

Expression and function of P2 receptors in bone

J.A. Gallagher and K.A. Buckley

Human Bone Cell Research Group, University of Liverpool, Liverpool, UK

Abstract

ATP (adenosine 5'-triphosphate) is one of the most important extracellular regulatory molecules in the skeleton. Extracellular ATP and other nucleotides signal through P2 receptors, a diverse group of receptors that are widely expressed by bone cells. P2 receptors are divided into two subclasses; P2Y G-protein coupled receptors, and P2X ligand-gated ion channels, and there is functional and molecular evidence for the expression of these receptors on both osteoblasts and osteoclasts. In order to activate P2 receptors, nucleotides must be released into the bone microenvironment. ATP is present in mmol concentrations in cells and can be released by cell lysis, cell trauma or physiological mechanisms, possibly through ABC transporters. Following co-activation of P2Y and PTH1 receptors on osteoblasts, there are multiple levels of interaction in downstream signalling that eventually lead to synergistic expression of osteoblastic genes, providing a mechanism for integrating local and systemic regulatory signals in bone particularly with regard to the activation of bone remodelling. Activation of P2Y₁ receptors on osteoblasts enhances expression of RANKL leading indirectly to an increase in osteoclast formation and resorption. Expression of P2X₇ inducible pores on osteoclast precursor cell membranes allows fusion to form multinucleated osteoclasts and blockade of this receptor inhibits resorption. The capacity of extracellular nucleotides to provide a highly localized and transient signal coupled with the profound effects of P2 receptor activation on osteoblastic and osteoclastic cells and the synergistic interactions with systemic hormones, indicate that nucleotides have a strong influence over bone tissue growth and regeneration.

Keywords: Extracellular ATP, Extracellular Nucleotides, P2 Receptors, Bone, Bone Remodelling, Osteoblasts, Osteoclasts

Introduction

Extracellular ATP (adenosine 5'-triphosphate) is one of the most important regulatory molecules in the skeleton. No other endocrine or paracrine factor has such a diverse and large number of receptors expressed so widely in bone cells. These receptors, formerly termed purinoceptors but now known as P2 receptors, are subdivided into two classes P2X and P2Y¹. They are expressed in a wide range of cells and tissues and are known to regulate diverse biological processes including neurotransmission, immune response, platelet aggregation, smooth muscle contraction and wound healing. Over the past few years it has emerged that P2 receptors play pivotal roles in the regulation of skeletal homeostasis and there is growing interest in these receptors as potential therapeutic targets in bone disease^{2,3}.

P2 receptors

The first recognition that purines played a major role as extracellular signalling molecules, followed the demonstration by Drury and Szent-Györgyi in 1929 that adenosine and adenosine 5'-monophosphate (AMP) were able to induce arterial dilation⁴. However it was the seminal work of Burnstock that led to the identification of a family of purinoceptors, which could be subdivided into P1 receptors (most responsive to adenosine) and P2 receptors (most responsive to ATP)⁵. The subdivision of P2 receptors into P2X and P2Y families is based on their mode of signal-transduction. P2Y receptors are G-protein coupled whereas P2X receptors are a diverse family of ligand-gated ion channels. Presently, six P2Y receptors, P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁ and P2Y₁₂, and seven mammalian P2X receptors, P2X₁₋₇, have been identified. The P2Y₄ and P2Y₆ receptors are preferentially activated by the pyrimidines UTP and UDP, respectively.

In common with other G-protein-coupled receptors, the tertiary structure of P2Y receptors consists of seven hydrophobic membrane-spanning domains, separated by alternating extracellular and intracellular hydrophilic loops.

Corresponding author: Jim Gallagher, University of Liverpool, Human Bone Cell Research Group, Sherrington Buildings, Ashton Street, Liverpool L69 3GE, UK

Email: jag1@liv.ac.uk

Accepted 27 June 2002

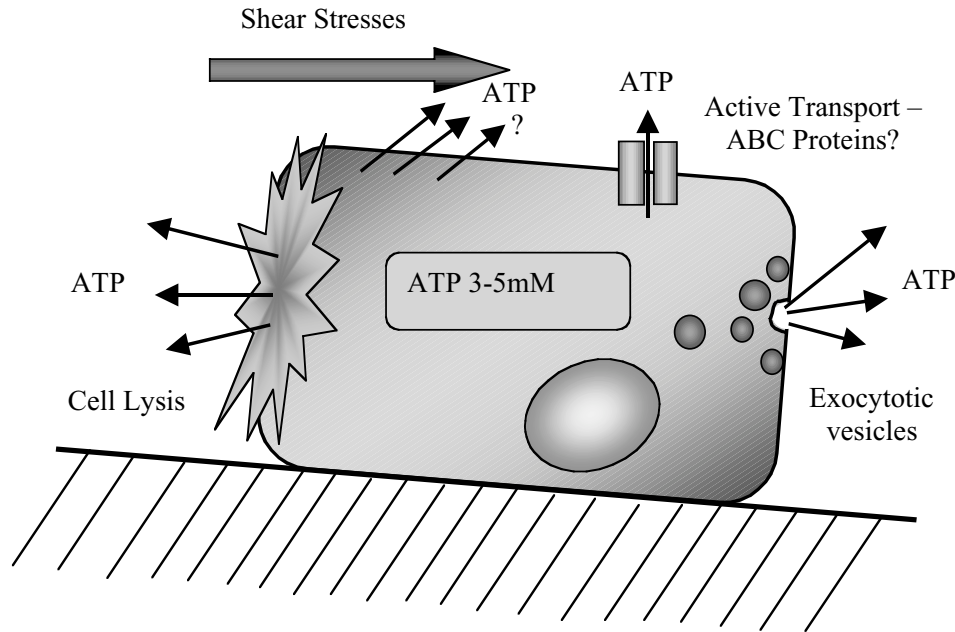


Figure 1. Potential mechanisms of ATP release from osteoblasts.

