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Purinergic signalling in osteoblasts

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1. ABSTRACT

The skeleton is maintained throughout life via the finely tuned actions of osteoblasts and osteoclasts, with disruption in this balance eventually leading to bone disease. The exact mechanisms balancing these actions are not fully known, although several regulatory systems are known to be involved. The involvement of purinergic signalling in bone has come to light over the past 20 years or so. This review will highlight the current knowledge of purinergic signalling in osteoblasts - covering expression of P2 receptors, mechanisms of ATP release and degradation, P2 receptor mediated signalling and finally the functional consequences of P2 receptor signalling in bone.

2. INTRODUCTION

2.1. Purinergic Signalling

The idea that purines can act as extracellular signalling molecules was first suggested in 1929, yet it was not until 1972 that the concept of purinergic neurotransmission was proposed (1, 2). It is now well recognised that extracellular nucleotides, signalling via purinergic receptors, participate in a wide number of biological processes in both neuronal and non-neuronal tissues. The receptors for purines and pyrimidines are classified into two groups; P1 receptors, primarily activated by adenosine, and P2 receptors. The P2 receptors are further subdivided into the P2X ligand-gated ion channels

Table 1. P2 receptor expression by osteoblasts

Receptor	Species	Osteoblast model	Evidence for expression	References
P2X1	Rat	primary cells	qPCR, ICC	(24)
P2X2	Rat	primary cells	ISH, RT-PCR, ICC	(16, 20)
	Mouse	MC3T3-E1	RT-PCR	(26)
	Human	MG-63 & SaOS-2	RT-PCR	(25)
P2X3	Rat	primary cells	qPCR, ICC	(24)
P2X4	Rat	primary cells	qPCR, ICC	(24)
	Human	SaM-1, MG-63 & SaOS-2	RT-PCR	(16, 20, 25)
P2X5	Rat	primary cells	RT-PCR, ICC	(16, 20)
	Mouse	MC3T3-E1	RT-PCR	(26)
	Human	SaM-1	RT-PCR	(18)
P2X6	Rat	primary cells	qPCR, ICC	(24)
	Human	SaM-1	RT-PCR	(18)
P2X7	Rat	primary cells	RT-PCR, ICC	(16)
	Human	MG-63, SaOS-2 primary cells	RT-PCR ICC, WB, pharmacology	(25-27)
	Mouse	primary cells	RT-PCR, pharmacology	(28)
$P2Y_1$	Rat	primary cells	ISH, RT-PCR, ICC	(16, 20)
	Rat	UMR-106	pharmacology	(77)
	Human	MG-63, HOBIT	RT-PCR	(19, 74)
$P2Y_2$	Rat	primary cells	ISH, RT-PCR, ICC, WB, pharmacology	(16, 19, 20)
	Human	primary cells, MG-63,OHS-2 SaOS-2, Te85, HOBIT, SaM1	RT-PCR, SB, ISH, Ca^{2+}imaging	(14, 18, 75)
	Mouse	MC3T3-E1	pharmacology	(74)
$P2Y_4$	Rat	primary cells	RT-PCR, ICC, WB	(16)
	Human	MG-63, OHS-2	RT-PCR	(19)
$P2Y_6$	Rat	primary	RT-PCR, ICC	(16)
	Human	MG-63, OHS-2 SaM-1	RT-PCR	(18, 19)
$P2Y_{12}$	Human	SaOS-2	RT-PCR	(17)
	Rat	primary cells	qPCR, WB	(24)
$P2Y_{13}$	Rat	primary cells	qPCR	(24)
$P2Y_{14}$	Rat	primary cells	qPCR, WB	(24)

Quantitative real time polymerase chain reaction (qPCR), immunocytochemistry (ICC), in situ hybridisation (ISH), reverse transcriptase polymerase chain reaction (RT-PCR), western blot (WB), southern blot (SB)

nd P2Y G-protein-coupled receptors (3-5). Currently, seven P2X receptors (P2X1-7) and eight P2Y receptors ($P2Y_{1,2,4,6,11,12,13,14}$) have been identified; each of these receptors has been cloned, characterised and shown to display distinct tissue expression and pharmacology (6). P2X receptors respond to adenosine triphosphate (ATP), whereas P2Y receptors respond to a number of nucleotides including ATP, adenosine diphosphate (ADP), uridine triphosphate (UTP) and uridine diphosphate (UDP). For a more detailed review please see "Introductory Overview of Purinergic Signalling" by Geoffrey Burnstock in this issue of FBS.

2.2. Osteoblast biology

Osteoblasts are the bone forming cells within the skeleton; they are plump and cuboidal in shape with a large nucleus, large Golgi apparatus and well developed rough endoplasmic reticulum for the synthesis of extracellular proteins and calcium secretion. Most of the protein secreted by osteoblasts is type I collagen (<90%) with the remaining ten percent divided between around 200 other proteins. This specialised protein matrix called the osteoid allows osteoblasts to extend long cytoplasmic processes within it. Calcium secreted by the osteoblast contributes to the formation of hydroxyapatite within the osteoid to then form bone (7, 8).

3. P2 RECEPTOR EXPRESSION IN OSTEOBLASTS

3.1. P2Y receptors

The first indication of P2 receptor expression by osteoblasts came around 20 years ago when fluorescence studies demonstrated that extracellular nucleotides could transiently increase $(Ca^{2+})_I$ and induce IP_3 formation (9, 10). Subsequent pharmacological studies demonstrated that extracellular nucleotides interacted with at least two P2 receptor subtypes ($P2Y_1$ and $P2Y_2$) on rat and human osteoblast-like cells (11-13). The first molecular evidence for P2Y receptor expression was shown in 1995 by Bowler *et al*, who used *in situ* hybridisation and RT-PCR to demonstrate $P2Y_2$ receptor expression in human osteoblasts from bone explants (14). A detailed investigation of both single cells and populations of human osteoblasts indicated that there was heterogeneity in P2 receptor expression in any one culture (15). This observation raised the possibility that P2 receptor expression was dependent on the differentiation status of the osteoblast; an idea which was later confirmed in a separate study (16).

