Journal of Cellular Biochemistry 86:413–419 (2002)

FAST TRACK

ATP and UTP At Low Concentrations Strongly Inhibit Bone Formation by Osteoblasts: A Novel Role for the $P2Y_2$ Receptor in Bone Remodeling

Astrid Hoebertz,[1,2] Siva Mahendran,[1] Geoffrey Burnstock,[1,2] and Timothy R. Arnett[1]*

[1]Department of Anatomy & Developmental Biology, University College London, Gower Street, London WC1E 6BT, United Kingdom
[2]Autonomic Neuroscience Institute, University College London, Gower Street, London WC1E 6BT, United Kingdom

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 $P2Y_1$ 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 $P2Y_1$ agonist 2-methylthioADP were without effect on bone nodule formation at concentrations between 1 and 125 μM, as was adenosine. However, UTP, a $P2Y_2$ and $P2Y_4$ 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 $P2Y_2$, but not $P2Y_4$ 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 $P2Y_1$ receptor on both osteoclasts and osteoblasts, is a powerful stimulator of osteoclast formation and activity, whereas UTP, signalling via the $P2Y_2$ 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.

Key words: P2 receptors; bone; osteoblast; $P2Y_2$; 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 ($[Ca^{2+}]_i$) [Ralevic and Burnstock, 1998]. There is increasing 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;

Grant sponsor: Arthritis Research Campaign.

Astrid Hoebertz's present address is Institute of Molecular Pathology, Dr. Bohr Gasse 7, A-1030 Vienna, Austria.

*Correspondence to: Timothy R. Arnett, Department of Anatomy & Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK.
E-mail: t.arnett@ucl.ac.uk

Received 29 April 2002; Accepted 2 May 2002

DOI 10.1002/jcb.10236

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 $P2Y_1$ 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_3 in osteoblast-like cells and non-transformed human bone-derived cells [Kumagai et al., 1989, 1991; Schoefl 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 $P2Y_1$- and $P2Y_2$-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 $P2Y_2$ 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 $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ 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 μM), 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® + 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (complete mixture abbreviated 'DMEM'), and cultured in a humidified atmosphere of 5% CO_2-95% air in 75-cm^2 flasks until confluence (2–4 days). At confluence, cells were subcultured into 24-well plates at a density of 10^4/well in DMEM supplemented with ascorbic acid (50 μg/ml), β-glycerophosphate (2 mM) and dexamethasone (10^{-8} M). After a 24-h preincubation period, nucleotides (adenosine, ADP, ATP, UTP at 0.2–125 μM) 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, GraphPAD Software).

RESULTS

Adenosine, ADP, ATP, and UTP, at concentrations between 0.1 and 500 μM, 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 μM, and was almost complete at 100 μM (Fig. 2). In the case of UTP,

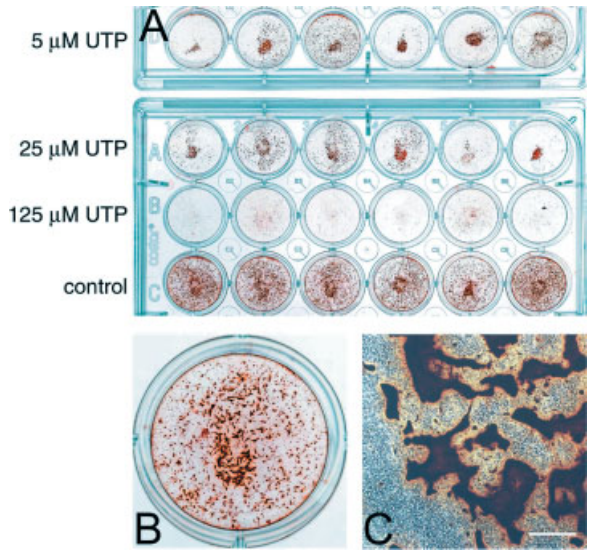

Fig. 1. Appearance of alizarin red-stained bone nodules in culture, illustrating inhibitory effect of UTP. (**A**) Rat calvarial osteoblasts cultured in 24-well plates for 15 days and stained with alizarin red to demonstrate mineralized nodules. Shown are one control row and three rows treated with UTP from 5 to 125 μM; 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 μM, again with near-complete inhibition at 100 μM (Fig. 3).