Stimulation of nearly all of the P2Y receptors leads to activation of G_q . The P2Y₁₁ receptor is also able to couple to the adenylyl cyclase pathway, and in this respect is unique amongst the P2Y family, and P2Y₂ receptors are able to couple to both G_i and G_q . P2Y₁₂ receptors couple only to G_i . Stimulation of G_q activates phospholipase C (PLC), which cleaves membrane-bound phosphatidyl-inositol-biphosphate (PIP₂) to generate inositol-triphosphate (IP3), and diacylglycerol (DAG). Elevated IP3 induces the release of intracellular calcium stores thereby stimulating a variety of signaling pathways, including phosphorylation of protein kinase C (PKC) and production of phospholipase A₂ (PLA₂). Diacylglycerol also phosphorylates PKC, which in turn may stimulate the MAPKinase pathways. The MAPKinase pathways are involved in cell metabolism, secretion, gene expression, and growth, illustrating the wide-ranging effects extracellular nucleotides may exert.

Expression of P2 receptors in osteoblasts

P2Y receptors

The first evidence for P2 receptors in bone was obtained from studies in which ATP was added to cultures of osteoblastic cells and shown to cause elevations in intracellular calcium ($[Ca^{2+}]_i$)⁶⁻⁹. Schöfl et al. demonstrated responses to ATP in primary osteoblasts derived from human bone and in the SaOS-2 osteosarcoma cell line. They concluded that the cells expressed the P2U receptor (subsequently redesignated P2Y₂). At this stage this receptor only existed as a pharmacological entity but following advances in molecular cloning, Bowler and colleagues confirmed the expres-

sion of P2Y₂ receptors in primary human osteoblasts¹⁰. Only low levels of this receptor were detected in SaOS-2 cells, which were subsequently shown to predominantly express P2Y₁. UTP is equipotent with ATP in activating P2Y₂ receptors, whereas ADP is a potent P2Y₁ agonist. Studies employing selective nucleotide agonists suggested that at least two P2Y receptor sub-types are expressed in UMR-106^{11,12}, the rat osteosarcoma cell line that is widely used as an osteoblast model *in vitro*. Subsequent investigation by Buckley et al. indicated that, in common with the human osteosarcoma line SaOS-2, the UMR-106 cell line predominantly expresses P2Y₁¹³. These initial studies were followed by a series of papers reporting that extracellular nucleotides elicit a range of proliferative and other responses in cultures of osteoblastic cells, and molecular studies have indicated that multiple subtypes of P2Y receptors are expressed in osteoblastic cells. P2Y₁, P2Y₂, P2Y₄, and P2Y₆ messenger RNA has been demonstrated to be present in human bone and osteoblastic cell lines by RT-PCR¹⁴ but to date functional evidence has only been conclusively provided for P2Y₁ and P2Y₂. The variable expression of P2Y receptors within and between osteoblastic populations and the disparity between non-transformed primary cultures and osteosarcoma cell populations indicates that expression may be differentiation-dependent¹⁵.

P2X receptors

In comparison with P2Y, the expression of P2X receptors by osteoblasts is less well documented. Nakamura and co-workers demonstrated both functional responses and RT-PCR evidence to indicate the presence of P2X₄, P2X₅, P2X₆

and P2X₇ receptors on human osteoblast-like MG-63 cells¹⁶. Gartland and colleagues reported the expression of the P2X₇ receptor in a sub-population of primary human osteoblasts by RT-PCR, immunohistochemistry and functional assays¹⁷. Interestingly SaOS-2 cells were shown to express the receptor whereas another osteosarcoma cell line, Te85, was negative. Hoebertz et al. detected P2X₂ and P2X₅ protein in rat calvarial osteoblasts *in situ* by immunohistochemistry and *in situ* hybridization¹⁸.

Expression of P2 receptors in osteoclasts

P2Y receptors

ATP and other extracellular nucleotides have been shown to induce elevations in [Ca²⁺]_i in osteoclasts¹⁹. Early studies revealed that these [Ca²⁺]_i elevations persisted in the absence of extracellular Ca²⁺, and could be blocked by inhibiting G-protein activation, implicating the involvement of P2Y receptors²⁰. P2Y₂ receptor messenger RNA was later shown, by RT-PCR, to be expressed by osteoclasts isolated from human giant cell tumours¹⁰. Although in these cells both ATP and UTP were unable to induce an elevation in [Ca²⁺]_i²¹, in a later study involving isolated rat osteoclasts, [Ca²⁺]_i elevations were observed in response to mmol concentrations of UTP, consistent with the presence of functional P2Y₂ receptors²². In this study, calcium responses elicited by other selective P2Y agonists, were consistent with the presence of additional P2Y receptor subtypes on these cells.