The expression of multiple P2 receptor subtypes by osteoblasts has now been reported by a number of groups (see table 1). Several studies have demonstrated the expression of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{12}$ receptor mRNA and protein in human osteosarcoma cell lines (MG-63, SaOS-2, OHS-4, SaM-1) (17-19) and primary rat osteoblasts (16, 20). In contrast, the rat osteosarcoma cell lines, ROS17/2.8 and UMR 106, do not appear to express $P2Y_2$ receptors (21, 22). Additionally, the expression of $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptor mRNA and protein has recently been detected in rat osteoblasts (23, 24).

3.2. P2X receptors

The expression of mRNA and protein for P2X2, P2X4 and P2X5 receptors has also been demonstrated in a

number of cell culture systems (16, 18, 20, 25, 26). More recently, expression of P2X1, P2X3, P2X4 and P2X6, receptor mRNA and protein by rat osteoblasts has been described (23). The P2X7 receptor, which was initially thought to be restricted to cells of haematopoietic origin, was found to be expressed on primary and clonal human osteoblasts, with only a subset of cells expressing the functional receptor protein (27). This finding was later confirmed by several studies which reported the expression of functional P2X7 receptors by primary mouse and rat osteoblasts (16, 28) and human osteosarcoma cell lines (25). Thus, osteoblasts appear to express all the known P2X receptor subunits. It is not known at present whether any of these subunits may combine to form functional heteromultimers (eg, P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/6, P2X4/6) in addition to homomultimers.

Although expression of most P2 receptor subtypes has been described for osteoblasts (see table 1), it should be noted that expression profiles are influenced by several factors, including: (1) species; (2) whether the osteoblasts in question are primary cells or immortalised lines; (3) the differentiation state of the osteoblasts.

4. ATP RELEASE AND TURNOVER IN OSTEOBLASTS

4.1. ATP release from osteoblasts

The expression of P2 receptors in osteoblasts and other bone cells indicates that ATP and other nucleotides are present in the bone microenviroment at sufficient concentrations for purinergic signalling in physiological and/or pathological conditions. ATP is a well characterised neurotransmitter but there is no evidence to date that neurones release ATP or other nucleotides in to the extracellular environment in bone. Extracellular ATP is likely to be derived from intracellular ATP pools, where concentrations are 1–5 mmol/L. As all cells have the capacity to release ATP following trauma and it is known that the general inflammatory response will increase the nucleotide concentration available upon platelet aggregation, it is apparent that high local ATP concentrations will be present to activate P2 receptors at sites of tissue injury, wounding, or fracture. However, given the widespread expression of P2 receptors on osteoblasts, it is unlikely that they are only activated during trauma states. For nucleotides to be physiologically relevant regulators of osteoblasts they must exist transiently in the bone microenvironment without cell damage. In other cell and tissue types non-lytic release of ATP had previously been demonstrated to occur (29) and released ATP could subsequently act back in an autocrine fashion on P2 receptors expressed on that particular cell and neighbouring cells. In order to determine if a similar system occurred in bone, early work focussed on using a custom-designed real-time detection system for the high-yield chemiluminescent reaction generated by luciferin and luciferase in the presence of ATP (30). Using that system it was successfully demonstrated that human osteoblasts constitutively release ATP into the bulk phase in the nanomolar range (31, 32). It is likely that the released ATP reaches much higher concentrations in the vicinity of the cell membrane than in the bulk phase measurement. This is because most cells express nucleotidases on the cell surface and there will be membrane trapping and then diffusion of ATP into the bulk phase. This concept was confirmed in the elegant study in which membrane-anchored luciferase revealed micromolar ATP concentrations at the surface of platelets (33). Subsequently, basal levels of ATP release from SaOS-2 osteoblastic cells was shown to increase in proportion with cell number in the absence of any change in cell death markers (17).

Several studies have been performed to try and elucidate the stimuli that direct osteoblasts to release ATP into the extracellular milieu. Of particular importance in the context of bone are mechanical stimuli including fluid flow, fluid shear stress, and compressive forces (34). ATP release from osteoblasts is increased in response to fluid flow (in the form of medium displacement) from HOBIT osteoblastic cells by 4-fold (35), SaOS-2 cells by 3-fold and from Te85 cells by 6-fold (36). ATP release from MC3T3-E1 osteoblasts increased 10-fold from basal levels in response to fluid shear stress in a Cytodyne parallel flow chamber within a closed loop system (12 dyne/cm^2, 5 minutes), with ATP release peaking after just 1 minute of fluid shear stress (37). A subsequent study using SaOS-2 cells in an Aberdeen Live Imaging Fluid Flow Chamber (ALIFFC) (38) measured ATP release at 1 minute intervals for 5 minutes at 3 different flow rates that fall just below and within the theoretical range thought to be engendered by shear stress inside bone canaliculi (i.e. between 6-30 dyne/cm^2) (39). No increase in ATP release was observed at 4.6 dyne/cm^2 (1.67ml/minute), whilst 2.5 times basal ATP was released at 13.8 dyne/cm^2 (5ml/minute) and 1.6 times basal at 23 dyne/cm^2 (8.33ml/minute) (36). The observation that no further increase in ATP release from SaOS-2 cells occurred above 13.8 dyne/cm^2 suggested that a ceiling for ATP release had been reached. In contrast to Genetos *et al* (2005), the study by Rumney *et al* (2010) observed a gradual increase in ATP release that took upwards of 3 minutes to show any significant increase compared to the static controls.