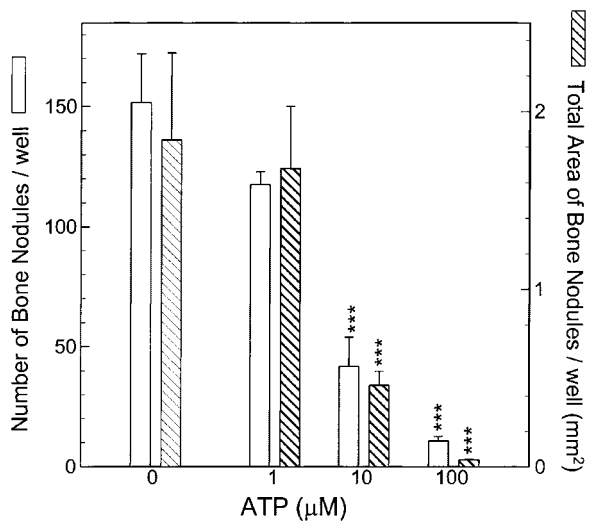

Fig. 2. Inhibitory effect of ATP on bone nodule formation. Concentrations of ATP ≥ 10 μM significantly inhibited number of bone nodules and total area of bone nodules/well. Values are means ± SEM (n = 6). Significantly different from control: ***$P < 0.001$.

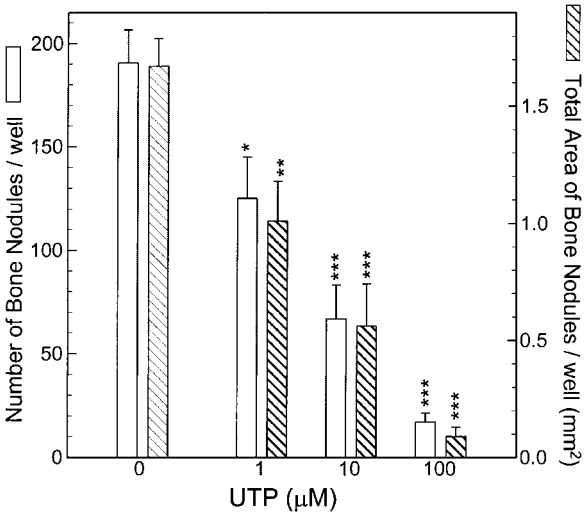

Fig. 3. Inhibitory effect of UTP on bone nodule formation. Concentrations of UTP ≥ 1 μM significantly inhibited number of bone nodules and total area of bone nodules/well. Values are means ± SEM (n = 6). Significantly different from control: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

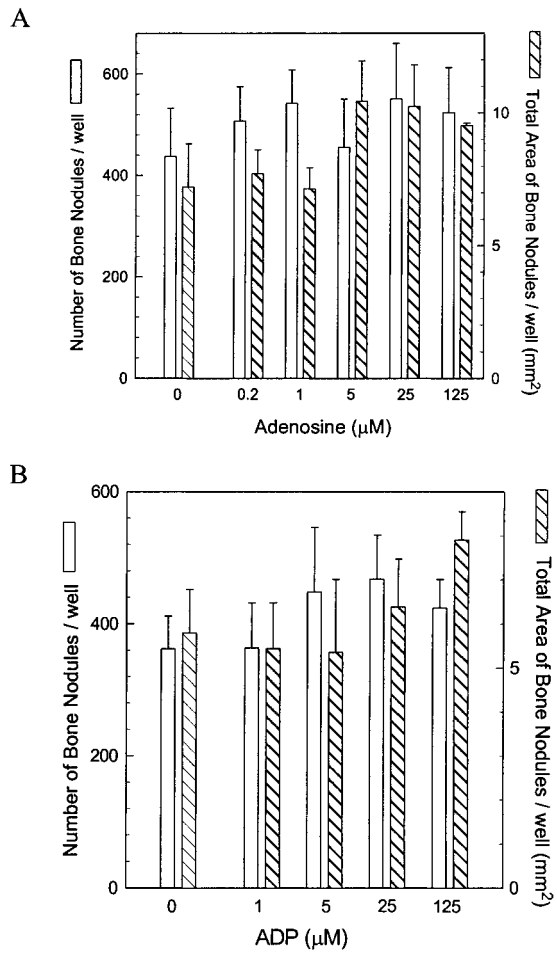

Fig. 4. Lack of effect of adenosine and ADP on bone nodule formation. Adenosine (**A**) and ADP (**B**) at concentrations between 0.2–1.25 μM had no effect on number of bone nodules and total area of bone nodules/well. Values are means ± SEM (n = 6).