P2X receptors

Both functional and molecular evidence exists for the presence of P2X receptors on osteoclasts. Sequences encoding the P2X₄ receptor have been identified in a rabbit osteoclast cDNA library, and by RT-PCR in purified preparations of rabbit osteoclasts²³. Application of ATP or ADP to these isolated rabbit osteoclasts activated an inward current, non-selective for cations, consistent with P2X receptor stimulation. A study involving both patch clamping and fluorescent labeling, demonstrated that elevations in [Ca²⁺]_i in rat osteoclasts were consistent with the presence of both P2X and P2Y receptors²⁴. P2X₂ and P2X₇ receptors have been detected by immunocytochemistry, in osteoclasts isolated from rat long bones, and mRNA encoding the P2X₄ receptor was also detected in these cells¹⁸. P2X₇ receptor in human osteoclasts has also been detected by RT-PCR, immunohistochemistry and functional assay in human osteoclasts generated from peripheral blood monocytes (PBMs)²⁵.

Extracellular ATP in the microenvironment of bone

The abundant and widespread expression of P2 receptors in bone cells suggests that ATP or other extracellular

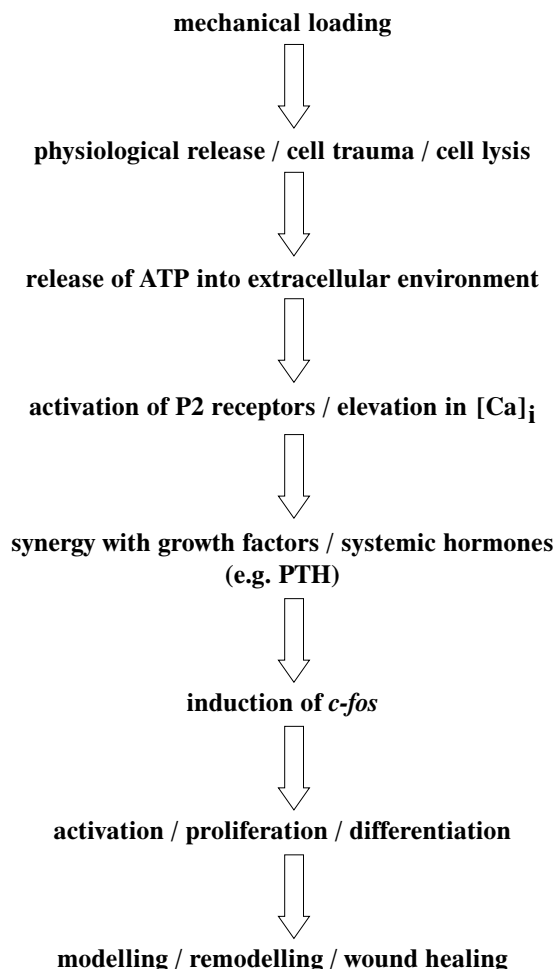


Figure 2. Potential role of extracellular ATP in tissue turnover and repair in response to mechanical loading. Cells respond to mechanical loading by releasing ATP into the extracellular environment through physiological mechanisms, possibly involving ABC transporters. Extreme loading, which results in tissue damage, such as bone fracture, gives rise to cell trauma and/or cell lysis and further release of ATP. Once in the extracellular environment, ATP activates P2 receptors in target cells, including osteoblasts and osteoclasts. Activation of P2 receptors elevates ([Ca²⁺]_i) and sensitizes the cells to activation by growth factors or systemic hormones including PTH. Co-activation results in a potentiated induction of the immediate early gene *c-fos*, which regulates the transcription of genes involved in cell proliferation, differentiation, tissue turnover and repair. The synergy between ATP and other hormones provides a molecular mechanism for integrating local and systemic responses in tissues including bone, particularly with regard to the activation of remodelling by PTH.

nucleotides are important regulators of bone cell function. To activate these receptors, ATP must be released into the bone microenvironment. Nucleotides can be released into the extracellular milieu from a variety of sources. ATP is present in mmol concentrations in cells and can be released by cell trauma or cell lysis. There is evidence that during trauma, extracellular concentrations of nucleotides can reach as high