In the majority of studies described to date, osteoblasts were cultured and mechanically stimulated in monolayer systems which do not best resemble the 3D structure of bone. To better replicate the bone microenvironment cultures of SaOS-2 and Te85 cells were grown in polyurethane scaffolds and subjected to either mechanical compressive forces or fluid flow. Cyclic compressive loading within a modified Electroforce 3200 powered BOSE biochamber (1Hz, 5% strain, sine wave) did not induce reproducible increases in ATP release in stark contrast to fluid flow (5 bouts, 80% medium displacement) which significantly increased ATP release from SaOS-2 cells in 3D scaffolds by 6-fold and Te85 cells by 4-fold (39) (Figure 1). Taken together, these data reinforce the theory that fluid forces are the most important for inducing bone remodelling (34).

4.2. Mechanisms of ATP release from osteoblasts

Knowing that certain physiological cues can induce ATP release from osteoblasts is of great importance

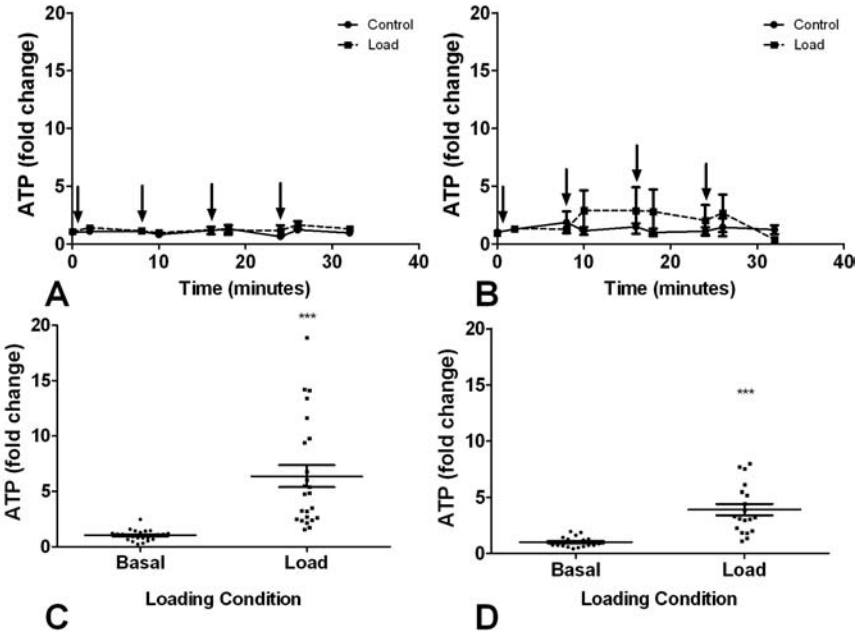


Figure 2. ATP release is inhibited by vesicular inhibitors. (A-C) MC3T3-E1 pre-osteoblasts were plated on type I collagen, allowed to proliferate to 90% confluency, and subjected to 12 dynes/cm^2 laminar fluid flow using a parallel plate flow chamber +/- Brefeldin A, Monensin and NEM and ATP release into the flow media was measured. (A) Brefeldin A, an agent that disrupts the Golgi and thereby prevents vesicle formation, attenuated FSS-induced increases in ATP release but had no effect on basal ATP release. (B) Monensin, which maintains the Golgi structure but prevents vesicle budding from the Golgi, similarly attenuated FSS-induced ATP release without affecting static ATP release. (C) N-ethylmaleimide prevents vesicular exocytosis by inhibiting NSF proteins. Addition of NEM also inhibited FSS-induced ATP release. (a: $p < 0.01$ vs. static cells in same conditions). (A-C © John Wiley and Sons. Reproduced from Genetos *et al*, Fluid shear-induced ATP secretion mediates prostaglandin release in MC3T3-E1 osteoblasts. Reproduced with permission from 37 D) Primary rat osteoblasts were treated with 1-100micromolar Monensin 1 hour prior to measurement of ATP release into the medium. Values are means +/- SEM (n=12); significantly different from controls:***=$P < 0.001$). E) SaOS-2 cultures in 24 well plates were serum starved for 1 hour and treated with 10nM NEM or DMEM vehicle. Loading was applied by 5 bouts of fluid displacement. N=3-6 wells per condition per experiment with 2-3 experimental repeats. ***=$P<0.001$ †††=$P<0.001$ *cf* load in DMEM.

in understanding the role of purinergic signalling in bone development and homeostasis. Pivotal to this is knowledge of the actual mechanism of ATP release, as this may present new therapeutic targets. Current debate exists within the field as to the mechanisms of ATP release from cells, with four likely mechanisms being suggested 1) vesicular release, 2) via connexins or pannexins, 3) via ABC-transporters and more recently 4) via the P2X7 receptor pore.

