflect concentrations occurring at the cell surface. These are probably in the micromolar range before breakdown by ectonucleotidases takes place. So far, release of UTP has been reported for a range of cells, such as vascular endothelial cells, astrocytoma cells, epithelial cells, and platelets, but not osteoblasts [Saiag et al., 1995; Lazarowski and Harden, 1999]. However, UTP could easily be generated extracellularly from other nucleotides through the action of ecto-nucleotidases, e.g., UDP + ATP → UTP + ADP via ecto-nucleoside diphosphokinase [Lazarowski et al., 2000]. Interestingly, UTP can also act through P2Y receptors to upregulate ATP release from human osteoblasts, providing a possible positive feedback mechanism [Bowler et al., 2001].

In conclusion, since ATP at physiological concentrations is both a potent stimulator of

bone (and also cartilage) resorption and a potent inhibitor of bone formation, the overall action of extracellular nucleotides on bone homeostasis would appear to be strongly negative, resulting in net bone loss. Our observations may have considerable significance because enhanced nucleotide release occurs in pathological states associated with local bone loss such as inflammation, tumors, and hypoxia [Pedersen et al., 1999; Bodin and Burnstock, 2001].

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