P2Y ₁	<p>↑[Ca²⁺]_i in UMR-106 rat osteosarcoma cells, <i>Reimer and Dixon 1992</i>⁽⁹⁾</p> <p>↑[Ca²⁺]_i in primary human osteoblasts, <i>Dixon et al. 1997</i>⁽¹⁵⁾</p> <p>RT-PCR of primary human osteoblasts, <i>Bowler et al. 1999</i>⁽²⁹⁾</p> <p>RT-PCR in RANKL derived human osteoclast cultures, <i>Buckley 2002 in press</i>⁽⁴¹⁾</p>
P2Y ₂	<p>↑[Ca²⁺]_i in primary human osteoblasts, <i>Schöfl 1992</i>⁽⁸⁾</p> <p>↑[Ca²⁺]_i in UMR 106 cells, <i>Gallinaro et al. 1995</i>⁽¹¹⁾</p> <p>RT-PCR and Northern analysis in primary human osteoblasts, whole bone, osteoclastoma, <i>Bowler et al. 1998</i>⁽²¹⁾</p> <p><i>In situ</i> hybridization of human osteoclasts, <i>Bowler et al. 1998</i>⁽²¹⁾</p> <p>RT-PCR in RANKL derived human osteoclast cultures, <i>Buckley 2002 in press</i>⁽⁴¹⁾</p>
P2Y ₄	<p>RT-PCR of human bone and bone cells, <i>Maier et al. 1997</i>⁽¹⁴⁾</p> <p>RT-PCR in RANKL derived human osteoclast cultures, <i>Buckley 2002 in press</i>⁽⁴²⁾</p>
P2Y ₆	<p>RT-PCR of human bone and bone cells, <i>Maier et al. 1997</i>⁽¹⁴⁾</p> <p>RT-PCR in RANKL derived human osteoclast cultures, <i>Buckley 2002 in press</i>⁽⁴¹⁾</p>
P2Y ₁₁	RT-PCR in RANKL derived human osteoclast cultures, <i>Buckley 2002 in press</i> ⁽⁴¹⁾
P2Y ₁₂	Not detected RT-PCR of primary human osteoblasts and SaOS cells (Buckley, unpublished observations)
P2X ₁	RT-PCR in RANKL derived human osteoclast cultures, <i>Buckley 2002 in press</i> ⁽⁴¹⁾
P2X ₂	Immunohistochemistry and <i>in situ</i> hybridization of osteoblasts and osteoclasts, <i>Hoebertz et al. 2000</i> ⁽¹⁸⁾
P2X ₃	Not detected
P2X ₄	<p>RT-PCR in RANKL derived human osteoclast cultures, <i>Buckley 2002 in press</i>⁽⁴¹⁾</p> <p>Immunohistochemistry and <i>in situ</i> hybridisation osteoclasts, <i>Hoebertz et al. 2000</i>⁽¹⁸⁾</p> <p>RT-PCR and activation of cation current in rabbit osteoclasts, <i>Naemsch et al. 1999</i>⁽²³⁾</p> <p>RT-PCR on MG63 cells, <i>Nakamura et al. 2000</i>⁽¹⁶⁾</p>
P2X ₅	<p>Immunostaining in rat osteoblasts, <i>Hoebertz et al. 2000</i>⁽¹⁸⁾</p> <p>RT-PCR on MG63 cells, <i>Nakamura et al. 2000</i>⁽¹⁶⁾</p> <p>RT-PCR in RANKL derived human osteoclast cultures, <i>Buckley 2002 in press</i>⁽⁴¹⁾</p>
P2X ₆	<p>RT-PCR on MG63 cells, <i>Nakamura et al. 2000</i>⁽¹⁶⁾</p> <p>RT-PCR in RANKL derived human osteoclast cultures, <i>Buckley 2002 in press</i>⁽⁴¹⁾</p>
P2X ₇	<p>RT-PCR on MG63 cells, <i>Nakamura et al. 2000</i>⁽¹⁶⁾</p> <p>RT-PCR, immunohistochemistry, pore formation in human osteoblasts and human osteoclasts, <i>Garland et al. 2001</i>⁽¹⁷⁾</p> <p>Immunohistochemistry of rat osteoclasts, <i>Hoebertz et al. 2000</i>⁽¹⁸⁾</p> <p>Pore formation in rat osteoclasts, <i>Modderman et al. 1994</i>⁽⁴⁶⁾</p> <p>RT-PCR in RANKL derived human osteoclast cultures, <i>Buckley 2002 in press</i>⁽⁴¹⁾</p>

Table 1. Evidence of expression of P2 receptors in bone.

as 20 mmol, which is sufficient to activate P2X and P2Y receptor subtypes. ATP and other nucleotides are also released in physiological conditions. ATP is recognised as a neurotransmitter and it is well established that activated platelets and leucocytes can release nucleotides at sites of tissue injury and inflammation. In addition, there is growing evidence that many cell types release ATP constitutively by physiological mechanisms. It has been suggested that ATP binding-cassette proteins (ABC transporters), a large family of membrane transporters including the multiple drug resistance protein, MDR, the cystic fibrosis transmembrane conductance regulator (CFTR) and the sulfonylurea receptor, act as mediators of ATP release^{26,27}. Interestingly, ABC transporters have been detected in bone cells by immunohistochemistry²⁸.

It is clear that high concentrations of ATP will be present transiently and locally in bone at sites of inflammation, tissue injury, wounding or fracture, thereby leading to activation of P2 receptors. In an endeavour to determine if bone cells can release extracellular nucleotides by a physiological, non-lytic mechanism, Bowler and co-workers developed a real-time detection system utilising the high yield chemiluminescent reaction of luciferin and luciferase. Using this system it was determined that primary human osteoblasts constitutively release ATP into the extracellular environment and that concentrations can rise into the mmol range²⁹. Released ATP is likely to be present at the highest concentrations adjacent to the cell membrane and in the vicinity of P2 receptors. This constitutive release of ATP is highly sensitive

to cell perturbation such that *in vitro* the fluid forces arising from gentle changing of medium are sufficient to amplify ATP release 10 – 100 fold³⁰.

Once in the bone microenvironment, ATP is rapidly broken down by ectonucleotidases present on the membranes of bone cells. The half-life of ATP in the presence of bone cells *in vitro* is around one minute; there is rapid conversion to ADP then AMP, and finally to adenosine.