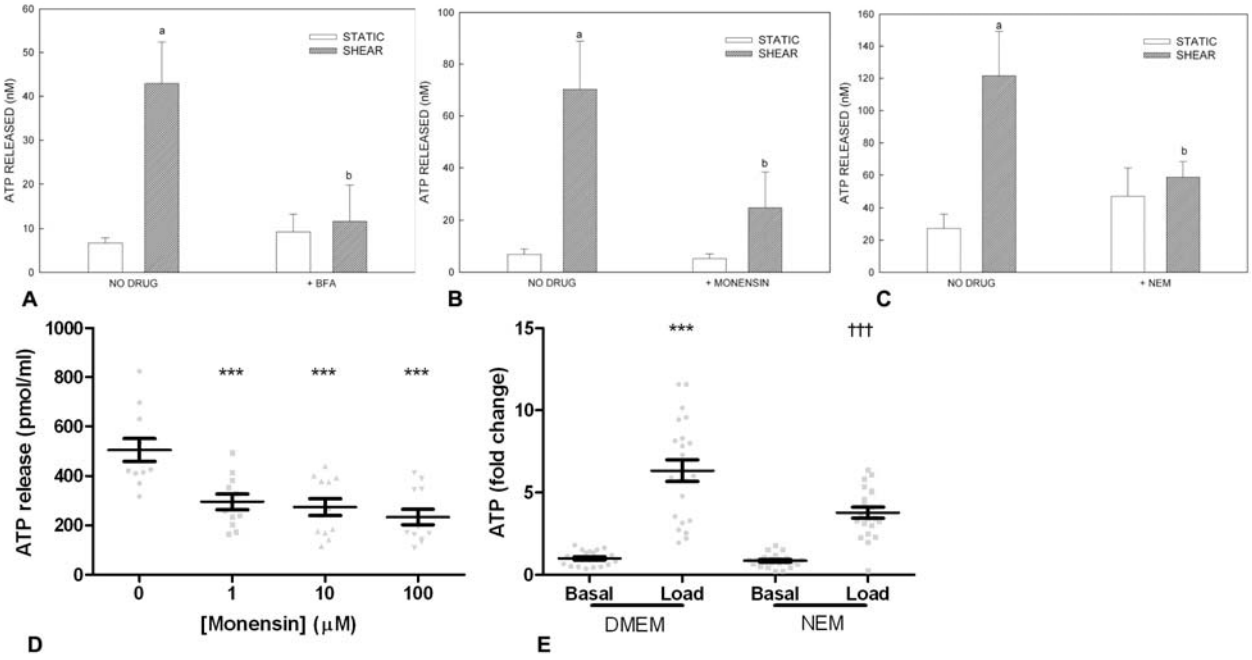
4.2.1. Vesicular release

Pharmacological evidence of a vesicular mechanism of ATP release from osteoblasts came from studies using MC3T3-E1 osteoblastic cells. When these cells were subjected to fluid shear stress in the presence of the vesicular inhibitors Brefeldin A, Monensin and N-ethylmaleimide (NEM) ATP release was inhibited (37). Similarly, we have shown that NEM inhibited ATP release from human osteoblastic SaOS-2 and Te85 cells in response to fluid flow (Figure 2) and rat osteoblasts in response to hypoxia (40-43). Further evidence for a vesicular mechanism of ATP transport in osteoblasts came from HOBIT osteoblastic cells stained with quinacrine to reveal fluorescent granular labelling which suggested ATP containing vesicles which were abolished in the presence of the vesicular inhibitor Monensin (44).

There is a concomitant role for calcium signalling in vesicular ATP release as extracellular Ca^{2+} increases ATP release from MC3T3-E1 cultures by threefold. Consistent with this finding, inhibition of L-voltage sensitive calcium channels by either nifedipine or verepamil attenuated shear induced ATP release (37). We have also confirmed that ATP release is calcium dependent as the calcium chelator BAPTA-AM inhibited flow-induced ATP release from SaOS-2 and Te85 cells (42).

4.2.2. Gap-junctions

Gap junctions, that is regions of physical continuity between cell membranes responsible for cell-to-cell movement of ions or small molecules (45), are composed of two apposing hemichannels (connexins or pannexins). Previous studies have shown that connexin hemichannels are required for intercellular ATP release between astrocytes (46), and forced expression of connexins in glioma cell lines increased ATP release by 5 to 15 fold (47). Although connexin hemichannels are well established in regulating extracellular calcium signalling



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between osteoblasts (22, 48), evidence for a direct role in ATP release from osteoblasts is lacking. Indeed a study using HOBIT osteoblastic cells over-expressing connexin43 showed that no increase in ATP release was observed compared to non-transfected controls (35), suggesting that they are not involved in ATP release. The more recently discovered pannexin hemichannels have been implicated in ATP secretion from mouse taste buds (49) and bovine retina (50). Pannexin expression is ubiquitous and *in silico* analysis has predicted elevated Panx3 gene expression in osteoblasts (50), however no study to date has demonstrated a direct role for pannexins in ATP release from osteoblasts.

4.2.3. ABC-Transporters

ATP-binding cassette (ABC) proteins, or ABC transporters, are a superfamily of membrane proteins that have two well-conserved nucleotide-binding folds in a functional unit. There are 48 different human ABC proteins subdivided into seven subfamilies termed ABCA-G. Of these the ABCB1 (*mdr1*) gene product P-glycoprotein, CFTR, and the Sulfonylurea receptors have been implicated in transmembrane ATP transport (51). Several of these proteins are involved in bone homeostasis - a polymorphism in the *ABCB1* gene encoding *P*-glycoprotein is associated with decreased bone mineral density in patients with Addison's disease (52). An immunolocalisation study demonstrated strong CFTR expression in osteoblasts *in situ* from human neonatal bone sections, and in primary human osteoblasts and MG63 osteoblasts cultured *in vitro* (53). A role for CFTR in regulating osteopontin and PGE_2 production in osteoblasts has been identified (55), whilst mice without the CFTR receptor suffer from osteopenia (54). Despite these observations there is no research to date that specifically links these ABC transporters to ATP release from osteoblasts.

4.2.4. P2X7 Receptor pore release

ATP release from osteoblasts may also be regulated by the ATP sensitive P2X7 receptor (P2X7R). A previous study using P2X7R-null mice demonstrated that the P2X7R is required for ATP release from astrocytes (56). Contrary to this, a study using bone cells from the same strain of P2X7R-null mice demonstrated that the P2X7R is not required for ATP release in response to mechanical loading (57). More recent results have been presented in support of P2X7R-regulated release whereby antagonists of the P2X7R inhibited fluid flow-induced ATP release from P2X7R expressing SaOS-2 cells but not from P2X7R-null Te85 cells (36).