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 $P2Y_2$ or $P2Y_4$ 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 $P2Y_4$ receptor subtype, it seems likely that the $P2Y_2$ 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 μM, respectively, a finding which differs from an 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 μM), although slight stimulation of bone formation was noted at lower concentrations (50 μM). Lower inhibitory concentrations (20 μM) were reported for 2-meSATP and the non-hydrolyzable ATP analogue ATPγS, whereas, consistent with our studies, adenosine

had no effect. ADP was not tested, and the effects of UTP were equivocal: in one experiment, 2 μM UTP stimulated bone formation, whereas in another experiment, 2–20 μM 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 ATPγS, a potent agonist at the $P2Y_2$ receptor, was also inhibitory is consistent with our conclusion that the effect is mediated via the $P2Y_2$ 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 $P2Y_1$ 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 $P2Y_1$ 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 $P2Y_2$ 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 $[Ca^{2+}]_i$ responses of osteoclasts and osteoblastic cells to nucleotides: application of extracellular ATP (50 or 100 μM) inhibited the Ca^{2+} 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^{2+} 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^{2+} through mobilization of $[Ca^{2+}]_i$, but not through Ca^{2+} 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 $[Ca^{2+}]_i$ at 1–100 μM, followed by an increase in IP_3 and IP_4 [Kumagai et al., 1991]. The presence of $P2Y_1$ and $P2Y_2$-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^{2+} signalling pathway with continued receptor occupation by ATP.

Kumagai et al. [1991] also observed a potentiated Ca^{2+} response to nucleotides in the presence of PTH. This is consistent with later studies showing that activation of $P2Y_1$ and $P2Y_2$ receptors potentiates subsequent PTH receptor-mediated Ca^{2+} 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_S

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 $P2Y_2$ receptor is required for the propagation of intercellular calcium waves between osteoblasts, whereas, surprisingly signalling to osteoclasts appears to require the $P2X_7$ 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 $P2X_5$ 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 ecto-nucleotidases 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].

ACKNOWLEDGMENT

The authors are grateful for the support of the Arthritis Research Campaign.

REFERENCES

Bodin P, Burnstock G. 2001. Purinergic signalling: ATP release. Neurochem Res 26:959–969.

Bowler WB, Birch MA, Gallagher JA, Bilbe G. 1995. Identification and cloning of human P2U purinoceptor present in osteoclastoma, bone, and osteoblasts. J Bone Miner Res 10:1137–1145.

Bowler WB, Littlewood Evans A, Bilbe G, Gallagher JA, Dixon CJ. 1998a. $P2Y_2$ receptors are expressed by human osteoclasts of giant cell tumor but do not mediate ATP-induced bone resorption. Bone 22:195–200.

Bowler WB, Tattersall JA, Hussein R, Dixon CJ, Cobbold PH, Gallagher JA. 1998b. Release of ATP by osteoblasts: Modulation by fluid shear forces. Bone 22:3S (abstract).

Bowler WB, Dixon CJ, Halleux C, Maier R, Bilbe G, Fraser WD, Gallagher JA, Hipskind RA. 1999. Signaling in human osteoblasts by extracellular nucleotides. J Biol Chem 274:14315–14324.

Bowler WB, Buckley KA, Gartland A, Hipskind RA, Bilbe G, Gallagher JA. 2001. Extracellular nucleotide signaling: A mechanism for integrating local and systemic responses in the activation of bone remodeling. Bone 28:507–512.

Buckley KA, Wagstaff SC, McKay G, Gaw A, Hipskind RA, Bilbe G, Gallagher JA, Bowler WB. 2001. Parathyroid hormone potentiates nucleotide-induced $[Ca^{2+}]_i$ release in rat osteoblasts independently of Gq activation or cyclic monophosphate accumulation. A mechanism for localizing systemic responses in bone. J Biol Chem 276:9565–9571.

Dixon SJ, Sims SM. 2000. P2 purinergic receptors on osteoblasts and osteoclasts: Potential targets for drug development. Drug Dev Res 49:187–200.

Dixon CJ, Bowler WB, Walsh CA, Gallagher JA. 1997. Effects of extracellular nucleotides on single cells and populations of human osteoblasts: Contribution of cell heterogeneity to relative potencies. Br J Pharmacol 120: 777–780.

Hoebertz A, Townsend-Nicholson A, Glass R, Burnstock G, Arnett TR. 2000. Expression of P2 receptors in bone and cultured bone cells. Bone 27:503–510.

Hoebertz A, Meghji S, Burnstock G, Arnett T. 2001. Extracellular ADP is a powerful osteolytic agent: Evidence for signaling through the $P2Y_1$ receptor on bone cells. FASEB J 15:1139–1148.

Hsu HH, Anderson HC. 1977. A simple and defined method to study calcification by isolated matrix vesicles. Effect of ATP and vesicle phosphatase. Biochim Biophys Acta 500: 162–172.

Jones SJ, Gray C, Boyde A, Burnstock G. 1997. Purinergic transmitters inhibit bone formation by cultured osteoblasts. Bone 21:393–399.

Jørgensen NR, Henriksen Z, Sørensen OH, Eriksen EF, Civitelli R, Steinberg T. 2002. Intercellular calcium signaling occurs between human osteoblasts and osteoclasts and requires activation of osteoclast $P2X_7$ receptors. J Biol Chem 277:7574–7580.