Consequences of P2 receptor expression by osteoblasts

Extracellular nucleotides signalling through P2 receptors on osteoblasts can influence many of the processes that govern skeletal growth and remodelling. ATP has been reported to stimulate the proliferation of MC3T3-E1 osteoblast-like cells³¹, and enhanced DNA synthesis has also been observed in osteoblast-like MG-63 cells following P2X receptor stimulation¹⁶. However, in an *in vitro* assay to measure authentic bone formation by osteoblasts, Jones et al. found that ATP and other nucleotide agonists had an inhibitory effect³². These apparently conflicting observations illustrate the complexity of extracellular nucleotide signalling in bone. In our view, one of the key observations is that activation of P2Y receptors in osteoblasts leads to an induction of *c-fos*. This immediate early gene plays a key role in the proliferation and differentiation of bone cells^{33,34}. Furthermore, whilst activation of P2 receptors alone leads to moderate inductions of *c-fos*, dual activation of P2 receptors and PTH1 receptors leads to a massive synergy in *c-fos* expression. In some populations of osteoblastic cells, e.g., UMR 106, the synergistic effect of extracellular ATP and PTH is associated with, and downstream of a synergistic elevation in $[Ca^{2+}]_i$ ¹³. However, in other cell types, e.g., SaOS-2 cells, the elevation in $[Ca^{2+}]_i$ in response to extracellular ATP is not enhanced by co-stimulation of the PTH1 receptor, but a synergistic induction of *c-fos* is observed nonetheless³⁵. These data demonstrate that following co-activation of P2 and PTH1 receptors there are multiple levels of interaction in downstream signalling, some calcium-dependent and some calcium-independent, which eventually lead to synergistic expression of osteoblastic genes. We hypothesize that this synergy provides a molecular mechanism for integrating local and systemic responses in bone, particularly with regard to the activation of remodelling

Extracellular nucleotides potentiate the action of parathyroid hormone

Parathyroid hormone (PTH) acting via the PTH1 receptor is amongst the most important systemic regulators of bone. PTH itself has complex effects on bone; administered PTH enhances bone resorption but can also have a profound anabolic action depending on the route and interval of delivery^{36,37}. One of the best-defined roles of PTH is to up-regu-

late bone remodelling by increasing the activation rate. However, it has never been clear how systemic hormones such as PTH can regulate bone remodelling, which is essentially a focal phenomenon. The discovery that co-activation of P2Y and PTH receptors leads to a profound synergism, provides a molecular mechanism whereby locally released extracellular nucleotides can sensitize cells to surrounding systemic PTH. The priming of bone cells to systemic hormones, such as PTH, by locally released nucleotides, may represent a mechanism by which systemic hormones are able to activate remodelling at discrete sites.

P2 receptors regulate formation and activity of osteoclasts

There is a growing body of evidence that ATP can stimulate bone resorption by enhancing the formation and activity of osteoclasts. Bowler and co-workers observed an increase in pit formation *in vitro* when ATP was added to cultures of human osteoclasts isolated from a giant cell tumour²¹. In contrast, UTP did not elevate resorption despite the demonstration by *in situ* hybridization that the resorbing cells expressed abundant P2Y₂ receptor mRNA. Morrison et al. found that extracellular ATP stimulated the resorptive activity of rat osteoclasts at mmol concentrations³⁸. The stimulatory effects of ATP appeared to be enhanced when osteoclasts were cultured in acidified media, implicating the involvement of the P2X₂ receptor, since it is sensitized by extracellular acidification³⁹. Nucleotide activation of P2 receptors has also been reported to enhance the Cl⁻/HCO₃⁻ exchange across the osteoclast cell membrane, leading to a transient decrease in intracellular pH⁴⁰. It has been suggested that an ATP-elicited pH decrease might favour resorption pit formation by facilitating the extrusion of H⁺.

The recent development of techniques to generate human osteoclasts *in vitro* from PBMs has facilitated closer examination of the effects of extracellular nucleotides on osteoclast formation and activity. Functional resorbing osteoclasts can be generated from PBMs in co-cultures with a stromal layer of UMR-106 cells or alternatively by direct supplementation of the PBM cultures with RANKL. Multinucleate resorbing cells first appear in these cultures between 10 and 14 days in culture, and there is aggressive resorption of mineralized substrates. Almost the full spectrum of P2 receptor mRNAs from the P2Y and P2X subclasses can be detected by RT-PCR in these cultures, except P2X₂ and P2X₃⁴¹. This receptor expression is constant throughout osteoclast development, apart from P2X₆, which is initially expressed at low levels, but later becomes up-regulated. Yet, when ATP or UTP is added to the stromal-cell free, RANKL-supplemented cultures, there is no obvious effect on the extent of lacunar resorption. In contrast, ATP at low mmol concentration greatly enhances the excavation of resorption pits in co-cultures containing UMR-106 cells, but UTP is without effect. These UMR-106 cells primarily express P2Y₁ receptor, with very low levels of P2Y₂,¹³ suggesting that ATP enhances resorption in these cul-

tures by acting via UMR-106-expressed P2Y₁. ATP, but not UTP up-regulates RANKL mRNA expression by UMR-106 cells⁴¹, therefore indicating that it is this nucleotide-induced increase in osteoblast RANKL expression that results in either enhanced osteoclast formation, activation of resorption by these cells, or both.