4.3. ATP degradation

Once ATP is released into the extracellular microenvironment it can be hydrolysed via a series of dephosphorylation steps catalyzed by a range of

extracellular enzymes liberating inorganic phosphate (Pi) at each step to yield adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine. Ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) acts upon ATP and ADP to generate AMP whereas ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) cleaves ATP into AMP and pyrophosphate (PPi). Nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1, also known as PC-1) is associated with matrix vesicles of SaOS-2 cells and plays an important role in mineralisation as it is responsible for generating inorganic pyrophosphate (PPi) (58). AMP is subsequently hydrolysed into adenosine and Pi by Ecto-5'-nucleotidase (59). The PPi generated by E-NPP is an inhibitor of bone mineralisation and is hydrolysed by alkaline phosphatase to inorganic Pi (60). The coexistence of ATP-consuming and ATP-generating activities on the osteoblast cell surface was demonstrated for the first time in a study by Buckley *et al* in 2003. By analysing ATP metabolism over time they demonstrated that inhibition of ecto-NDPK activity increased the overall degradation of ATP (17). More recently exogenous ATP has been shown to be rapidly hydrolysed in cultures of SaOS-2 and Te85 cells with a half life of 2-3 minutes, which is extended in the presence of the alkaline phosphatase (ALP) inhibitor levamisole (42). ALP is particularly important in bone where it is required for mineralisation (60). ALPs are encoded by four human genes: intestinal, placental, placental-like and bone/liver/kidney (tissue non-specific ALP; TNAP) (61, 62). The different forms of TNAP have the same amino acid sequence but have different post-translational modifications (63). Despite the close relationship between the different forms of TNAP, bone specific ALP can be differentiated by using a monoclonal antibody against ALP from SaOS-2 cells. The same study demonstrated that bone derived ALP had distinctive enzyme kinetics as it had a lower heat inactivation time than total ALP (64). ALP activity is not uniform throughout bone as osteoblasts on the superior surface of calvaria express more ALP than osteoblasts on the inferior surface which is reflective of the direction of bone growth (65). ALP has broad substrate specificity and targets ATP, ADP, AMP, PPi and other phosphates (66). This is particularly important in bone where ALP hydrolyses the mineralisation inhibitor PPi which is presented into the extracellular space through ATP hydrolysis by E-NPP1 or by transport from inside the cell via the multiple pass membrane protein ANK (67). Mice deficient in TNAP have elevated PPi and poor bone mineralisation whereas mice deficient in the cell surface PPi transporter ANK have decreased PPi and hypermineralisation in bone (60).

As ALP rapidly hydrolyses ATP, any factor which modulates the expression and/or activity of ALP will ultimately influence the concentration of extracellular nucleotides and any downstream purinergic signalling. ALP activity has been demonstrated to be lower compared to controls in rodent calvaria osteoblasts treated with 10microM ATP every 3 days for up to 10 days (68). Whilst there is contrary data as to the effects of fluid shear stress upon ALP with one study suggesting that it increased the expression of ALP mRNA in rat calvaria osteoblasts (69) and another study suggesting that it decreased ALP activity in MG63 cells (70). Clearly regulation of ALP activity in osteoblasts is of timely importance and interest.

5. P2 RECEPTOR SIGNALLING

5.1. Signalling cascades downstream of P2-Receptor activation in osteoblasts

As stated earlier, P2Y receptors are G-protein coupled receptors and can be further divided into subgroups according to the G-protein they couple to. The $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors couple to $G_{q/11}$, with the $P2Y_{11}$ receptor being distinct as it also couples G_s. Stimulation of these receptors leads to activation of phospholipase C, which hydrolyses phosphatidyl inositol 4,5-bisphosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG) resulting in Ca^{2+} release from internal stores. In contrast, the $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors couple to G_i, activation of these receptors inhibit adenylyl cyclase and alter cyclic adenosine monophosphate (cAMP) levels. A number of studies have demonstrated that $P2Y_2$ receptor activation in osteoblast-like cells activates a number of intracellular signalling pathways including p38 mitogen-activated protein kinase (p38 MAPK), protein kinase C (PKC) and c-Jun NH_2-terminal protein kinase (JNK) (71-74). $P2Y_2$ receptors have also been shown to mediate the Ca^{2+} mobilisation induced by oscillatory fluid flow in mouse osteoblasts (75). An early study demonstrated that mechanically stimulated human osteoblasts propagate fast intercellular Ca^{2+} waves between osteoblasts via the autocrine activation of $P2Y_2$ receptors (22). In a follow-up study, the same authors demonstrated that intercellular signalling between osteoblasts and osteoclasts was not mediated via P2Y receptors but instead appeared to require the P2X7 receptor (76). The P2X7 receptor is also thought to play a role in mediating the ERK1/2 activation caused by fluid shear stress in osteoblast-like cells. In addition, ATP release was required for ERK1/2 phosphorylation and appeared to be dependent on both elevation of $(Ca^{2+})_i$ and activation of protein kinase C (PKC) (77, 78). More recently a role for lipid-signalling pathways downstream of the P2X7 receptor activation in osteoblasts has been demonstrated, with activation of P2X7 receptors resulting in stimulation of PLD and PLA_2, resulting in production of the potent lipid mediator lysophosphatidic acid (LPA) (79). LPA then acts through its G protein-coupled receptor to induce membrane blebbing via a pathway dependent on Rho-associated kinase. Thus, a number of the effects of P2X7 receptor activation in osteoblasts may be mediated by prostaglandins and LPA.

5.2. P2 receptor signalling synergy with growth factors

Bone remodelling is known to be tightly controlled via systemic factors, in particular PTH. Yet remodelling occurs in discrete foci – suggesting that this process is sensitive to local stimuli, including mechanical strain. We believe that one important local stimulus is ATP release and activation of P2 receptors which sensitizes cells to the action of PTH and thus is one mechanism for integrating local and systemic responses in bone (80).