Kaplan AD, Reimer WJ, Feldman RD, Dixon SJ. 1995. Extracellular nucleotides potentiate the cytosolic Ca^{2+}, but not cyclic adenosine 3′,5′-monophosphate response to parathyroid hormone in rat osteoblastic cells. Endocrinology 136:1674–1685.

King BF, Townsend-Nicholson A, Burnstock G. 1998. Metabotropic receptors for ATP and UTP: Exploring the correspondence between native and recombinant nucleotide receptors. Trends Pharmacol Sci 19:506–514.

Komarova SV, Ataullakhanov FI, Globus RK. 2000. Bioenergetics and mitochondrial transmembrane potential during differentiation of cultured osteoblasts. Am J Physiol Cell Physiol 279:C1220–C1229.

Kumagai H, Sakamoto H, Guggino S, Filburn CR, Sacktor B. 1989. Neurotransmitter regulation of cytosolic calcium in osteoblast-like bone cells. Calcif Tissue Int 45:251–254.

Kumagai H, Sacktor B, Filburn CR. 1991. Purinergic regulation of cytosolic calcium and phosphoinositide metabolism in rat osteoblast-like osteosarcoma cells. J Bone Miner Res 6:697–708.

Lazarowski ER, Harden TK. 1999. Quantitation of extracellular UTP using a sensitive enzymatic assay. Br J Pharmacol 127:1272–1278.

Lazarowski ER, Boucher RC, Harden TK. 2000. Constitutive release of ATP and evidence for major contribution of ecto-nucleotide pyrophosphatase and nucleoside diphosphokinase to extracellular nucleotide concentrations. J Biol Chem 275:31061–31068.

Luo LC, Yu H, Ferrier J. 1997. Differential purinergic receptor signalling in osteoclasts and osteoblastic cells. Cell Signal 9:603–607.

Maier R, Glatz A, Mosbacher J, Bilbe G. 1997. Cloning of $P2Y_6$ cDNAs and identification of a pseudogene: Comparison of P2Y receptor subtype expression in bone and brain tissues. Biochem Biophys Res Commun 240:298–302.

Morrison MS, Turin L, King BF, Burnstock G, Arnett TR. 1998. ATP is a potent stimulator of the activation and formation of rodent osteoclasts. J Physiol Lond 511:495–500.

Nakamura E, Uezono Y, Narusawa K, Shibuya I, Oishi Y, Tanaka M, Yanagihara N, Nakamura T, Izumi F. 2000. ATP activates DNA synthesis by acting on P2X receptors in human osteoblast-like MG-63 cells. Am J Physiol Cell Physiol 279:C510–C519.

Pedersen S, Pedersen SF, Nilius B, Lambert IH, Hoffmann EK. 1999. Mechanical stress induces release of ATP from Ehrlich ascites tumor cells. Biochim Biophys Acta 1416: 271–284.

Ralevic V, Burnstock G. 1998. Receptors for purines & pyrimidines. Pharmacol Rev 50:413–492.

Reimer WJ, Dixon SJ. 1992. Extracellular nucleotides elevate $[Ca^{2+}]_i$ in rat osteoblastic cells by interaction with two receptor subtypes. Am J Physiol 263:C1040–C1048.

Saiag B, Bodin P, Shacoori V, Catheline M, Rault B, Burnstock G. 1995. Uptake and flow-induced release of uridine nucleotides from isolated vascular endothelial cells. Endothelium 2:279–285.

Schoefl C, Cuthbertson KS, Walsh CA, Mayne C, Cobbold P, von zur Muhlen A, Hesch RD, Gallagher JA. 1992. Evidence for P2-purinoceptors on human osteoblast-like cells. J Bone Miner Res 7:485–491.

Shimegi S. 1996. ATP and adenosine act as a mitogen for osteoblast-like cells (MC3T3-E1). Calcif Tissue Int 58: 109–113.

Sistare FD, Rosenzweig BA, Contrera JG, Jordan B. 1994. Separate P2T and P2U purinergic receptors with similar second messenger signaling pathways in UMR-106 osteoblasts. J Pharmacol Exp Ther 269:1049–1061.

Sistare FD, Rosenzweig BA, Contrera JG. 1995. P2 purinergic receptors potentiate parathyroid hormone receptor-mediated increases in intracellular calcium and inositol trisphosphate in UMR-106 rat osteoblasts. Endocrinology 136:4489–4497.

Suzuki A, Kotoyori J, Oiso Y, Kozawa O. 1993. Prostaglandin E_2 is a potential mediator of extracellular ATP action in osteoblast-like cells. Cell Adhes Commun 1:113–118.

Yu H, Ferrier J. 1993. Osteoblast-like cells have a variable mixed population of purino/nucleotide receptors. FEBS Lett 328:209–214.