The apparent lack of effect of P2 agonists on the formation and/or activity of osteoclasts in osteoblast-free cultures, despite the expression of P2 receptor mRNA, is perplexing. At least some of the species of mRNA are transcribed and functional P2 receptors expressed in the osteoclasts since it is possible to demonstrate the activation of signalling cascades^{42,43}. Nucleotides signal via MAPKinase pathways to transduce signals from activated P2 receptors to the nucleus in osteoclasts. Nucleotide-induced activation of CREB demonstrates that functional P2Y receptors are expressed by both recombinant RANKL- and co-culture-generated human osteoclasts. These results indicate that extracellular nucleotides regulate other activities of osteoclasts rather than simply the rate of resorption. *In situ*, osteoclasts respond to signals that determine the direction, pattern and cessation of resorption in addition to the rate and it is likely that extracellular nucleotides play a part in all of these controls.

P2X₇ receptors and osteoclast formation

There is now strong evidence that the P2X₇ receptor is highly influential in osteoclast formation. In common with other members of the P2X family, this is an ATP-gated ion channel but in addition, the receptor has the unique ability to form pores that are permeable to molecules of up to 900Da, when exposed to repeated or prolonged application of nucleotide agonist. This receptor is expressed by human osteoblasts and osteoclasts and its potential functions include regulation of cytokine release, induction of apoptosis and fusion of osteoclasts. It is likely that formation of P2X₇ inducible pores allows fusion of osteoclast precursor cell membranes to form multinucleated osteoclasts. Blockade of the receptor with the antagonist oxidized ATP or a specific blocking monoclonal antibody inhibits formation of multinucleated osteoclasts in RANKL-supplemented cultures of human PBMs⁴⁴. The mononuclear cells differentiate and become adherent but do not form multinucleated cells presumably because of a specific failure in the fusion process. These findings are consistent with the observations that P2X₇ receptors are highly expressed on macrophages prior to formation of multinucleated giant cells⁴⁵. It is also possible that nucleotides can regulate osteoclast number by P2X₇-induced apoptosis⁴⁶.

Extracellular ATP and mechanotransduction in bone

The capacity of extracellular nucleotides to provide a highly localized and transient signal coupled with the pro-

found effects of P2 receptor activation on osteoblastic and osteoclastic cells and the synergistic interactions with systemic hormones, indicate that nucleotides are ideal candidates to play a role in mechanotransduction in bone. This is the process by which detection of mechanical deformation or fluid shear by skeletal cells results in modelling or remodelling. Bowler and co-workers have demonstrated that an enhancement of ATP release from SaOS-2 cells results following fluid shear³⁰. Similar findings have also been reported in other cell types⁴⁷. In fact, responses arising from mechanical stimulation of skeletal cells bear many similarities to those resulting from P2 receptor stimulation. These include release of Ca²⁺ from intracellular stores, activation of PKC and analogous patterns of Ca²⁺ transients. Therefore, it is possible that remodelling events result following mechanical stimuli due to enhanced localized extracellular nucleotide release, which then act in an autocrine or paracrine manner to transduce the mechanical signal into a functional response. Jorgensen and colleagues demonstrated that human osteoblasts propagate intercellular calcium signals via P2 receptor stimulation following mechanical stimulation, providing further evidence for the importance of extracellular nucleotides as mediators of mechanotransduction⁴⁸. A similar finding of localized nucleotide release in response to mechanical stress that allows intercellular communication has been reported in epithelial cells⁴⁹.

Conclusions

In recent years, reports of functional effects of extracellular nucleotides in bone have rapidly accumulated, providing strong evidence for the significance of these molecules in the bone microenvironment. Numerous investigators have shown that multiple subtypes of P2X and P2Y receptors are expressed by both osteoblasts and osteoclasts, where they are activated by nucleotides released from surrounding cells by lytic and non-lytic mechanisms. In osteoblasts, this results in elevated gene transcription and differentiation, and these signals can be integrated with those arising from systemic activators, providing a mechanism of localizing responses to these systemic factors. Nucleotides can also influence bone remodelling by both inducing osteoclast formation and stimulating osteoclast resorption via induction of RANKL expression by osteoblasts. In addition, activation of the P2X₇ receptor appears to be required for osteoclast formation. Extracellular nucleotides have also been implicated as mediators of mechanotransduction in bone.

References

1. Abbracchio MP, Burnstock G. Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharmacol Ther* 1994; 64:445-475.
2. Dixon SJ, Sims SM. P2 purinergic receptors on osteoblasts and osteoclasts: potential targets for drug development. *Drug*