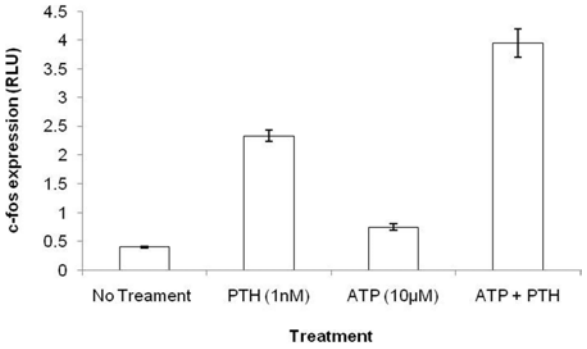

Figure 3. c-*fos* expression in SaOS-2 cells after varying treatments. SaOS-2 cells transfected with a luciferase c-*fos* reporter system was used to study c-*fos* expression. Cells were serum starved for 24 hours prior to experiment, then treated with either PTH (1nM), ATP (10 micromolar) or both simultaneously. Cells were left for 4 hours before cell lysis and measurements taken. n=10. ***=$p<0.0001$

When ATP and parathyroid hormone (PTH) stimulate their respective receptors a synergistic increase in c-*fos* expression is observed (see Figure 3). Therefore it is reasonable to presume that in areas where both ATP and PTH are present more remodelling will occur. The intracellular mechanism behind this synergy is a complex one, which differs between cell lines. It can be achieved either through a calcium dependent or calcium independent method, depending on which P2 receptor is activated. ATP acting at either the $P2Y_1$ or $P2Y_2$ receptor causes an increase of intracellular calcium. Via its $G_{q/11}$ subunit, the stimulated P2 receptor activates PLC that cleaves PIP_2 into IP_3. IP_3 then acts on its receptors to release calcium from intracellular stores. Meanwhile PTH can act alone to cause an increase in c-*fos* via activation of the G_s subunit. This will activate adenylate cyclase, causing an increase in cAMP. cAMP then acts on PKA which can phosphorylate CREB. CREB can then cause transcription of c-*fos* via the Ca/CRE part of the promoter (21). In the rat osteosarcoma cell line UMR-106, ATP, acting at the $P2Y_1$ receptor, achieves this synergistic increase in c-*fos* expression via a potentiation of calcium release from intracellular stores (21). This increased calcium will then target the Ca/CRE part of the promoter, which, as well as responding to CREB will also respond to calcium signalling pathways (Figure 4). Thus, with these two pathways acting on the same region of the promoter, an increase of c-*fos* transcription is observed.

However in SaOS-2 cells, there is no potentiation of the calcium signal, instead synergy is achieved via two separate pathways converging on two separate parts of the same promoter. The PTH pathway again acts at the Ca/CRE, whereas the ATP pathway acts at the SRE region (Figure 5). Interestingly the ATP pathway is independent of the classic ERK or TCF MAPK kinase pathways (81).

Further to this, more recent studies have revealed that ATP can sensitize the osteoblasts to the effect of PTH. ATP only has a half life of up to 3 minutes *in vitro*. Despite this, the synergistic effects are still observed when it is added to the cells 8 hours before PTH addition, suggesting ATP also has a long term effect on the osteoblast. Given that ATP has been shown to be released when osteoblasts are subjected to various forms of mechanical stress (35, 37, 40, 82) this synergy provides a mechanism whereby the action of the system wide PTH can be focused to a specific, mechanically stressed, area. This may provide an insight on the observed effect of increased remodelling in areas of the skeleton that undergo higher loading.

PTH is not the only hormone to synergize with ATP; a similar effect is observed when osteoblasts are co-stimulated with ATP and glucose insulinotropic polypeptide (GIP) (83). This hormone is released from the duodenum and jejunum of the GI tract when glucose is present. Its primary function is to induce insulin secretion. But receptors for GIP have been found on various osteoblastic cell lines. Recent studies have shown that when GIP and ATP are both present at the osteoblast cell membrane a synergistic increase is again observed in c-*fos* expression. This suggests a mechanism where the body is able to signal to the skeleton that nutrients are available and thus remodelling can proceed, again in areas where ATP is present.

6. FUNCTIONAL CONSEQUENCE OF P2 RECEPTOR ACTIVATION IN OSTEOBLASTS

The functional consequence of P2 receptor activation on osteoblasts is varied, and at times contradictory, and has been shown to influence many of the processes that govern skeletal growth and remodelling. A proliferative role for ATP was first reported using MC3T3-E1 osteoblast-like cells (84), and enhanced DNA synthesis has also been observed in osteoblast-like MG-63 cells following P2X receptor stimulation (26). However, the first report that ATP and other nucleotide agonists had an inhibitory effect on bone formation was made using an *in vitro* assay to measure authentic bone formation by primary rat osteoblasts (85). This was subsequently supported by the observations that at concentrations of between 1 and 100 micromolar, ATP and UTP strongly inhibited bone formation (86). In a follow up investigation, light microscopy revealed that ATP and UTP-treated osteoblasts deposited abundant collagenous matrix with the characteristic morphology of bone nodules, but that mineralisation had failed to occur (68). ALP activity was decreased up to 65% in cultures treated with 10 micromolar ATP or UTP, whilst collagen deposition was unaffected. These inhibitory actions of ATP and UTP were consistent pharmacologically with mediation via the $P2Y_2$ or $P2Y_4$ receptor subtypes. The $P2Y_4$ receptor antagonist, reactive blue 2, failed to prevent the nucleotide-induced block of mineralisation, suggesting that $P2Y_2$ receptor stimulation mediates the functional effects of ATP and UTP (68). Skeletal analysis of $P2Y_2$ knockout mice by dual energy x-ray absorptiometry (DEXA) and microCT demonstrated large increases in trabecular and cortical bone parameters in the long bones (68, 87).