- Dev Res 2000; 49:187-200.
3. Bowler WB, Buckley KA, Gartland A, Hipskind RA, Bilbe G, Gallagher JA. Extracellular nucleotide signaling: a mechanism for integrating local and systemic responses in the activation of bone remodeling. *Bone* 2001; 28:507-512.
 4. Drury AN, Szent-Györgyi AY. The physiological activity of adenosine compounds with a special reference to their action upon the mammalian heart. *J Physiol (Lond)* 1929; 68:213-237.
 5. Burnstock G. A basis for distinguishing two types of purinergic receptor. In: Bolis L and Straub RW (eds) *Cell membrane receptors for drugs and hormones*. Raven Press, NY; 1978:107-118.
 6. Kumagai H, Sacktor B, Filburn CR. Purinergic regulation of cytosolic calcium and phosphoinositide metabolism in rat osteoblast-like osteosarcoma cells. *J Bone Miner Res* 1991; 6:697-708.
 7. Kumagai H, Sakamoto H, Guggino S, Filburn CR, Sacktor B. Neurotransmitter regulation of cytosolic calcium in osteoblast-like bone cells. *Calcif Tissue Int* 1989; 45:251-254.
 8. Schöfl C, Cuthbertson KS, Walsh CA, Mayne C, Cobbold P, von zur Mühlen A, Hesch RD, Gallagher JA. Evidence for P2-purinoreceptors on human osteoblast-like cells. *J Bone Miner Res* 1992; 7:485-491.
 9. Reimer WJ, Dixon SJ. Extracellular nucleotides elevate $[Ca^{2+}]_i$ in rat osteoblastic cells by interaction with two receptor subtypes. *Am J Physiol* 1992; 263:C1040-C1048.
 10. Bowler WB, Birch MA, Gallagher JA, Bilbe G. Identification and cloning of human P2U purinoreceptor present in osteoclastoma, bone and osteoblasts. *J Bone Miner Res* 1995; 10:1137-1145.
 11. Gallinaro BJ, Reimer WJ, Dixon SJ. Activation of protein kinase C inhibits ATP-induced $[Ca^{2+}]_i$ elevation in rat osteoblastic cells: selective effects on P2Y and P2U signalling pathways. *J Cell Physiol* 1995; 162:305-314.
 12. Yu H, Ferrier J. Osteoblast-like cells have a variable mixed population of purino/nucleotide receptors. *FEBS Lett* 1993; 328:209-214.
 13. Buckley KA, Wagstaff SC, McKay G, Gaw A, Hipskind RA, Bilbe G, Gallagher JA, Bowler WB. 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* 2001; 276:9565-9571.
 14. Maier R, Glatz A, Mosbacher J, Bilbe G. Cloning of P2Y6 cDNAs and identification of a pseudogene: comparison of P2Y receptor subtype expression in bone and brain tissues. *Biochem Biophys Res Commun* 1997; 237:297-302.
 15. Dixon CJ, Bowler WB, Walsh CA, Gallagher JA. Effects of extracellular nucleotides on single cells and populations of human osteoblasts: contribution of cell heterogeneity to relative potencies. *Br J Pharmacol* 1997; 120:777-780.
 16. Nakamura E, Uezono Y, Narusawa K, Shibuya I, Oishi Y, Tanaka M, Yanagihara N, Nakamura T, Izumi F. ATP activates DNA synthesis by acting on P2X receptors in human osteoblast-like MG-63 cells. *Am J Physiol Cell Physiol* 2000; 279:C510-C519.
 17. Gartland A, Hipskind RA, Gallagher JA, Bowler WB. Expression of a P2X7 receptor by a subpopulation of human osteoblasts. *J Bone Miner Res* 2001; 16:846-856.
 18. Hoebertz A, Townsend-Nicholson A, Glass R, Burnstock G, Arnett TR. Expression of P2 receptors in bone and cultured bone cells. *Bone* 2000; 27:503-510.
 19. Yu H, Ferrier J. ATP induces an intracellular calcium pulse in osteoclasts. *Biochem Biophys Res Commun* 1993; 191:357-363.
 20. Yu H, Ferrier J. Mechanisms of ATP-induced Ca^{2+} signalling in osteoclasts. *Cell Signal* 1994; 6:905-914.
 21. Bowler WB, Littlewood-Evans A, Bilbe G, Gallagher JA, Dixon CJ. P2Y2 receptors are expressed by human osteoclasts of giant cell tumour but do not mediate ATP-induced bone resorption. *Bone* 1998; 22:195-200.
 22. Wiebe SH, Sims SM, Dixon SJ. Calcium signalling via multiple P2 purinoreceptor subtypes in rat osteoclasts. *Cell Physiol Biochem* 1999; 9:323-337.
 23. Naemsch LN, Weidema AF, Sims SM, Underhill TM, Dixon SJ. P2X4 purinoreceptors mediate an ATP-activated, non-selective cation current in rabbit osteoclasts. *J Cell Sci* 1999; 112:4425-4435.
 24. Weideman AF, Barbera J, Dixon SJ, Sims SM. Extracellular nucleotides activate non-selective cation and Ca^{2+} -dependent K^+ channels in rat osteoclasts. *J Physiol (Lond)* 1997; 503:303-315.
 25. Gartland A, Ginty AF, Gallagher JA, Bowler WB. Activation of P2X7 receptors expressed by human osteoclastoma modulates bone resorption. *Calcif Tissue Int* 1999; 64(Suppl.1): S56 (abstract).
 26. Roman RM, Wang Y, Lidofsky SD, Feranchak AP, Lomri N, Scharschmidt BF, Fitz JG. Hepatocellular ATP-binding cassette protein expression enhances ATP release and autocrine regulation of cell volume. *J Biol Chem* 1997; 272:21970-21976.
 27. Abraham EH, Prat AG, Gerweck L, Seneveratne T, Arceci RJ, Kramer D, Guidotti G, Cantiello HF. The multidrug resistance (mdr1) gene product functions as an ATP channel. *Proc Natl Acad Sci USA* 1993; 90:312-316.
 28. Wagstaff SC, Bowler WB, Gartland A, Birch MA, Caddick MX, Dillon JP, Wlodarski B, Bilbe G, Gallagher JA. A novel subfamily of osteoclast-specific ATP binding cassette proteins implicated in resorption. *Calcif Tissue Int* 1999; 64:S44 (abstract).
 29. Bowler WB, Tattersall JA, Hussein R, Dixon CJ, Cobbold PH, Gallagher JA. Real time measurement of ATP release from human osteoblasts. *J Bone Miner Res* 1998; 13:524 (abstract).
 30. Bowler WB, Tattersall JA, Hussein R, Dixon CJ, Cobbold PH, Gallagher JA. Release of ATP by osteoblasts: modulation by fluid shear forces. *Bone* 1998; 22:3S (abstract).
 31. Shimegi S. ATP and adenosine act as a mitogen for osteoblast-like cells (MC3T3-E1). *Calcif Tissue Int* 1996; 58:109-113.
 32. Jones SJ, Gray C, Boyde A, Burnstock G. Purinergic transmitters inhibit bone formation by cultured osteoblasts. *Bone* 1997; 21:393-399.
 33. Grigoriadis AE, Wang Z-Q, Cecchini MG, Hofstetter W, Felix R, Fleish HA, Wagner EF. C-fos – a key regulator of osteoclast-macrophage lineage determination and bone remodeling. *Science* 1994; 266:443-448.
 34. Johnson RS, Speigelman BM, Papaioannou V. Pleiotropic effects of a null mutation in the c-fos proto-oncogene. *Cell* 1992; 71:577-586.
 35. Bowler WB, Dixon CJ, Halleux C, Maier R, Bilbe G, Fraser WD, Gallagher JA, Hipskind RA. Signaling in human osteoblasts by extracellular nucleotides. Their weak induction of the c-fos proto-oncogene via Ca^{2+} mobilization is strongly