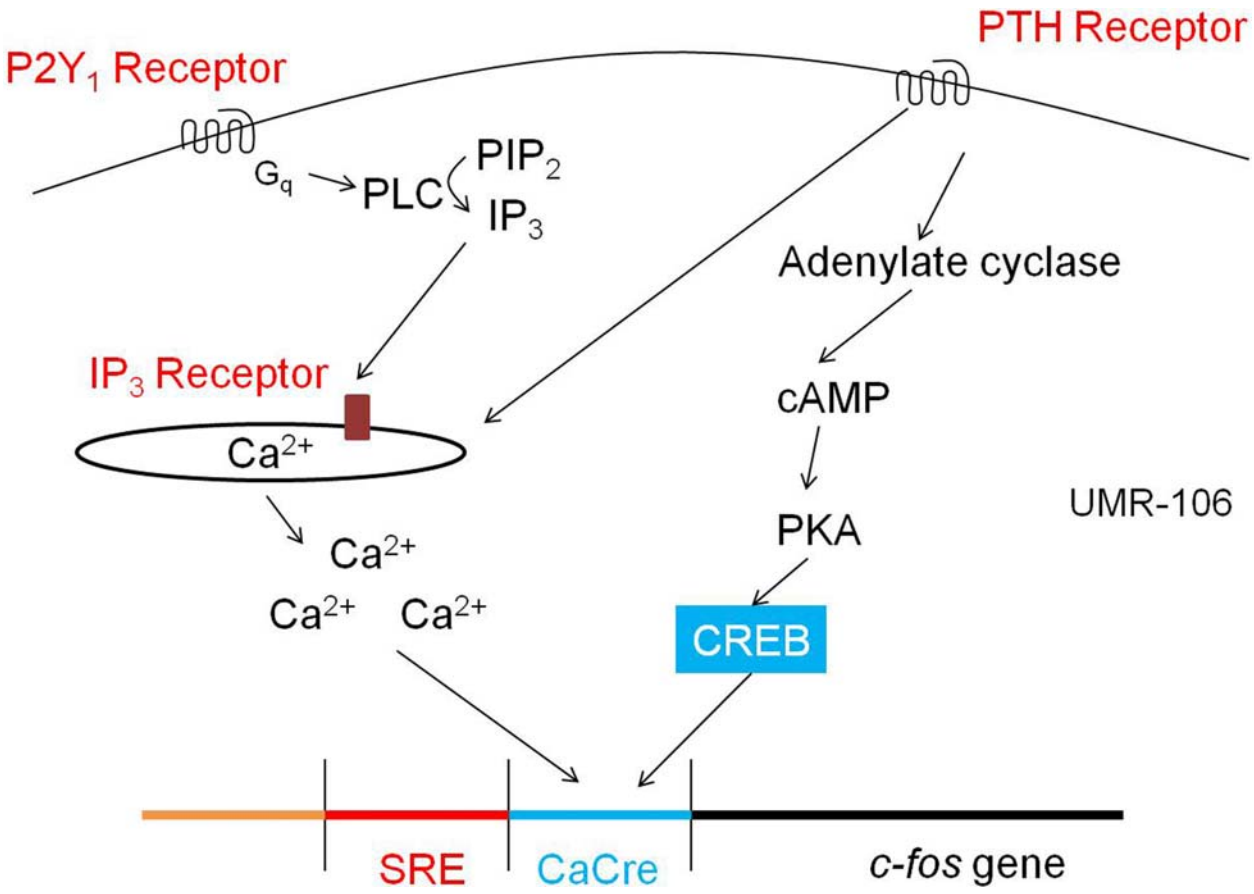

Figure 4. Intracellular mechanisms of ATP and PTH synergy in SaOS-2 cells. Activation of the PTH receptor leads to an increase in *c-fos* production via the G_s subunit. This activates a downstream signalling cascade involving adenylate cyclase, cAMP, PKA, and will result in the phosphoylation of CREB. CREB will then act on the Ca/CRE part of the promoter to cause *c-fos* transcription. When both the $P2Y_1$ and PTH receptors are activated there is a potentiation of calcium release from intracellular stores. This increase in calcium will also target the Ca/CRE part of the promoter leading to a synergistic increase in *c-fos* transcription.

The P2X7 receptor is distinct from other P2X receptors in that it has a relatively low affinity for ATP (> 100 micromolar) and preferentially binds 2',3'-*O*-(benzoyl-4-benzoyl)-ATP (Bz-ATP). Prolonged or repeated exposure to high agonist concentrations mediates the formation of cytolytic pores, whereas, transient receptor stimulation causes the formation of non-selective membrane pores permeable to molecules up to 900KDa in size (88). The first report describing the expression of the P2X7 receptor on a sub-population of osteoblasts demonstrated that P2X7-receptor activation caused membrane blebbing, cytoskeleton re-arrangements and ultimately apoptosis (27). The authors commented that it was most likely that the expression of P2X7 receptors on osteoblasts would have much more subtle and pertinent functions such as the controlled release of cytoplasmic components via rapid reversible pore formation. Indeed, recent studies have supported this hypothesis as P2X7 stimulation has been demonstrated to lead to increased bone formation and ALP activity *in vitro*; an effect thought to be mediated via enhanced production of prostaglandin E_2 (PGE_2) and lypophosphatidic acid (LPA) (79, 89). In agreement with this stimulatory role, P2X7 receptor knockout mice have decreased periosteal bone formation in the tibia (28) and osteoblasts cultured from these animals displayed reduced osteogenesis and ALP activity (79). These stimulatory effects of Bz-ATP are opposite to those seen with ATP and UTP; thus, it seems that the effects of extracellular nucleotides on osteoblast function are likely to vary depending on the ATP concentration in the microenvironment and the subsequent P2 receptor subtype activated.

A number of other effects of P2 receptor activation on osteoblast functions have also been reported. ATP acting via the P2X5 receptor has been reported to stimulate proliferation in osteoblast-like cells (26). Furthermore, P2Y receptor stimulation by ATP has been associated with increased interleukin-6 synthesis (18) and arachidonic acid release (90). Both of these factors have been shown to play a major role in bone remodelling.