- potentiated by a parathyroid hormone/cAMP-dependent protein kinase pathway independently of mitogen-activated protein kinase. *J Biol Chem* 1999; 274:14315-14324.
36. Podbesek R, Edouard C, Meunier PJ, Parsons JA, Reeve J, Stevenson RW, Zanelli JM. Effects of two treatment regimes with synthetic human parathyroid hormone fragment on bone formation and the tissue balance of trabecular bone in greyhounds. *Endocrinology* 1983; 112:1000-1006.
 37. Uzawa T, Hori M, Ejiri S, Ozawa H. Comparison of the effects of intermittent and continuous administration of human parathyroid hormone (1-34) on rat bone. *Bone* 1995; 16:477-484.
 38. Morrison MS, Turin L, King BF, Burnstock G, Arnett TR. ATP is a potent stimulator of the activation and formation of rodent osteoclasts. *J Physiol* 1998; 511:495-500.
 39. King BF, Ziganshina LE, Pintor J, Burnstock G. Full sensitivity of P2X2 purinoceptor to ATP revealed by changing extracellular pH. *Br J Pharmacol* 1996; 117:1371-1373.
 40. Yu H, Ferrier J. Osteoclast ATP receptor activation leads to a transient decrease in intracellular pH. *J Cell Sci* 1995; 108:3051-3058.
 41. Buckley KA, Hipskind RA, Gartland A., Bowler WB, Gallagher JA. ATP stimulates human osteoclast activity via upregulation of osteoblast-expressed RANKL. Manuscript accepted by *Bone* 2002.
 42. Buckley KA, Hipskind RA, Gaw A, Gallagher JA, Bowler WB. Nucleotide effects on human osteoclast formation and transcription factor activation. *J Bone Miner Res* 2000; 15:1214 (abstract).
 43. Buckley KA, Hipskind RA, Gaw A, Gallagher JA, Bowler WB. Effects of extracellular ATP on resorption and transcription factor activation in co-culture and RANK ligand-derived human osteoclasts. *Calcif Tissue Int* 2000; 66 (Suppl.1):O51/S62 (abstract).
 44. Gartland A, Buckley KA, Gallagher JA, Bowler WB. Blockade of the pore-forming P2X7 receptor inhibits formation of multinucleated human osteoclasts. Manuscript submitted to *J Cell Sci* 2002.
 45. Chiozzi P, Sanz JM, Ferrari D, Falzoni S, Aleotti A, Buell GN, Collo G, Di Virgilio F. Spontaneous cell fusion in macrophage cultures expressing high levels of the P2Z/P2X7 receptor. *J Cell Biol* 1997; 138:697-706.
 46. Modderman WE, Weidema AF, Vrijheid-Lammers T, Wassenaar AM, Nijweide PJ. Permeabilization of cells of hemopoietic origin by extracellular ATP⁴⁺: elimination of osteoclasts, macrophages, and their precursors from isolated bone cell populations and fetal bone rudiments. *Calcif Tissue Int* 1994; 55:141-150.
 47. Burnstock G. Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. *J Anat* 1999; 194:335-342.
 48. Jorgensen NR, Geist ST, Civitelli R, Steinberg TH. ATP- and gap junction-dependent intercellular calcium signaling in osteoblastic cells. *J Cell Biol* 1997; 139:497-506.
 49. Homolya L, Steinberg TH, Boucher RC. Cell to cell communication in response to mechanical stress via bilateral release of ATP and UTP in polarized epithelia. *J Cell Biol* 2000; 150:1349-1359.