It is worthy of note that ATP could also exert effects on bone independently of P2 receptor activation via

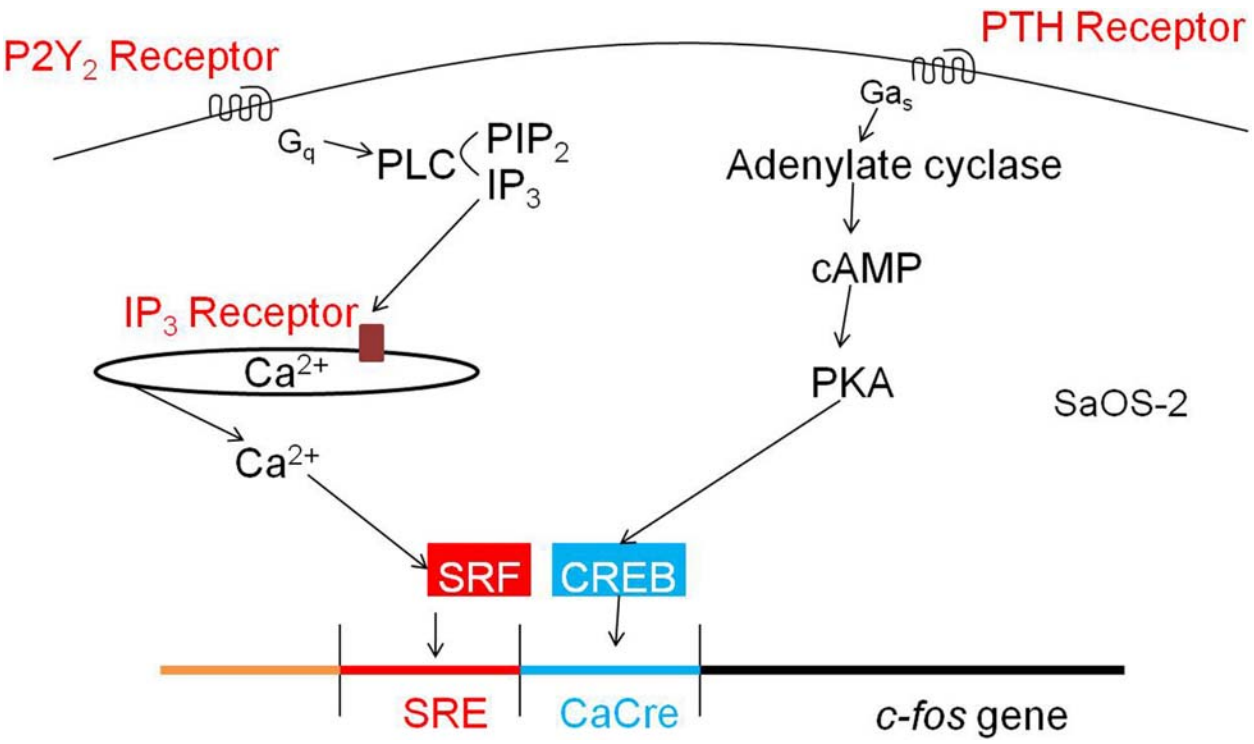

Figure 5. Intracellular mechanisms of ATP and PTH synergy in the UMR-106 rat osteosarcoma cell line. Similar to UMR-106 cells, activation of the PTH receptor leads to an increase in c-*fos* production via the G_s subunit. This activates a downstream signalling cascade involving adenylate cyclase, cAMP, PKA, and will result in phosphoylation of CREB, CREB will then act on the Ca/CRE part of the promoter to cause c-*fos* transcription. However in SaOS-2 cells there is no potentiation of calcium release from intracellular stores. Instead, intracellular calcium release, as a result of $P2Y_2$ receptor activation, will act on the SRE region of the promoter. However this is not through the classical MAPK pathways involving ERK or TCF. Thus the synergistic increase is achieved through two pathways acting at two different parts of the same promoter.

PPi. PPi is generated by the hydrolysis of the phosphodiester bond in purine and pyrimidine nucleoside triphosphates by E-NPPs. Osteoblasts express three members of the E-NPP family, E-NPP1, E-NPP2 and E-NPP3 (68, 91). Since PPi is a potent inhibitor of bone mineralisation it is possible that some of the actions of ATP and UTP could occur independently of P2 receptors. A recent study demonstrated that the nucleotide triphosphates, CTP and GTP [which are also hydrolysed to produce PPi although they do have very weak agonist activity at the rat $P2Y_2$/$P2Y_4$ receptors (92)], similarly inhibited bone mineralisation between 1 and 100micromolar but did not affect ALP activity or expression (68). The same study also demonstrated that osteoblastic E-NPP activity was capable of generating biologically significant concentrations of PPi *in vitro*. Thus, nucleotide triphosphates can exert a dual inhibitory action on bone mineralisation via both P2 receptor-mediated signalling and direct hydrolysis to PPi (68).

Finally, probably one of the most significant observations made on the functional consequences of ATP and P2 receptor signalling in osteoblasts came from Buckley *et al* in a study looking at the effects of extracellular nucleotides on human osteoclast activity. In this study they found that ATP modulated osteoclast activity only when applied to osteoblast-osteoclast precursor co-cultures. The mechanism behind this activation of osteoclasts was shown to be via up-regulation of RANKL expression both at the mRNA and protein level (93). The observations that ATP can modulate the expression of the osteoclastogenic factors IL-6 and RANKL by osteoblasts (18, 93) highlights the delicate balance and interplay between osteoclast and osteoblast activity, and that the local actions of ATP are ideally placed to co-ordinate focal bone remodelling.

7. FUTURE PERSPECTIVE

The availability of mouse KO models to study global knock-down of P2 receptors has enabled us to advance our understanding of purinergic signalling in bone (see Orriss *et al.*, "Bone phenotypes displayed by P2 receptor knockout mice" in this issue of FBS.), whilst generation of gene/tissue specific KO models will present many new and exciting findings and future directions for of the role of specific P2 receptors in bone. The continual development and availability of novel, specific agonists and antagonists for individual receptors will also provide researchers with a better means to elucidate the exact roles for these receptors in bone disease. Currently denufosol and diquafosol, $P2Y_2$ agonists, are in clinical trial for cystic

fibrosis and dry eye syndrome respectively whilst several $P2Y_{12}$ antagonists are in clinical development for thrombosis (for an overview of clinical development within the purinergic field see Jacobson *et al* (94). Thus the realisation of purinergic signalling as therapeutic targets for treating bone diseases will not be too far into the future.

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